DISSERTATION

THE USE OF CHRONIC MODELS OF TEMPORAL LOBE EPILEPSY IN ANTIEPILEPTIC DRUG DEVELOPMENT

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY HEIDI GRABENSTATTER ENTITLED "THE USE OF CHRONIC MODELS OF TEMPORAL LOBE EPILEPSY IN ANTIEPILEPTIC DRUG DEVELOPMENT" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

THE USE OF CHRONIC MODELS OF TEMPORAL LOBE EPILEPSY IN ANTIEPILEPTIC DRUG DEVELOPMENT

A chronic animal model with altered ion channels, transmitter receptors and/or neural circuitry similar to temporal lobe epilepsy (TLE) may be useful in the discovery of new antiepileptic drugs (AEDs). The hypothesis was that rats with kainate-induced epilepsy are pharmacosensitive to AEDs, but high doses do not block all spontaneous seizures (i.e., these rats are "pharmacoresistant"). A repeated-measures cross-over protocol was used to show single intraperitoneal injections of topiramate, RWJ-333369, and carbamazepine reduced the frequency of spontaneous motor seizures. The same protocol with administration of 30 mg/kg and 100 mg/kg carbamazepine in specially-formulated food pellets was as effective as intraperitoneal injections, and 100 mg/kg carbamazepine administered in food three times per day completely suppressed motor seizures in 50% of the animals for a prolonged time period (i.e., 24 h) while reducing any stress to the animals. Video-EEG showed carbamazepine preferentially reduced spontaneous *convulsive* seizures compared to *nonconvulsive* seizures at 100 mg/kg, reduced seizure duration in some animals at 100 mg/kg, and caused a subtle decrease in the maximum frequency of

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population spikes during seizures at 30 mg/kg. These data suggest that animal models of TLE with spontaneous seizures can be used efficiently to test AEDs, and that this repeated-measures cross-over protocol is amenable to both dose-effect and time-course-of-recovery studies for the direct comparison of AEDs. This approach can provide statistical power to compensate for seizure clusters and variability across animals. These experiments also show that rats with kainate-induced epilepsy are pharmacosensitive to standard and experimental AEDs; additional studies are required to determine if this model is also pharmacoresistant.

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CHAPTER 1- Background and significance

Temporal lobe epilepsy (TLE) is one of the most common neurological disorders in humans, afflicting more than 1% of the population (Engel, 1989). Over 30% of patients with temporal lobe epilepsy have seizures that are refractory to commonly used antiepileptic drugs (AEDs) (Bauer and Burr, 2001). For many years, a limited number of AEDs (e.g., phenytoin, valproate, carbamazepine, phenobarbital) were available as therapy for epilepsy patients. A new generation of AEDs has been developed over the past decade to treat the refractory population. Now, roughly two dozen AEDs are clinically available (Rogawski and Loscher, 2004). These drugs are potentially more effective, although the primary benefit of the new AEDs appears to be that they suppress seizures at lower doses and with fewer side effects. Despite the progress made in recent years and the availability of new AEDs, the percentage of patients that have ineffective seizure control has failed to decline.

Temporal lobe epilepsy is typically an injury-induced, acquired condition characterized by a latent period and a progressive increase in frequency and severity of chronic seizures. Epileptogenesis refers to all the anatomical, biochemical, and physiological changes that occur in cortical tissue following a brain injury that progressively lead to the emergence of epilepsy. The study of

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epileptogenesis is based around surrogate markers that are hypothesized to be predictive of neuronal or systemic changes, and may appear before the onset of seizure activity. The appearance of appropriate markers (e.g., reduced inhibitory responses, shifts in subunit composition of receptors and/or ion channels, mossy fiber sprouting) may be causally linked with the development of spontaneous seizures. Animal models of injury-induced epilepsy have been used to test several hypotheses regarding mechanisms of epileptogenesis, but additional neurophysiological analysis is needed to understand the network interactions involved in the development of epileptic seizures. A recent NIH-sponsored workshop came to the conclusion that models of injury-induced epilepsy, particularly those that involve chemically-induced status epilepticus, should be tested for pharmacoresistance and pharmacosensitivity (Stables et al., 2002).

1.1 Overview of animal models used in AED testing.

An ideal model of epilepsy for use in the identification of new antiepileptic drugs should exhibit the following characteristics: (1) spontaneously recurrent seizures, (2) a seizure type that mimics the clinical condition in human epilepsy, (3) a time-dependent onset of epilepsy (i.e., a latent period) similar to that observed in epileptic syndromes in humans, (4) clinical seizures should have associated epileptiform activity recorded in the EEG, (5) pharmacokinetics of AEDS should mirror those in humans to allow for maintenance of therapeutic drug levels during chronic drug treatment, and (6) effective plasma concentrations of AEDs similar to those required to control the modeled seizure

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type in human patients. No model presently meets all criteria and there is an overwhelming need to develop new animal models of epilepsy and to approach the preclinical AED development process using multiple models exploiting both acute seizure models and chronic models of epilepsy for their strengths (Loscher and Schmidt, 1988).

Traditional acute seizure models.

The main goal of AED testing in animal models of seizures and epilepsy is to determine whether a compound is active or effective against the endpoints (e.g., experimental seizures, spontaneous seizures, after discharge threshold) elicited in the model in question. For decades, the primary models used to elucidate the effectiveness of a potential AED were the maximal electroshock (MES) and the subcutaneous pentylenetetrazol (s.c. PTZ) acute seizure models. PTZ is a $GABA_A$ antagonist and is a test for absence seizures. The dose of PTZ that is determined to be convulsive in 97% of the animals is administered subcutaneously (~70 mg/kg in rats) approximately 10 min. prior to the anticipated peak effect of the drug being tested. The animals are generally observed for an additional 30 min for the occurrence of clonic seizures. The dose required to suppress clonic seizures in 50% of the animals (usually 8-10 animals per dose) is calculated as the ED_{50} and subsequent dose-response curves are also determined. Ethosuximide, valproic acid, and the benzodiazepines are effective in the absence-type seizures evoked in this model, but phenytoin and carbamazepine are not. Primidone and phenobarbital block PTZ-evoked

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seizures more effectively than ethosuximide which raises the concern (based on the clinical seizure type for which these drugs are effective) that the s.c. PTZ seizure model may more closely resemble myoclonic epilepsy rather than absence epilepsy (Loscher and Schmidt, 1988; Velisek., 2006).

MES is a supramaximal electrical stimulus-induced seizure model that predicts drugs effective in generalized seizures. In addition to identifying drugs that may be effective against generalized tonic-clonic seizures, it has also been proposed that the MES test may predict AEDs that suppress partial seizures. A supramaximal stimulus is applied using constant current corneal or ear electrodes for 0.2 sec at 50 mA for mice and 150 mA for rats. The efficacy of a potential AED is determined using the supramaximal MES test by calculating the dose required for suppression of tonic hindlimb extension in 50% of the animals (i.e., ED_{50}). Phenytoin, carbamazepine, phenobarbital, and primidone are very effective in this model, while ethosuximide is ineffective (Loscher and Schmidt, 1988; Mares and Kubova., 2006). The traditional acute seizure models are a rapid screening tool that have been used to develop the two dozen compounds marketed as (AEDs) worldwide, nine of which were developed since 1993. However, they lend themselves to false positive and false negative results. These tests are primarily used as the first-line tests of the NIH-sponsored Anticonvulsant Drug Development (ADD) program because of their moderately high throughput [(averaging 1,000 compunds/year) Stables et al., 2002]. There is doubt, however, whether these tests will identify all potential AEDs or whether highly effective drugs will go unrecognized because their mechanisms of action

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are not those tested by these acute models. The MES test pre-selects drugs with specific mechanisms of action and misses others. Electroconvulsive seizures are particularly sensitive to sodium channel antagonists so it is not surprising that most of the AEDs that were identified using this test function, at least in part, via this mechanism. The successfully marketed drug, levetiracetam, which acts by a different mechanism, had a strong antiepileptic effect in the amygdala-kindled model of temporal lobe epilepsy, but the AED did not increase seizure threshold in either the maximal electroshock or pentylenetetrazol seizure tests. (Loscher et al., 1988). Glutamate antagonists that block *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors are highly effective in the MES test against tonic seizures, but fail to block kindled seizures or to demonstrate clinical efficacy (Loscher and Schmidt, 1988). The percentage of therapy-resistant individuals has failed to decrease in the past decade even with the introduction of nine new drugs discovered using these traditional seizure tests. This suggests that new approaches are needed to test the efficacy of recently and not yet discovered AEDs. The central limitation of these acute seizure models is that the evoked seizures are elicited in *normal* animals. The lack of efficacy of new drugs in pharmacoresistant patients suggests that development of future AEDs should mandate that efficacy is defined by the ability to limit seizures that evolve spontaneously from an altered CNS substrate not only evoked, symptomatic seizures (Stables et al., 2003).

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Acute threshold seizure models.

Threshold models are also commonly used in AED screening, in which the current (or voltage) necessary to evoke a minimal (clonic) or maximal (tonic extension) seizure is quantified after stimulating via corneal or ear electrodes. Constant current or constant voltage is typically delivered as sinusoidal or rectangular pulses at a frequency of 50-60/sec for 0.2 sec. In this test, the effect of a drug on an individual animal is determined instead of using a fixed electrical or chemical stimulation parameter for all animals throughout the protocol and ignoring the inter-animal differences in seizure susceptibility. The threshold is defined as the current or voltage that causes hindlimb extension in 50% of the animals tested. Maximal electro-convulsions evoked in mice are a sensitive test to determine a drug's potency against generalized tonic-clonic seizures (Swinyard, 1972).

Threshold tests can also be conducted using PTZ administered via intravenous infusion (i.v. PTZ). The threshold dose of i.v. PTZ necessary to evoke clonic seizures is quantified in this test. Three seizure components are used as efficacy endpoints in this screening test: (1) isolated jerks, (2) generalized clonic seizures with loss of righting reflex, and (3) maximal tonic-clonic seizures (Swinyard, 1973). There is an interanimal variability to time of onset of the tonic component making the i.v. PTZ threshold test poorly suited for rapid AED testing. Threshold doses of PTZ for each endpoint are dependent on rate of i.v. infusion and concentration of PTZ used (Nutt et al, 1986). However, the i.v. PTZ threshold test is more sensitive than s.c. PTZ tests for clonic

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seizures, and is predictive of drugs that are clinically effective against generalized seizures of the absence type. This is supported by an increase in seizure threshold by ethosuxomide and valproate, but a lack of effect with phenytoin and carbamazepine when tested in this model. Using supramaximal tests (e.g., MES and s.c. PTZ) in combination with threshold tests, the antiepileptic effect of the drug can be attributed to reduction of seizure spread or an increase in seizure threshold. Threshold tests are also more likely than traditional tests to detect any proconvulsive effects of a potential AED (Loscher and Schmidt, 1988).

Genetic (idiopathic) animal models of epilepsy.

Genetic animal models of epilepsy are likely candidates for the development of AEDs to treat specific epileptic syndromes. However, they are rarely used in preclinical testing of AEDs. There are two types of inherited epilepsy models, those that display recurrent spontaneous seizures and models in which seizures are induced by specific sensory stimuli in genetically susceptible animals (e.g., baboons, DBA/2 mice, gerbils). Most of the animals with naturally occurring spontaneous seizures have a relatively low seizure frequency, and are not well suited for AED efficacy trials. The prevalence of epilepsy in dogs is 0.6% which is similar to the incidence in the human population. AED trials have been conducted in *epileptic dogs* which present with focal and primary and/or secondary generalized tonic-clonic seizures. Symptomatic epilepsies have also been noted in dogs primarily due to brain

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tumors. Interestingly, 20-40% of epileptic dogs with tonic-clonic seizures tested are refractory to high-dose AEDs. These numbers parallel the percentage of human patients that are refractory to current antiepileptic therapy. Despite this interesting fact, epileptic dogs are not well suited for AED efficacy trials because the naturally occurring seizures are relatively low in number and it is difficult to obtain an adequately high number of epileptic dogs to conduct any long-term studies (Loscher, 1984; 1997; Loscher et al., 1985).

Rats and mice with spontaneously occurring petit mal seizures have advantages in this respect because seizure frequencies in these models are high enough that it is not necessary to use unnatural means to evoke seizures. The rat petit mal model exhibits EEG paroxysms and spike-discharges associated with behavioral arrest and myoclonic twitching of the facial musculature. The pharmacological profile of these animals suggests that they are appropriate to use for the identification of drugs for the treatment of absence epilepsy, and can be used in both chronic and acute studies (Loscher, 1984).

The tottering mouse model exhibits focal spontaneous seizures characterized by clonic jerks of the limbs with secondary generalization. The focal seizures respond to treatment with diazepam, but are not affected by ethosuximide or valproate. A second distinct seizure pattern that is found in tottering mice is spike-wave petit mal seizures which are much more frequent and resemble those seen in the petit mal rat. The petit mal seizures are affected by ethosuximide, diazepam and phenobarbital, but not phenytoin. A disadvantage of the seizure expressing tottering mouse is that it is difficult to

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obtain enough homozygous mice for pharmacological studies (Green et al., 1962; Noebels, 1979a; 1979b; Loscher, 1984).

The *AE mouse*, a genetic model of spontaneous generalized tonic-clonic seizures has seizures too infrequently to be used in AED efficacy studies. However, the animals are more susceptible to induction of seizures by MES and PTZ, but less responsive to AEDs than *normal* animals (Loscher et al., 1986). This suggests that testing drugs in non-epileptic animals by evoking seizures via MES or PTZ may exaggerate the potential clinical effectiveness. Other mouse strains that exhibit spontaneous seizures include the *C57BL/10Bg* strain and *quaking mice*. Homozygous *quaking mice* have been pharmacologically characterized and have either spontaneous or evoked myoclonic and generalized tonic-clonic seizures. Induced seizures are caused by handling for the purpose of AED evaluation, and can be blocked by phenobarbital, phenytoin, carbamazepine and valproic acid, but ethosuximide and diazepam are ineffective. Quaking mice may be an inexpensive, efficient means for testing AEDs for the treatment of partial epilepsy, however, it is still uncertain whether this model may prove to predict clinical efficacy for human complex partial epilepsy (Seyfreid et al., 1985; Taylor et al., 1985).

Other species also demonstrate inherited epilepsies. The inbred line BIO 86.93 of Syrian hamsters experience spontaneous myoclonic and generalized tonic seizures, but mild stress can also induce the seizures in this model. The behavioral stages of the seizures occur as follows: hyperkinetic-ataxic gait, straub tail, falling, tonic hind limb extension, wading pool crawl, head bob

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followed by a paralytic state of extended hind limbs, and finally, myoclonus of forelimbs and jaws (Yoon et al., 1976). Unfortunately, these behavioral symptoms do not correlate with any known human epilepsy which suggests this model would have little predictive value for AED development. Benzodiazepines are effective against these seizures, but phenytoin, phenobarbital, ethosuximide, and trimethadione fail to block seizures (Loscher and Schmidt, 1988).

There are several animal models that are predisposed to reflex epilepsies such that seizures can be induced by specific sensory stimuli (e.g., photic, audiogenic, vestibular). Examples of some of these models include (1) the baboon *Papio papio* which responds to photic stimulation to have myoclonic and tonic-clonic type seizures (Killam et al., 1979), (2) *photosensitive fowls* which exhibit grand mal epilepsy (Johnson and Davis, 1984), (3) *audiogenic susceptible mice* which have violent generalized seizures (Seyfried, 1979), (4) the *genetically epilepsy-prone rat (GEPR)* which has sound-induced tonic–clonic seizures (Consroe et al., 1979; Loscher, 1984), (5) the *Mongolian gerbil* which responds to various stimuli to have myoclonic and tonic-clonic seizures (Buckmaster, 1994), and (6) the *El mouse* which has generalized tonic-clonic seizures in response to vestibular stimulation (Seyfried, 1985). These genetic models with reflex seizures have the main limitation that human epilepsies characterized by these seizure types are very rare. Those that do occur are typically of an absence or generalized tonic-clonic type, and complex partial "reflex" seizures are very infrequent. Additionally, photic stimulation can induce seizures in non-epileptic patients suggesting that animals that have seizures in response to such stimuli

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are not necessarily models of epilepsy (Loscher and Schmidt, 1988). The main advantage of genetic models with reflex seizures for efficacy studies of AEDs is that seizures are easily reproduced by simple sensory stimulation and the seizures that occur are similar in type to those observed in human epilepsies. The genetic models with reflex seizures have not been pharmacologically characterized in most cases so their predictive value is unclear or they do not show an advantage over the traditional acute AED tests, such as MES and PTZ.

6-Hz corneal stimulation seizure model.

The 6-Hz psychomotor mouse seizure model has been proposed to be a potential model of therapy-resistant epilepsy based on the similarity of its pharmacological profile to that of human TLE. This model was abandoned shortly after its description (Brown, 1953) due to insensitivity to phenytoin, but was revisited later based on this fact to determine if the psychomotor seizures were pharmacoresistant. Unlike the short-duration, high frequency stimuli (50 Hz) used in the acute MES model, the 6-Hz model uses the alternative paradigm of long-duration, low-frequency corneal stimulation (i.e., 6 Hz, 0.2 ms rectangular pulse width, 3 s duration). The seizures evoked by this method involve stun, forelimb clonus, vibrissae twitching, and Straub-tail (Barton et al., 2001). The current required to produce a seizure in 50% and 97% of the animals is calculated and defined as the CC50 and CC97, respectively. Drug screening protocols used in the NIH-sponsored ADD program utilize the original stimulus parameters or 1.5 x CC50 (Brown et al., 1953), CC97, and 2 x CC97 (e.g., 32,

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22, and 44 mA). At a stimulus intensity of 32 mA, clonazepam, phenobarbital, trimethadione, ethosuximide, valproic acid, felbamate, tiagabine, and levetiracetam all displayed dose-dependent protection against 6-Hz seizures (i.e., highest dose reduced seizures by 100%). However, phenytoin, carbamazepine, and lamotrigine showed sub-maximal efficacy and topiramate was ineffective at blocking 6-Hz seizures. AED efficacy is dependent on the current employed to evoke seizures. At a stimulus intensity of 22 mA, lamotrigine and leviteracitam are the most effective drugs tested; and at 44 mA, only leviteracitam and valproic acid are protective but with a reduced potency than at lower stimulus intensities. Additionally, c-Fos staining shows that increasing the stimulus intensity to 44mA causes recruitment of brain regions in addition to the amygdala, neocortex, piriform, cingulate, and perirhinal cortices, specifically the dentate gyrus. An advantage of this model for AED screening is that it does not discriminate between clinical types of AEDs at the moderate stimulus intensity of CC97 and thus, it is useful as a secondary screen for identification of potential AEDs that are found inactive in the MES and s.c. PTZ tests. Compounds that are found active in the 6-Hz model are subsequently evaluated in the kindling model (Barton, 2001; White et al., 2006).

Kindling model.

The kindling model is a well-studied model of focal seizures that may hold the most predictive value as a test for potential AEDs to treat TLE and complex partial seizures. Focal stimulation of the hippocampus or amygdala (the

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amygdala is the most responsive structure) is utilized in the kindling model to elicit acute seizures and if it is a desired end-point, status epilepticus. Repeated (daily for 10-15 days) stimulation of the specific brain region initially only produces subconvulsive responses, but gradually leads to the development of focal and secondary generalized seizures. The method is based on the fact that "seizures beget seizures" or "epilepsy induces epilepsy." A fixed current is used (e.g., 400-500 μA 1 msec monophasic square-wave pulses for 1 sec with 50 or 60/sec) for stimulation or is determined based on the required current necessary to induce afterdischarges. Seizure-like activity develops in stereotypic behavioral classes as follows: (1) behavioral arrest, eye closure, vibrissae twitching, sniffing: (2) facial clonus and head bobbing; (3) unilateral forelimb clonus; (4) rearing with continued forelimb clonus (bilateral); and (5) rearing with loss of motor control and falling. For experimental purposes the behavior observed in classes 1, 2, and 3 are compared to the complex partial or limbic seizures found in human epilepsies. The latter classes (i.e., class 4 and 5) are consistent with secondary generalized seizures. Once the animals begin to have class 5 seizures, they are considered fully kindled. Continued periodic stimulation of a fully kindled rat leads to eventual spontaneous seizures (Racine, 1972). The benefit of the kindling model is that it provides the ability to test AEDs efficacy throughout the well-controlled process leading to epileptogenesis and also on the fully kindled animal with spontaneous seizures. Chronic efficacy studies can be conducted in animals that have developed spontaneous seizures (Loscher and Schwark, 1985; Loscher et al., 1993; Loscher, 2002). Additionally, acute AED tests can be

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conducted in the kindling model to differentiate between drugs that increase seizure threshold or decrease seizure spread if the kindling process is completed more rapidly (i.e., 1 day vs. 10 days of repeated stimulation) or if suprathreshold simulation parameters are used. Efficacy endpoints tested in acute tests using the kindling model include: (1) seizure latency; (2) seizure severity based on behavioral seizure classes (see above); (3) seizure duration; and (4) afterdischarge duration (Loscher et al., 1986; Loscher and Rundfeldt, 1991). Chronic AED testing is also possible in a small number of fully kindled animals because daily stimulation is possible at this stage without alteration of seizure parameters. Two main approaches using suprathreshold stimulation in fully kindled animal to test AEDS are possible. One method is to measure the effect of drug on mean latency, severity, and duration of behavioral seizures and mean duration of amygdalar afterdischarges after administration of a single dose of drug or control. This method does not allow differentiation between the affect of drug on partial versus generalized seizures. A second method considers this potential difference by calculating separate ED_{50} values for partial seizures, generalized seizures, and afterdischarges. Phenytoin, carbamazepine, valproic acid, and primidone are effective against the complex partial seizures, as is phenobarbital after prolonged administration. Ethosuximide is ineffective and benzodiazepines are only moderately effective in the amygdala-kindled model. The ED_{50} values calculated for carbamazepine, phenytoin, and phenobarbital showed that each of these drugs was more effective at reducing secondary generalized seizures than in blocking focal seizures. Advantages of this model

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are that neuronal degeneration occurs in limbic brain areas, spontaneous seizures occur after animals are fully kindled, and although these factors define kindling as a chronic model of epilepsy, acute seizures can be elicited at will via stimulation through chronically implanted electrodes (Loscher et al., 1986).

Fully kindled rats are the only established model of pharmacoresistant epilepsy. The focal and secondary generalized seizures observed in these animals are less sensitive to traditional AEDs than seizures evoked in the MES test. Following repeated testing of phenytoin on the afterdischarges of fully kindled rats, the animals can be separated into two distinct groups of phenytoin responsive, and phenytoin nonresponsive. Phenytoin nonresponders also show a reduced effect compared to the responsive group when challenged with other established AEDs (Loscher and Rundfeldt, 1991; Loscher et al., 1993).

Electrically-induced, self-sustained status epilepticus.

After an episode of sustained electrical stimulation to the hippocampus, the lateral or basolateral nucleus of the amygdala, or other excitatory limbic pathways, subsequent self-sustaining status epilepticus (SSSE) independent of the initial seizure-inducing stimulus and eventual spontaneous seizures develop in this model of chronic, acquired epilepsy. SSSE is induced in freely behaving rats at least two weeks after the surgeries are performed to implant a bipolar stimulating electrode and a recording electrode. Adult rats are stimulated for 30 minutes according to the following paradigm: 10-s trains of 1-ms square wave monophasic stimuli at 20 Hz, delivered every minute (30 trains total).

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Continuous 2 Hz stimulation (with otherwise identical parameters) is delivered simultaneously with the 10-s trains. Initial behavioral changes are in response to trains and are characterized by motor arrest and facial myoclonus. Gradually, seizure intensity increases to from forelimb clonus to loss of motor control and falling, first in response to trains and eventually independent of the stimulus trains. After perforant path stimulation is stopped, the self-sustained seizures continue and stimulation is considered successful if seizures occur for an additional 10 minutes. Generally, electrographic seizures occur for 6 to 10 hours after perforant path stimulation is stopped. Despite the severity of the SSSE, the survival rate is high (90-100%) and 80% of the animals start developing spontaneous seizures 3 weeks after SSSE. The long-term consequences of electrically-induced, SSSE include bilateral neuronal injury to the thalamus, neocortex, hippocampus and other limbic areas. Mossy fiber sprouting is evident 2 weeks after SSSE. This model can be used to evaluate drugs that may potentially terminate status epilepticus (i.e., drugs may prevent SSSE induced by 60 min perforant path stimulation in addition to 30 min of stimulation), or AEDs that may exert efficacy against the spontaneous seizures that develop during the chronic state (Lothman et al., 1989; 1990; Mazarati et al., 2006).

Chemically-induced status epilepticus and kainate-induced epilepsy.

Chemical convulsants, such as the glutamate analogue kainate or the cholinergic (muscarinic) agonist pilocarpine, are injected systemically or focally (i.e., intracerebral injections) to induce status epilepticus. A high mortality is

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associated with the systemic injection of each of these compounds at convulsant doses, and most investigators either limit the status epilepticus to 90-120 min using diazepam or pentobarbital or administer the convulsant drugs in repeated, low doses to maintain low mortality percentages (e.g., 5 mg/kg kainate every hour, Hellier et al.,1998; Hellier and Dudek, 2005). The brain damage that develops after kainate- or pilocarpine-induced status epilepticus is widespread and involves the hippocampus, piriform cortex, and amygdala. Other brain regions are also damaged that are not typically damaged in patients with TLE. The remainder of this review of chemically-induced status epilepticus models will focus on the use of systemically administered kainic acid to induce acute seizures during status epilecticus and the eventual chronic epileptic state characterized by spontaneous convulsive seizures.

In addition to the epileptic syndrome characterized by an acute limbic status epilepticus and subsequent neuronal brain damage qualitatively similar to that found in human temporal lobe epilepsy, kainate-treated rats also develop a permanent decrease in seizure threshold similar to kindled animals and chronic recurrent spontaneous seizures. Behavioral changes following systemic administration of kainic acid can be divided into several distinct stages. The animals enter behavioral arrest as early as 5 minutes after the first intraperitoneal (or subcutaneous) injection. This behavior is usually accompanied by staring and can persist for up to 1 hr. Additional kainate-induced behavioral manifestations include masticatory movements with some salivation, head nodding with myoclonic jerks, and wet dog shakes. These behaviors also persist

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for about 1 hr until the initiaton of generalized seizures. About an hour after kainate administration (or the first low-dose injection), animals begin to experience forelimb clonus; and between 90 and 120 minutes, repeated limbic seizures involving the whole body with rearing and loss of motor control. Eventually, these limbic seizures culminate in status epilepticus lasting for several hours and having histopathological consequences for susceptible brain regions. Some of the symptoms, especially during generalized seizures, are remarkably similar to those seen in kindling (Racine, 1972; Goddard et al., 1969). There is a relatively low mortality rate (15%) using repeated, low-dose IP injections of kainic acid to induce status epilepticus and 97% of kainate-treated rats develop spontaneous seizures (Hellier, 1998).

The electrographic activity of kainate-treated rats correlates closely with the behavioral changes. Epileptiform activity may arise in the entorhinal cortex, propogate to the CA3 layer of the hippocampus, and to the amygdala during wet dog shakes (Ben-Ari et al., 1981). This early epileptiform activity is then proposed to progress to other limbic structures, including the median thalamic complex, the region superior to CA1 and the medial frontal cortex before diffusely spreading to the cortical EEG. Intense recurrent seizure-like discharges typically occur for up to 70 minutes before any generalized motor seizures are detected. During severe status epilepticus, electrographic seizures are synchronized in the entorhinal cortex, amygdala, and hippocampus, generalize to other non-limbic regions and coincide with behavioral manifestations. Once acute status epilepticus has ended, rhythmic spiking decreases but can still be observed

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several days and potentially weeks later in the amygdala (Ben-Ari et al., 1981; Lothman and Collins., 1981).

The expression of late spontaneous seizures as a consequence of kainate-induced acute seizures has been attributed to hippocampal damage and synaptic plasticity (Ben-Ari and Represa, 1990). Kainate-treated rats also show a general decrease in seizure threshold which is not dependent on the motor expression of a full limbic seizure syndrome or on extended brain damage in the limbic structures. Thus, antiepileptic treatments may efficiently interrupt the kainate-induced acute seizures and neuropathological sequelae, but do not protect against the development of spontaneous seizures (i.e., epileptogenesis). Rats with kainate-induced epilepsy and spontaneous seizures, although more labor-intensive to use, share similarities with human patients with temporal lobe epilepsy. These include: 1) the presence of spontaneous seizures, 2) histopathologic changes similar to hippocampal sclerosis (Margerison and Corsellis, 1966), and 3) molecular changes that may occur as a result of time-dependent epileptogenesis. Hypothetically, chronic epilepsy models with spontaneous seizures (e.g., the kindling model, electrically and chemically-induced status epilepticus models) will more successfully predict a potential AED's clinical efficacy (Heinemann et al., 1994; Kupferberg, 2001; Leite et al., 2002; Levy et al., 2002; Loscher and Schmidt., 1988; Schmidt and Rogawski, 2002; Stables et al., 2003; White, 2003; 2004).

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ADD program AED screening process.

Since 1974, the NINDS has sponsored the Anticonvulsant Screening Project, which, since its inception, has tested over 25,000 investigational AEDs. Through a contract with the University of Utah Anticonvulsant Drug Development Program, an investigational AED is initially screened for efficacy in both MES and PTZ tests. Compounds that demonstrate anticonvulsant efficacy with minimal behavioral toxicity are characterized based on endpoint measures of time to peak effect, ED_{50}, and TD_{50}. Compounds found inactive in the initial identification tests (PTZ and MES) are screened in the 6-Hz psychomotor seizure test. The activity of those drugs that are found to be active in the 6-Hz seizure test is quantified (i.e., ED_{50} and TD_{50}) at their respective time to peak effect. All compounds that are found to be active in any of the three initial high-throughput tests are then differentiated on the basis of their activity. This is achieved using a battery of tests to characterize the drugs potential to block (1) acute clonic seizures in the subcutaneous bicuculline test ($GABA_A$ receptor antagonist) and the subcutaneous picrotoxin test (Cl^--channel blocker); (2) audiogenic seizures in the Frings audiogenic seizure-susceptible mouse; and (3) limbic seizures in the hippocampal kindled rat model of partial epilepsy. Currently, the kindled rat model offers the highest predictive value of all of the tests utilized by the program, but as a chronic model is used as a secondary screen. The screening process of the ADD program is constantly evolving to include well characterized models that may provide more clinically relevant information. Future evaluation of new AEDs for treatment of refractory epilepsy may utilize the spontaneous

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seizure models as a preclinical screen that closely mimics the human pharmacological situation (White et al., 2006).

AED testing in chronically epileptic animal models.

New AEDs that would be effective in pharmacoresistant epilepsy may be discovered by testing them in animal models with actual spontaneous *epileptic* seizures, and these new AEDs may be ineffective in the acute seizure models in otherwise normal animals. An important issue to consider while testing an AED is whether the animal model mimics the condition for which the drug is a potential therapy (i.e., clinical pathology and associated seizure type). Little research has been conducted studying the effects of AEDs on spontaneous seizures in animals with injury-induced epilepsy. If epileptogenesis involves new mechanisms not present in the normal brain (e.g., altered receptor subunits or new circuits), then traditional AED testing in acute seizure models will not identify effective versus ineffective drugs, because they are being tested on animals whose brains have not undergone epileptogenic changes. The NIH-sponsored "Models II Workshop" recommended potential AEDs be tested on animals that model the chronic, epileptic disorder (Stables et al., 2003). Although imperfect, these animals aim to "model" the condition of temporal lobe epilepsy. What is epileptogenic in these animal models and how these alterations may apply to human temporal lobe epilepsy is unknown. It has been hypothesized that the use of animals that have experienced epileptogenesis (and any changes that occur during this process) will more effectively detect new drugs (Heinemann et

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al., 1994; Kupferberg, 2001; Leite et al., 2002; Levy et al., 2002; Loscher and Schmidt., 1988; Schmidt and Rogawski, 2002; Stables et al., 2003; White, 2003; 2004). Chronic epilepsy models may be better able to predict the clinical success of experimental drugs because they produce spontaneous seizures, a chronic epileptic state, and histopathological alterations qualitatively similar to the mesial temporal sclerosis observed in human temporal lobe epilepsy (Margerison and Corsellis, 1966). The following chapters provide evidence that chronic epilepsy models with spontaneous seizures, such as the kainate-induced epilepsy model, can be used to test the efficacy of AEDs.

Epileptogenic mechanisms triggered by kainate-induced status epilepticus

Many different hypothetical mechanisms of epileptogenesis involving molecular, cellular, and integrative levels have evolved from electrophysiologic experiments on slices from rats with kainate-induced epilepsy. Potential mechanisms of epileptogenesis include: (1) decreases in synaptic inhibition, (2) increases in excitatory synaptic mechanisms including new excitatory circuits, and (3) alterations of transmitter receptors and ion channels in compromised brain structures. These experiments and the development of these hypotheses are possible because of the existence of an experimental model that mimics hypothesized components of the human epilepsy (i.e., rats with kainate-induced epilepsy).

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Susceptible brain regions and resulting lesions.

Systemic injection of kainic acid may directly reach susceptible brain areas and cause direct axon-sparing lesions. Small amounts of the toxin are capable of causing damage in vulnerable regions like the hippocampus, but diffusion is likely a mechanism causing distant secondary damage. Very similar amounts of damage are caused despite whether kainate is delivered via systemic or intrahippocampal injection (Sperk, 1994). The most vulnerable areas to the neurodegenerative actions of kainate are the CA3 pyramidal layer neurons of the hippocampus, interneurons of dentate hilus, and CA1 pyramidal cells. The same areas are most severely affected by chronic kainate-induced seizures. This is a difference from typical hippocampal sclerosis in humans, where CA1 is usually more damaged than CA3 (Ben-Ari, 1985; Tauck and Nadler, 1985). This may be explained by the high number of kainate receptors present on these structures (Berger and (Ben-Ari, 1983). Kainate receptors located presynaptically on mossy fibers may indirectly contribute to the neurotoxicity of kainate by releasing glutamate (Repressa et al., 1987). The sustained stimulation of excitatory pathways in the brain may also lead to neurodegeneration after kainate treatment. Neuronal stimulation during seizures may be largely mediated by excitatory amino acid mechanisms (Ben-Ari, 1981; Schwob et al., 1980; Meldrum, 1991). Thus, seizure-induced lesions depend on presynaptic innervation from glutamatergic and cholinergic afferents (Kohler et al., 1979; Nadler, 1981; Okasaki and Nadler, 1988). Nonselective neuronal lesions, which are unrelated to specific pathways or regions and are not restricted to neurons

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are also observed following kainate treatment. These include massive edema, hemorrhages, and nonspecific progressive necrosis of the piriform cortex and amygdala (Sperk et al., 1983; Sperk et al., 1992).

Neuronal cell death and reactive gliosis

Hallmarks of human mesial temporal sclerosis include neuronal degeneration and gliosis in temperolimbic structures. Kainate injections and seizure activity progressively lead to loss of neurons in the hilus, CA3, and CA1 layers of the hippocampus and associated gliosis that is qualitatively similar to tissue from patients with TLE (Ben-Ari and Lagowska, 1978; Nitecka et al., 1984; Tremblay et al., 1984; Ben-Ari, 1985; Nadler et al., 1978; Nadler, 1981). Neurons of the dentate gyrus and CA2 are relatively spared (as seen in humans). Granule cell dispersion has been observed in the intrahippocampal-kainate injection model, which is also a characteristic of human mesial temporal sclerosis (Bouilleret et al., 1999; Gouder et al., 2003; Weiser et al., 2004). After systemic kainate injections, neuronal damage can occur in many areas in addition to the hippocampus and amygdala. Although the kainate model replicates characteristics of mesial temporal lobe sclerosis, this alone does not make it a valid model of chronic epilepsy or an appropriate model for AED testing.

Mossy-fiber sprouting

A potential basis for chronic epileptogenesis is the time-dependent changes that occur following kainate-treatment including the formation of new

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synaptic circuits, and the alteration of neurotransmitter receptors and ion channels. Hippocampal tissue removed from epileptic animals with spontaneous seizures show dentate granule cells form new axon collaterals in the inner molecular layer as evidenced by intracellular Timm's staining (Nadler et al., 1980; Buckmaster, 2004; Dudek and Shao, 2004; Dudek et al., 2002; Nadler, 2003, Buckmaster and Dudek, 1997a, 1997b, 1999). This mossy-fiber sprouting has also been observed in tissue from patients with intractable TLE (Babb et al., 1991; de Lanerlle et al., 1989; Houser et al., 1990; Sutula et al., 1989). Additional studies provide evidence that similar mechanisms occur throughout the temporal pole and in other structures, namely in CA1 pyramidal cells (Esclapez et al., 1999; Meier and Dudek, 1996; Perez et al., 1996; Shao and Dudek, 2004; Smith and Dudek, 2001).

Metabolic changes induced by kainate injection.

Measured using 2-deoxyglucose radiography, changes in metabolic activity (i.e., time-course and regional distribution of local glucose consumption) during and after kainate treatment can be correlated with seizure activity and subsequent neuronal damage. CA3 pyramidal cells demonstrate the most sensitivity to kainate based on 2-deoxyglucose accumulation. In response to an intraperitoneal kainate injection, animals had mild limbic seizures and a four-fold increase in 2-deoxyglucose was observed in ventral hippocampus. Increasing doses of kainate, showed metabolic activity in CA1, the subiculum, dentate gyrus, lateral septum, the amygdala, the entorhinal and piriform cortices,

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thalamic nuclei, and substantia nigra (Lothman and Collins, 1981). Metabolic activity spreads in stages. Accumulation of 2-deoxyglucose was restricted to the hippocampal formation and the septum prior to motor seizures, but during limbic convulsions, activity spread to other limbic areas and eventually extralimbic areas. After several days, metabolic activity decreased in most brain areas. In parallel with EEG observations, levels of 2-deoxyglucose remained high in the amygdala and the amygdalo-hippocampal transitional areas suggesting ongoing neuronal activity in these regions Ben-Ari et al., 1981).

MRI Imaging has demonstrated time-dependent lesion progression in the hippocampus , amygdala, and piriform cortex following kainate-treatment in both short- and long-term studies (Lester et al., 1999; Pirttila et al., 2001). In vivo magnetic resonance spectroscopic imaging (MRSI) with N-acetylaspartate was used monitor neuronal loss for 3 days following kainate-induced status epilepticus (Ebisu et al., 1994; Hasegawa et al., 2003). Neuronal changes have been followed for up to 4.5 months after status-epilepticus using multi-parametric MRI (Nairismagi, 2004).

Genetic and molecular changes.

The variety of changes that occur relative to time and brain region during epileptogenesis suggests a high degree of complexity regarding kainate-induced changes in gene expression. The most-studied genes affected by seizures are the immediate-early genes and the growth factors. Immediate-early genes are induced by seizures in several different animal models of epilepsy (Morgan and

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Curran, 1991). Kainate-induced status epilepticus has been shown to induce: *c-fos, fos* B, *jun* B, *egr-1, fra-2* and *c-jun*; *fra-2* and *c-jun* may be more specific to damaged areas (Goodenough et al., 1997). Status epilepticus increases mRNA levels for brain-derived growth factor (BDNF) and nerve growth factor (NGF), but a decrease or no change in mRNA levels of neurotrophin-3 is observed during epileptogenesis (Gall, 1993). These changes seem to be consistent in other seizure models in addition to the kainate model of chronic epilepsy.

Potential mechanisms of pharmacoresistance

Despite the development of ten new AEDs in the past decade, pharmacoresistance remains an issue for the treatment of TLE. Over 30-40% of patients with epilepsy are considered intractable, and only 10-15% of these patients responded to any of the newly released AEDs (Perucca, 2002). Pharmacoresistance remains poorly understood, but some hypotheses of potential mechanisms of drug resistance include (1) time-dependent, seizure-induced alterations in receptors and/or ion channels, (2) overexpression of multi-drug resistant transporters (e.g., p-glycoprotein [Pgp], MDR1); (3) genetic predisposition towards pharmacoresistance (i.e., refractory from day one); and (4) development of AED tolerance with long-term administration (Schmidt and Loscher, 2005; Elger, 2003).

Disease-related mechanisms of pharmacoresistance may be a consequence or the etiology of epileptogenesis, disease progression despite treatment, structural brain alterations and/or network changes, and alterations in

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drug targets. An example of one of these potential changes is the observed alteration of sodium channels in both epileptic animals and epileptic patients. The interaction between sodium currents and voltage- and ligand–gated ion channels are critical to neuronal excitation and epileptogenicity. The density, distribution, and the molecular structure and function of the ion channels are altered during epileptogenesis (Remy et al., 2003). Voltage-gated sodium channels show a reduced effect from carbamazepine and phenytoin treatment compared with the drugs' effect in normal controls. In patients with drug resistant epilepsy, carbamazepine had no effect on the sodium channels or on the high frequency discharges of the channel. Conversely, in drug-responsive patients, the high-frequency responses were deactivated.

Drug efflux transporters that are expressed at the blood-brain barrier function to protect cells from endogenous and exogenous toxins, but also limit the ability of many drugs to access the brain. Multidrug transporters (e.g., Pgp, MRP1, and MRP2) and their genes are overexpressed in epileptogenic brain tissue from patients with medically intractable epilepsy (Tishler et al., 1995; Sisodiya et al., 2002) and chronically epileptic animals (Potschka et al., 2004; Volk et al, 2004). A number of commonly used AEDs are substrates for the multidrug transporters found in the blood brain barrier. Hypothetically, the overexpression of Pgp or the MRPs during epileptogenesis may reduce the amount of drug that reaches the epileptic neurons and contributes to multidrug resistance in epilepsy.

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Genome variability between epilepsy patients may explain why two patients with the same disease differ in their initial response to drug treatment. Gene polymorphisms can contribute to alterations in drug metabolism, drug targets, or drug transporters. Sequence variants (e.g., polymorphisms) in drug transporter genes have been discovered that can affect the expression and function of the corresponding proteins (Ferraro and Buono, 2005). For example, low biotransformation capacity due to metabolic enzyme polymorphisms may result in inadequate drug levels for AEDS that are metabolically activated (Eadie, 1991). Polymorphisms of sodium channel α subunit genes (e.g., SCN1A, SCN2A), which are targets for AED binding, have been associated with the use of maximal doses of carbamazepine and phenytoin (Tate et al., 2005). The polymorphism, C3435T, found on exon 25 of the gene encoding ABCB1 (MDR1) is associated with increased expression of the protein and drug resistance (Siddiqui et al., 2003).

Drug-related mechanisms based on pharmacokinetics and pharmacodynamics may play a role in drug resistance in epilepsy. An acquired loss of therapeutic efficacy (i.e., functional tolerance); induction of drug-metabolized enzymes (i.e., metabolic tolerance) or drug transporters; and ineffective mechanisms of action can all contribute to pharmacoresistance to AEDs (Loscher and Schmidt, 2006). Pharmacokinetic (metabolic) tolerance occurs due to the induction of AED-metabolizing enzymes, and has been demonstrated for most traditional AEDs. Physicians simply increase the dosage administered to overcome pharmacokinetic tolerance. Pharmacodynamic or

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functional tolerance is due to adaptation of AED targets. For example, AEDs lose activity during prolonged treatment periods due to loss of receptor sensitivity. Functional tolerance occurs for both traditional and new-generation AEDs, but the extent of tolerance depends on the drug and individual differences between patients. Cross-tolerance can also develop such that repeated use of AEDs in a given category may confer tolerance to themselves, but also to other drugs in the same category that may be attempted as therapeutics later in the same patient.

General mechanisms of available antiepileptic drugs

Several cellular and synaptic mechanisms contribute to the initiation of seizures, synchronization of surrounding neurons, and propagation of the seizure discharge to surrounding brain areas. The basic causes of epileptic seizure genesis and spread are (1) abnormal voltage-gated ion channels; (2) abnormal receptor-operated ion channels (i.e., increase in excitation or decrease in inhibition); (3) alterations in extracellular ionic environment; and (4) recruitment of normal neurons via aberrant anatomical circuits. Each of these mechanisms can be disrupted by drugs, and it is convenient to categorize AED actions according to those that involve (1) modulation of voltage-gated ion channels; (2) augmentation of synaptic inhibition; or (3) attenuation of synaptic excitation.

Voltage-gated ion channels (including sodium, calcium, and potassium channels) are integral to action potential generation and the regulation of a

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neurons responsiveness to synaptic signals. Additionally, they contribute to paroxysmal depolarization shifts and ultimately, seizure generation. Voltage-gated ion channels also play a crucial role in neurotransmitter release, which is required for synaptic transmission. For these reasons, conventional AEDs generally inhibit voltage-gated ion channels. For example, use-dependent sodium channel blockers (e.g., carbamazepine, phenytoin, and lamotrigine) reduce repetitive firing of neurons by prolonging the inactivated state of the sodium channel and thus the relative refractory period (Rogawski and Loscher, 2004; Brody et al., 1998; Levy et al., 2002).

Neurotransmitter-regulated channels mediate synaptic inhibition and excitation. These channels permit neuronal synchronization and propagation of abnormal discharges to local and distal sites. Benzodiazepines (e.g., diazepam, clonazepam, and lorazepam) modulate the GABA receptor-channel complex increasing the frequency of chloride-channel opening when GABA combines with its receptor. Barbituates with seizure-suppressing action such as phenobarbital interact with the GABA receptor at a different binding site adjacent to the chloride channel and prolong the opening of chloride channels achieved by a given amount of GABA (i.e., GABA-potentiating). In addition, blockade of glutamate receptors (including NMDA, AMPA, kainate, and group I metabotropic mGluR1 and mGluR5 types) can interfere with seizure activity by reducing excitation, specifically high-frequency discharges that may play a role in seizure onset. Hypothetically, a drug that blocks NMDA receptor activity reducing abnormal excitatory neurotransmission could reduce seizure genesis, slow neuronal

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synchronization, and slow seizure spread. Although NMDA receptor antagonists reduce seizures in animal models of seizures, side effects have limited their use in humans (Rogawski and Loscher, 2004; Brody et al., 1998; Levy et al., 2002).

Ion channels and glutamate receptors are the primary targets of currently available AEDs to exert their antiepileptic action either individually or via multiple mechanisms in combination (i.e., broad-spectrum drugs such as topiramate). Seizure genesis and spread is a multifactorial process and targeting one contributing mechanism has failed to control seizures in a high proportion of patients with temporal lobe epilepsy (Rogawski and Loscher, 2004). In addition to developing new potential AEDs with multiple mechanisms of action and testing novel neuromodulators (e.g., RWJ-333369), a goal should be to develop drugs that are more effective in the *abnormal* brain and potentially target the *epileptic* glutamate receptor or ion channel which requires screening new drugs in epileptic animals with spontaneous seizures.

Pharmacokinetics

Each AED has a different pharmacokinetic profile and this must be taken into consideration during experimental design. Ideally, the metabolism of traditional AEDs in the chronically epileptic animals will be similar to that seen in humans. During preclinical development of potential AEDs, the pharmacokinetics of the drugs may be unknown. However, the AEDs ultimate use will be to treat a chronic condition so they must be absorbed orally and cross the blood-brain barrier. All AEDs are metabolized to a certain extent, and all bind plasma

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proteins, many exert significant plasma protein binding. The half-life of AEDs can vary dramatically based on the patient's age, drug in question, and exposure to other drugs. Some AEDs demonstrate linear kinetics or a direct relationship between dose and serum concentration of the drug, while others have nonlinear kinetics in which there is a much greater rise in serum concentration for a given increase in dose (especially at high doses). Nonlinear relationships are due to the liver enzymes that catalyze the drug becoming saturated when serum concentrations rise above a certain value (Brody et al., 1998).

AED metabolism occurs in two phases: (1) reactions involving the cytochrome P450 (CYP) family of mixed functional oxidases and (2) conjugation reactions that increase hydrophilicity and facilitate renal excretion. Four P450 family members are involved in AED metabolism: CYP1, CYP2, CYP3, and CYP4. CYP enzymes incorporate one atom of molecular oxygen into the substrate and the other atom into water. A functional group is added during phase 1 reactions, and is subsequently used as a site of conjugation in phase 2 reactions. Acetyl and sulfate moieties, glucuronic acid, glycine, and glutathione are common molecules conjugated to AEDs during this step (Ferraro and Buono, 2005).

Conventional AEDs have pharmacokinetic-related limitations, including bioequivalence problems (e.g., phenytoin, carbamazepine), linear kinetics (e.g., phenytoin), time-dependent kinetics (e.g., carbamazepine), and short half-life (e.g., carbamazepine and valproic acid). The metabolism of some AEDs is susceptible to enzyme induction or inhibition. In addition, some AEDs are

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themselves enzyme inducers or inhibitors (e.g., phenobarbital, phenytoin, valproic acid, [Bialer, 2005]). Carbamazepine, a traditional AED, is metabolized in the liver to produce its major metabolite 10,11-epoxide, which is relatively stable and accumulates in the blood. This metabolite has been shown to have antiepileptic effects and may contribute to the toxicity attributed to carbamazepine use. Carbamazepine induces its own metabolism, and higher doses are required over time to maintain constant serum concentrations and continuous antiepileptic effects (Brody et al., 1998).

New-generation AEDs offer certain pharmacokinetic advantages in terms of linear and less variable kinetics, complete oral absorption, and lower interaction potential. The pharmacokinetics of new-generation AEDs differs for each drug. Topiramate is eliminated unchanged in the urine, and does not appear to be involved in drug interactions due to enzyme inhibition. However, during polytherapy, topiramate is involved in drug interactions due to enzyme induction by traditional AEDs (e.g., phenobarbital, phenytoin, and carbamazepine). When these AEDs are administered with topiramate, they induce the cytochrome P450-mediated metabolism of topiramate, and increase both total clearance and the fraction of the drug that is metabolized. These traditional AEDs (e.g., Phenobarbital, phenytoin, and carbamazepine) are also enzyme inducers of felbamate, lamotrigine, oxcarbazepine, tiagabine and zonisamide. Conversely, gabapentin, pregabalin, vigabatrin, and leviteracetam have little or no interaction potential. (Bialer et al., 2004; Bialer, 2005)

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Clinical significance of proposed research.

Experiments in this thesis address the pharmacosensitivity of animals that have undergone time-dependent epileptogenesis following an episode of status epilepticus. This type of injury in humans leads to TLE that is commonly refractory to AEDs. Pharmacoresistance can be defined as the persistence of seizures with all possible therapies at maximally tolerated doses. This suggests that the concept of pharmacoresistance must take into account issues such as AED dose, seizure frequency and severity, increased probability of seizure clusters, and behavioral side effects associated with AED treatment. Relatively little work using animal models has been done to study pharmacoresistance and pharmacosensitivity, but such studies would provide unique insights, yet they would need to involve a careful neurophysiological analysis of time- and dose-dependent AED effects.

Uncontrolled seizures are associated with a wide range of pathological consequences. Calcium entry into cells and changes in protein synthesis due to prolonged excitation triggers a cascade of events that causes neuronal damage. Damage potentially contributes to the progression of temporal lobe epilepsy and the occurrence of secondary foci. Refractory epilepsies can result in unexpected death and are associated with secondary symptoms such as central and obstructive apnea, cardiac arrhythmias, and autonomic and hormonal changes. The idea that seizure-induced neuronal damage contributes to further seizure activity and physiologic compromise suggests that it is necessary to understand

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the mechanisms underlying pharmacoresistance and epileptogenesis and to develop therapies to impede each process (Engel, 1989).

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CHAPTER 2- General Methods

Kainate treatment and status epilepticus insult.

Kainic acid was administered intraperitoneally (IP) in repeated, low doses (5 mg/kg) every hour until each rat experiences convulsive status epilepticus for >3 h. Injection concentrations were calculated based on animals weights (180-200 g), and a concentration of 2.5 mg/ml kainic acid (in 0.9% NaCl), administered in a volume of 0.2 ml per 100 g of rat. For control rats, normal saline was administered in the same volume as the kainate solution (0.2 ml/100 g rat). Each injection time and the dose administered was recorded. The times of motor seizures were recorded and scored on a scale from III to V using a modified Racine scale (Racine, 1972). A class III seizure included an erect tail, lordotic posturing, and forelimb clonus; a class IV seizure included the above mentioned behaviors, rearing on hind limbs, and continued forelimb clonus; and finally, a class V seizure was characterized by the behaviors ascribed to a class IV followed by a fall. Every hour the severity and behavior of the rats were used to determine whether the dose of kainate administered during the next round of injections would be given as (a) a full dose (5 mg/kg) or titrated to (b) a half dose (2.5 mg/kg) or (c) omitted (no injection). If a rat exhibited inactivity, hyperactivity, or had ≥10 seizures per hour the injection was skipped until the next hour's

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assessment. After rats had been in status epilepticus for >3 h, injections were stopped, and (3 ml/ 100 g, subcutaneous) warmed lactated Ringers solution (or normal saline) and fresh fruit was administered to the rats. This method decreased the rate of mortality during kainate-induced status epilepticus.

Surgical implantation of electrodes.

The head of the rat was placed in a stereotaxic apparatus and the rat was anesthetized with 2% isoflurane and pretreated with 0.8 ml atropine (0.54 mg/kg), 0.2 ml dexamethasone (2 mg/kg) and 0.2 ml penicillin (300,000 IU, IP). Prior to making the incision, 0.5 ml of 0.5% bupivicaine was injected at the incision site. The surgical site was clipped and prepped with betadine scrub and solution. A midsagittal incision was made along the scalp. The skin was retracted using hemostats, and the bone and dura over the hippocampus was removed as necessary to implant stimulating and recording electrodes. Nine holes were drilled in the skull to implant electrodes (recording, stimulating, and ground) using Bregma as a reference point. Support screws were placed into the skull above the frontal lobe and occipital lobe. The EEG recording electrode was positioned on the dura of the left hemisphere and ground electrodes were placed on both hemispheres (for exact coordinates see chapter 6). To activate the perforant path afferents to the dentate gyrus of the hippocampus, an indifferent electrode was placed on the dura of the left hemisphere. The depth-recording electrode was lowered 2500 μm below the level of the dura to the CA1 pyramidal cells. An audio monitor was used to localize the cell layer, and the electrode was lowered

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another 500 μm to position it in the granule cell layer of the dentate gyrus. A monopolar, Teflon-insulated stainless-steel stimulating electrode was lowered 2300-3500 μm into the right entorhinal cortex or the angular bundle projecting to the hippocampus. The exact location of the electrode with respect to the dentate granule cells was determined using electrophysiological criteria (e.g., positive extracellular synaptic potentials and negative population spikes). Once the appropriate electrophysiological responses were located, dentate acrylic was used to fasten the electrodes in place and to cover and seal all exposed skull surfaces. The wire leads were gathered into a plastic connector that was cemented to the skull of the rat. If necessary, a stitch was applied to improve the interface between the skin and the electrode implant. Topical antibiotic was applied to the wound edges. No further experimentation was undertaken until the animals regained body weight (at least 3 days). Subcutaneous buprenorphrine at 0.01 mg/kg will be administered daily for 72 h or as needed following surgery.

Repeated-measures, cross-over protocol for AED testing.

Each AED trial involved approximately six treatments of AED and six vehicle treatments administered on alternated days (e.g., Sun., Tues., Thurs., Sat.) with a recovery day between the treatment days to control for possible persistent effects of high doses (chapters 3,4,5, and 6 contain detailed explanations of individual trials). IP injections of the assigned treatment (AED or vehicle) were administered at 9:00 AM on treatment days to rats with kainate-

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induced epilepsy. Analysis of video recordings (8:00 AM to 4:00 PM) began 1 h after the injections were conducted to control for possible effects that animal handling and injections may have had on seizure activity. Each AED trial lasted at least 4 weeks to complete 6 AED-versus-vehicle tests for each rat. Solutions for IP injections of 30 mg/kg of topiramate were prepared based on the animals' weight, a 1 ml per 500 g rat injection ratio, and injection concentrations of 15 mg/ml topiramate (in 0.9% NaCl, pH 8). For lower doses, the concentration was simply titrated. Topiramate (100 mg/kg) was prepared at a concentration of 25 mg/kg and injected at a volume of 2 ml/ 500 g rat. The injections of 30 mg/kg RWJ-333369 were based on a concentration of 15 mg/ml RWJ-333369 (in 10% Solutol-HS-15), administered in a volume of 1 ml per 500 g of rat and titrated for lower doses. Carbamazepine ([CBZ],30 mg/kg) was dissolved at 15 mg/kg CBZ (in 20% (2-Hydroxypropyl)-β-cyclodextrin), administered at 1 ml/ 500 g rat. Lower doses were titrated from this base concentration and administered in the same volume, but 100 mg/kg CBZ was prepared at a concentration of 25 mg/ml CBZ (in 20% (2-Hydroxypropyl)-β-cyclodextrin), and injected at 2 ml/500 g rat. All of the above solutions were heated on low (40-60°C) in a warm water bath while stirring vigorously.

Drug-in-food method of AED administration.

Using the same repeated-measures, cross-over protocol, animals were fasted overnight (i.e., 5:00 PM to 9:00 AM) on recovery days and AED in food was administered at 9:00 AM on treatment days. CBZ was formulated into dose-

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specific 1-g pellets by Bioserve (i.e., each 1-g pellet contained 5 mg CBZ. The mean daily food intake for epileptic rats was about 60 g/kg/day. Therefore, for a 100 mg/kg dose of CBZ administered as 5 mg CBZ/1-g pellet is approximately 10 pellets for a 500 g rat (or 0.02 pellets per X g rat). Rats were provided with supplemental food at 5:00 PM after it was clear they had consumed all the drug-in-food treatment or control pellets. The repeated-measures, cross-over protocol was modified to evaluate periodic administration of AED in food administered three times per day for 5 days. For this experiment, the three drug administrations of 100 mg/kg CBZ (i.e., 300 mg/kg CBZ) were equal to the daily caloric needs of the animals. Therefore, the animals were not food deprived or provided supplemental food (see chapter 3 for additional details).

Continuous video-monitoring of spontaneous seizures.

Each rat was video-monitored for the entirety of the protocol on 8-h videotapes by a Panasonic WV-BP334 black and white camera, either in conjunction with EEG-monitoring or for continuous detection of motor seizures during 24-h periods. While the animals were in the recording chamber, their behavior was video-monitored to associate motor behavior with electrographic seizure activity. A modified Racine scale was implemented as in the kainate treatment to score seizure severity (Racine, 1972). A trained technician blinded to the treatments and dates corresponding to the recordings viewed the animals' behavior. The videotapes were observed in the fast-forward mode for any activity suggestive of a seizure (running, jumping, rearing, lordosis, erect tail,

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etc.). If any seizure-like activity was seen, the tape was stopped, rewound, and watched in real time to evaluate any possible seizures (as defined above).

Blood Analyses

Blood concentrations were analyzed from single injections of vehicle, 3, 10, and 30 mg/kg RWJ-333369. RWJ-333369 blood-concentrations of each treatment were analyzed in the same set of 8 vehicle-control rats with an appropriate recovery time for drug clearance and re-accumulation of blood volume. The same experiments were repeated in 8 rats with kainate-induced epilepsy. Blood samples were collected 4 hours after vehicle, 3 mg/kg, and 10 mg/kg injections in 8 kainate-treated animals and 8 age-matched controls. Two time points were be analyzed: 4 h and 24 h after 30 mg/kg injections.

Blood samples from 8 animals (2 groups of 4) were obtained at intervals of 24 hours or more after a single injection. For example, following a single injection of 30 mg/kg RWJ-333369, the 4-h blood samples could safely be removed. However, a second injection of drug was administered after an appropriate recovery period (e.g. 48 hours) to obtain the 24-h blood sample. The second group of 4 animals received injections and blood samples were collected during the recovery days of the first group. These precautions were taken to assure that time points could be accurately managed and a safe percent of total blood volume was removed at any one time. In separator tubes with lithium heparin, 0.4 ml whole blood was collected via tail vein. The tubes were inverted several times and iced immediately. From these samples, 200 µl plasma (exact)

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was collected into Eppendorf tubes after centrifuging at 3000 rpm at 4°C for 5 min. The plasma samples were stored at -80°C until shipped on dry ice for mass spectral analysis. Acetonitrile containing 1μM internal standard was added to the sample for mass spectral analysis (this procedure was conducted by technicians at Johnson and Johnson).

Chronic in vivo recordings and data analysis of electrophysiology.

Chronic field-potental recordings were conducted during vehicle and CBZ treatments. Rats were monitored both behaviorally and electrographically daily for 8-h periods. Seizure quantification was conducted based on seizure number, duration, and type (i.e., convulsive and nonconvulsive [Racine, 1972]). The duration of the interictal interval was measured to determine if the AED reduced the occurrence of seizure clusters. The EEG data was amplified (100x), digitized (Neuro-Corder, Neuro Data Instruments), and stored on videotapes for off-line analysis. Field-potential recordings were digitized at 2 kHz and analyzed using commercially available software (MP150, Biopac Systems, Inc., Goleta, CA).

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CHAPTER 3- Use of chronic epilepsy modes in antiepileptic drug discovery: The effects of topiramate on spontaneous motor seizures in rats with kainate-induced epilepsy[1]

Relative contributions

Heidi Grabenstatter was responsible for conducting all experiments and data analysis described in this chapter. Dr. Phil Williams and D.J. Ferraro trained her in the techniques used to complete these experiments. Dr. Phil Chapman served as a statistical consultant and assisted in the development of the repeated-measures ANOVA used to determine any potential significant differences in seizure frequency between vehicle and AED treatments. The concepts included within evolved from discussions with Dr. F. Edward Dudek, who offered suggestions and criticisms on early drafts. Dr. H.S. White, the director of the Anticonvulsant Drug Discovery (ADD) program at the University of Utah, generously offered suggestions and comments on the final draft of this chapter.

Introduction

Traditional antiepileptic drug (AED) testing has utilized acute-seizure models based on chemical and electrical induction of seizures in otherwise

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normal animals. Efficacious AEDs may be ineffective in these models (Stables et al., 2002). Hypothetically, new AEDs that would be effective in pharmacoresistant epilepsy may be discovered by testing them in animal models with epileptic seizures, and these new AEDs may be ineffective in the acute-seizure models (Stables et al., 2002; Loscher and Honack, 1993; White, 2003). Relatively little research has been conducted studying the effects of AEDs on spontaneous seizures in animals with injury-induced epilepsy. If epileptogenesis involves new mechanisms not present in the normal brain (e.g., altered receptor subunits or new circuits), then traditional AED testing in acute-seizure models may not identify effective versus ineffective drugs, because they are being tested on animals whose brains have not undergone the epileptogenic changes.

The NIH sponsored "Models II Workshop" recommended potential AEDs be tested on animals with chronic epilepsy (Stables et al., 2003). Although imperfect, these animals aim to "model" the condition of temporal lobe epilepsy. What is epileptogenic in these animal models and how these alterations may apply to human temporal lobe epilepsy is unknown. It has been hypothesized that the use of animals that have experienced epileptogenesis (and any changes that occur during this process) will more effectively detect new drugs (White, 2003; Kupferberg, 2001; Leite, et al., 2002; Loscher, 2002; Schmidt and Rogawski, 2002). Chronic epilepsy models may be better able to predict the clinical success of experimental drugs because they produce spontaneous seizures, a chronic epileptic state, and histopathological alterations qualitatively similar to the mesial temporal sclerosis observed in human temporal lobe

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epilepsy (Margerison et al., 1966). Our laboratory has recently generated evidence that chronic epilepsy models with spontaneous seizures, such as the kainate- and pilocarpine-induced epilepsy models, can be used to test the efficacy of AEDs (Hernandez et al., 2002). The present study in chronically epileptic rats attempted to develop an improved paradigm for testing AEDs that would not only provide dose-effect data, but would also allow time-course-of-recovery analyses.

Topiramate is a broad spectrum AED with multiple proposed uses and mechanisms of drug action (White, 1997; Shank et al., 1994; Shank et al., 2000; Bourgeois, 2000; Garnett, 2000; Maryanoff et al., 1998). Possible mechanisms include antagonism of AMPA/kainate-type glutamate receptor-mediated inward currents (Schmidt and Rogawski, 2002; Gibbs et al., 2000), attenuation of voltage-dependent Na^+ channels (Taverna et al., 1999), negative modulation of L-type Ca^{2+} channels (Zhang et al., 2000), augmentation of $GABA_A$ receptor-mediated Cl^- currents (White et al., 1997), activation of K^+ conductance (Herrrero et al., 2002), and inhibition of carbonic anhydrase (Dodgson et al., 2000). The diverse mechanisms of drug action exhibited by topiramate allow for a variety of clinical uses of the drug. In these studies, we conducted a proof-of-principle experiment to determine if a repeated-measures, cross-over protocol could be used to perform both dose-effect and time-course-of-recovery analyses for intraperitoneal injections of topiramate.

The experimental design in our previous study in rats with pilocarpine-induced epilepsy proved useful for comparing the effects of different AEDs

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(Hernandez et al., 2002). The important conceptual and practical problem in this previous study, however, was that the three drugs - TFMPP, phenobarbital, and fluoxetine - each affected the frequency of spontaneous seizures for substantially different periods at the doses tested (i.e., TFMPP for about 6 hours, phenobarbital for slightly less than a day, and fluoxetine for substantially more than a day). In the present study, we have designed a new protocol that would also enable us to evaluate the duration of the anticonvulsant effect. We used a different but similar chemoconvulsant model of chronic epilepsy (i.e., the kainate-treated rat). As before, a repeated-measures, cross-over protocol would allow each animal to be used as its own control, in spite of differences in baseline seizure frequency. In the present study, *single* AED injections were alternated with *single* vehicle-control injections, compared to the previous study where the animals received each treatment for five consecutive days (Hernandez et al., 2002). In addition to being able to determine the dose-effect relations, we were also able to estimate recovery from the drug effect.

Methods

Kainate treatment and status epilepticus.

Adult Sprague-Dawley rats (150-200g) were given intraperitoneal injections of kainate to induce status epilepticus, a model of injury-induced epilepsy. Kainate was administered in repeated, low doses (5 mg/kg) every hour until each rat (n= 8 rats per treatment series, 8 separate kainate treatments)

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experienced convulsive status epilepticus for >3 h. Motor seizures were scored from III to V using the Racine scale (Racine, 1972). A class III seizure was defined to include an erect tail, lordotic posturing, and forelimb clonus; a class IV seizure was defined by the above mentioned behaviors, rearing on hind limbs, and continued forelimb clonus; and a class V seizure was characterized by the behaviors ascribed to a class IV seizure followed by a fall.

Each rat was injected subcutaneously with ~2.5 ml lactated Ringers and received fresh fruit following the kainate-induced status epilepticus. The rats were housed in the vivarium with a standard 12-h light/dark cycle and provided food and water ad libitum for 5-6 months during direct monitoring of spontaneous seizures. The temperature of the vivarium was kept between 17° and 20°C, with a humidity of 44–78%. A trained technician monitored epileptic animals for intervals of 1-2 h (during a period when lights were on) for a total of 6 h per week before the rats were selected for the actual study on the effects of topiramate. The Racine scale was used to score spontaneous seizures during direct observation in the same manner as it was previously during the kainate treatment. Rats with spontaneous, recurrent seizures were selected for drug trials. Once selected, animals to be included in the trials were video-monitored for 24 h per day prior to initiation of the testing protocol to eliminate rats that had infrequent spontaneous motor seizures.

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Topiramate treatment and cross-over protocol.

Six 1-month trials were conducted using a cross-over protocol (Fig. 3.1) to assess the effect of 0.3, 1, 3, 10, 30 and 100 mg/kg topiramate (n= 6-10 rats per trial). A total of 22 animals were used in all experiments, and some rats were tested in more than one trial. The 3, 10, and 100 mg/kg trials included 8 rats, 6 of which were ovariectomized females and 2 additional males. All other trials were comprised solely of male Sprague-Dawley rats. The mean duration of epileptogenesis (i.e., time from kainate treatment to initiation of protocol) for rats involved in each trial was 199 ± 19, 198 ± 15, 359 ± 11, 305 ± 11, 199 ± 4, and 331 ± 11 days for the 0.3, 1, 3, 10, 30, and 100 mg/kg trials respectively.

Each trial involved six treatments of topiramate and six saline treatments administered on alternate days (e.g., Sun., Tues., Thurs., Sat.) with a recovery day between the treatment days to allow for possible persistent effects of high doses. Topiramate or saline was administered via intraperitoneal injection at 9:00 a.m. on treatment days. Analysis of seizure frequencies for the first 6-h epoch (10:00 a.m. to 4:00p.m.) began 1 h after the injections were conducted to allow for possible effects that animal handling and injection may have on seizure frequency. To prepare topiramate for injections, appropriate quantities (depending on dose) were mixed with 0.9% saline (pH=8) and stirred vigorously in a warm water bath (40-60°C) until dissolved. Both drug treatments and saline-control (0.9% saline, pH=8) treatments were administered to rats based on the weight of the animal.

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Continuous video-monitoring of spontaneous seizures.

Each rat was video-monitored continuously for the entire protocol on 8-h videotapes by a Panasonic WV-BP334 black-and-white camera. The Racine

THREE-TRIAL AED PROTOCOL

RAT #	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY	SUNDAY
1				CONT			
2	B		R	CONT	R		R
3	A		E	CONT	E		E
4	S		C	CONT	C		C
5	E		O	CONT	O		O
6	L	CONT	V		V	CONT	V
7	I	CONT	E		E	CONT	E
8	N	CONT	R		R	CONT	R
9	E	CONT	Y		Y	CONT	Y
10		CONT				CONT	

RAT #	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY	SUNDAY
1	CONT				CONT		
2	CONT	R		R	CONT	R	
3	CONT	E		E	CONT	E	
4	CONT	C		C	CONT	C	
5	CONT	O		O	CONT	O	
6		V	CONT	V		V	
7		E	CONT	E		E	
8		R	CONT	R		R	
9		Y	CONT	Y		Y	
10			CONT				

Fig. 3.1. Experimental Protocol. Topiramate or saline was administered every other day for 12 days (e.g., Tuesday, Thursday, Saturday, Monday, Wednesday, and Friday); each kainate-treated rat served as its own control. The protocol shown above was doubled in length to include six topiramate vs. vehicle-control tests. Video recordings were made for 7 days per week, 24 h per day for the duration of the protocol (25 days) for each dose. The tapes that contained the actual injection began 1 h before and ended 7 h after the injection (i.e., tape was started at 8:00 a.m., injection was at 9:00 a.m.)

scale (Racine, 1972) was implemented, as before, to score seizure severity. A trained technician blinded to the treatments and dates viewed the animals'

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behavior. The videotapes were observed in the fast-forward mode for any activity suggestive of a seizure (running, jumping, rearing, lordosis, erect tail, etc.). If any seizure-like activity was seen, the tape was stopped, rewound, and watched in real time to evaluate any possible seizures (defined as above). All seizures meeting the criteria of a class III seizure (Racine, 1972) or greater were counted. The continuous video-monitoring did not detect sub-convulsive seizures.

Seizure clusters.

The occurrence of clusters of seizures during either saline or AED treatments would be expected to result in heterogeneous variance and an asymmetric distribution of the data. Others have defined normal seizure distributions using the Poisson model (Balish et al., 1991; Tauboll et al., 1991), which describes the distribution of "random" events. By this definition, deviations from the Poisson model are representative of nonrandom seizure aggregations or seizure clusters. Seizures occasionally appeared to occur in clusters (Fig. 3.2), where an increased seizure frequency was observed within a specific time period (i.e., 6-18 seizures per 6-h epoch). Average interictal intervals within apparent clusters of seizures, regardless of dose or time, ranged between 9 min and 44 min. Seizure clusters were seen to varying degrees at several doses of topiramate and during saline treatments.

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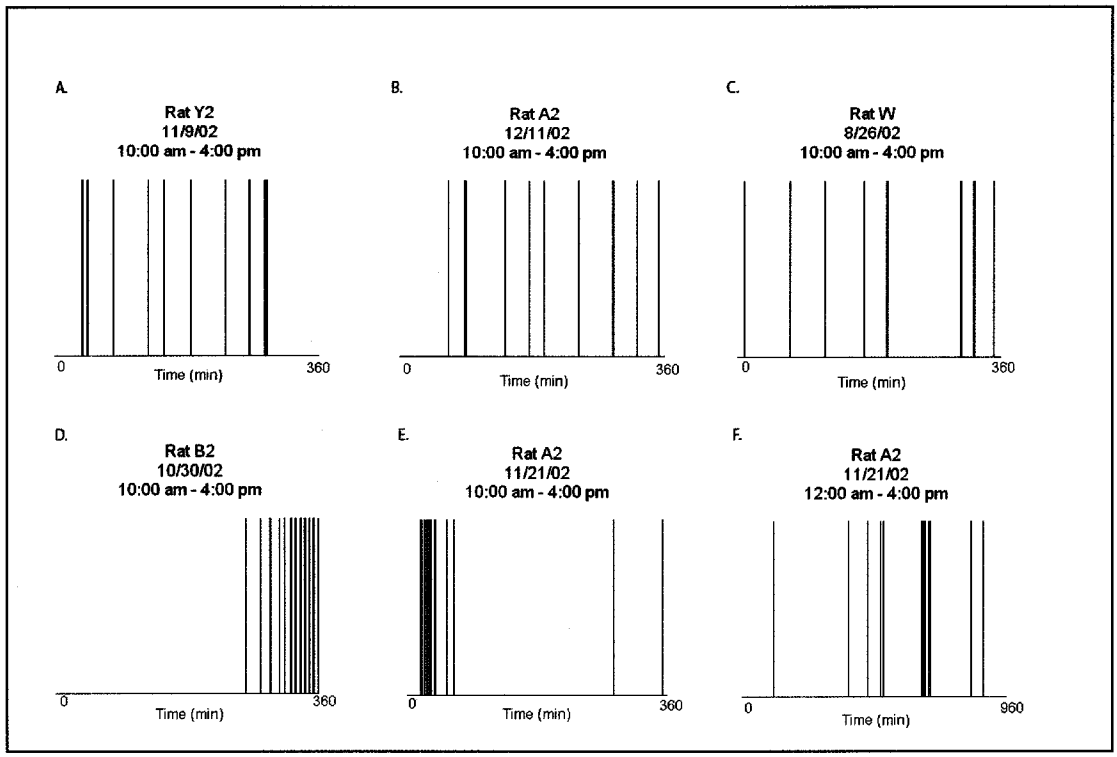

Fig. 3.2. Seizure clusters. A-F are raster plots depicting the time of occurrence of seizures. A-C show that some seizures did not occur in clusters, even during high seizure frequency. D and E show high seizure frequency with short interictal intervals suggesting nonrandom seizure clusters, which supported the hypothesis that these seizures occurred in clusters. F is a 16-h extension of the 6-h figure shown in E.

The log transformation and justifications for its use.

Log transformations are commonly used in the analysis of seizure frequency to compensate for the asymmetric distribution of data and heterogeneous variance between groups, which presumably occurs because of seizure clustering (French, 2001). The data to be analyzed should meet a set of criteria for the use of a log transformation (Keene, 1995). These criteria include: the largest value of a set must be more that three times larger than the smallest

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value, and data are bound below by zero. A log transformation leads to a more symmetric distribution of the data where the variances are more homogenous between groups.

The observed seizure clusters within the data and the criteria described above suggested the data did not have a normal distribution. A plot of residuals versus predicted values from the original scale indicated that the variance increased with the mean; therefore, the data were analyzed in the $\log_{10}$ (y+0.1) scale. The relationship between mean and variance (i.e., larger mean implies larger variance) identified in the original data permits the log transformation, and the transformed data more closely satisfies the assumption of homogeneity of variances. Following log transformation, seizure frequencies for the topiramate and saline treatments of individual AED tests were compared using a repeated measures analysis of variance that included fixed effects for treatment, time, and the treatment by time interaction, as well as random effects for rats, rat by treatment, rat by time, and rat by treatment by time interactions. The analysis in SAS Proc Mixed (SAS Institute, 1999) utilized the restricted maximum likelihood estimation method, which pools random effects having negative variance estimates with the higher-order random interactions. Denominator degrees of freedom for F-tests of effects and individual comparisons were estimated using the Satterthwaite method. A repeated-measures analysis controlled for the effects of time within the protocol, since the kainate-induced epilepsy tends to become more severe over time (Hellier et al., 1998). This approach essentially compared each seizure frequency for a particular AED treatment to the seizure

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frequency of a saline treatment directly before or after the AED treatment in question. Estimated relative seizure frequencies (i.e., a ratio of seizure frequency following AED injection per seizure frequency following vehicle injection) in the log scale were back-transformed following analysis to the original linear scale for presentation of results.

Results

The time course of recovery from 30-mg/kg injections of topiramate.

The first issue addressed in these experiments was to determine the duration of the effect of a 30-mg/kg injection of topiramate on relative seizure frequency (Fig. 3.3). When each 6-h epoch was analyzed directly, a significant effect of topiramate on seizure occurrence ($p<0.0001$ and $p=0.03$) was observed for the first two 6-h epochs (i.e., 12 h after the 30-mg/kg injection). The single injections of 30 mg/kg during the first 6-h epoch (Fig. 3A) reduced spontaneous motor seizures by approximately one half, producing a relative seizure frequency of 0.51 (±0.20). In the second 6-h epoch (Fig. 3A), seizure frequency was still reduced, with a relative seizure frequency of 0.68 (±0.24). No significant differences between topiramate and saline treatments were found at any of the later time intervals. A linear regression analysis was performed to generate a "best-fit" line through the means of the relative seizure frequency of all epochs (Fig. 3.3B). Using these data, I determined the time point (i.e., x-values) at which the effect of topiramate was completely recovered based upon where the

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"best-fit" line intersected with a y-value equivalent to1.0. These data suggest that the topiramate injections did not have a persistent effect that interfered with the next sequential saline test.

Topiramate reduced seizure activity in a dose-dependent manner.

The effect of different doses of topiramate relative to saline treatment was compared utilizing a log transformation of the seizure frequency and a repeated-measures ANOVA analysis. Six doses were tested, and they ranged from 0.3 mg/kg to 100 mg/kg. Topiramate exerted a significant effect relative to saline at the doses of 10 ($p=0.02$), 30 ($p<0.0001$), and 100 mg/kg ($P<0.0001$). A dose of 30 mg/kg reduced spontaneous seizure frequency by approximately one half, while a dose of 100 mg/kg caused a slightly greater reduction in seizure frequency of 0.44 (±0.15). Therefore, the effects of topiramate (0.3-100 mg/kg) were found to be dose-dependent (Fig. 3.4).

An important end-point of AED analysis in addition to seizure frequency is seizure severity. We addressed the question of whether the different doses of topiramate reduced seizure severity.

An analysis of the sum of seizure severity scores (i.e., intensity-weighted seizure number) was conducted using the same method as described above, but the data were analyzed in the $\log_{10}(y+1)$ scale. This analysis was performed on the seizure severity score (Racine, 1972) for every seizure occurring in the first 6-h epoch (0.3-100 mg/kg). The doses of 10 mg/kg ($p=0.0024$), 30 mg/kg ($p=0.0022$) and 100 mg/kg ($p=0.0053$) significantly reduced relative intensity-

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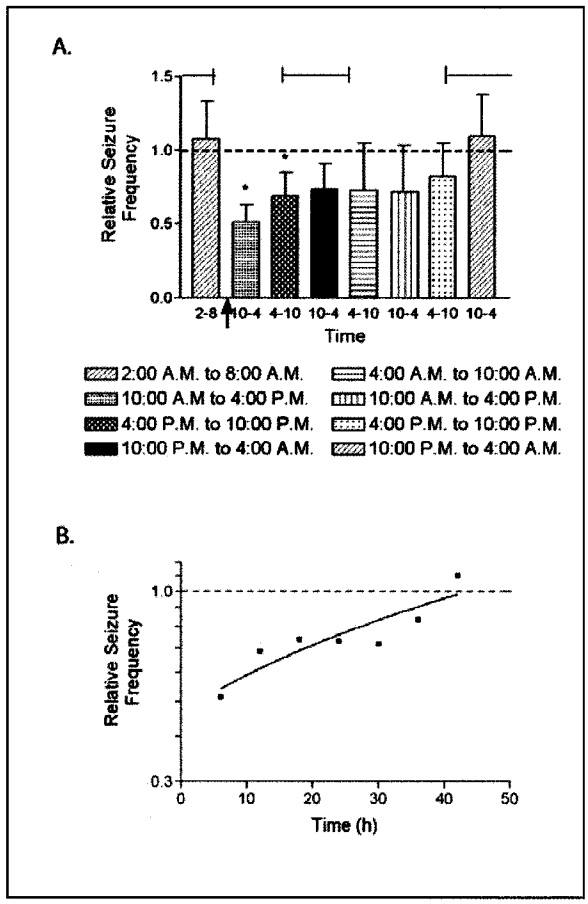

Fig. 3.3. Time course of recovery from 30 mg/kg injections of topiramate.
Topiramate was significantly different ($p<0.05$) from saline for the first and second 6-h epoch, 10:00 a.m. to 4:00 p.m. ($p<0.0001$) and 4:00 p.m. to 10 p.m. ($p=0.0322$). In this and the subsequent figure, the dashed line shows the baseline (i.e., no effect). The bracketed bars represent the periods of darkness (6:00 p.m. to 6:00 a.m.). Arrow indicates time of drug/saline administration (9:00 a.m., every other day). Vertical bars, ±SEM. As shown with diagonal lines, the first epoch (2:00 a.m. to 8:00 a.m.) shares data with the last epoch (10:00 p.m. to 4:00 a.m.). B. A semi-log plot of the best-fit line through the mean values of the relative seizure frequency for the 6-hr epochs (described above). The equation of the line is $y= 0.01224x + 0.4673$. Thus, extrapolation of the line suggests that complete recovery from the effects of topiramate occurred after about 43 h. Ticks represent intervals of 0.1 on the y-axis. In this figure and in Figure 4, an asterisk (*) indicates significant effects.

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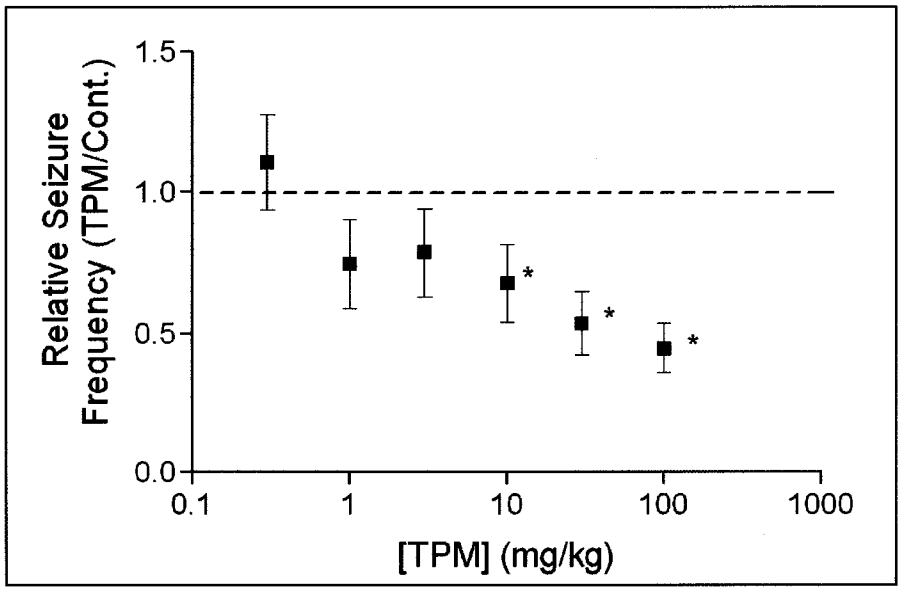

Fig. 3.4. Dose-dependent effect of topiramate. A repeated-measures ANOVA was conducted to calculate significant differences ($p<0.05$) between topiramate and saline treatments. To compensate for clustering of seizures, a log transformation was performed. Topiramate had significant effects relative to saline at the concentrations of 10 ($p=0.02$), 30 ($p<0.0001$), and 100 ($p<0.0001$) mg/kg.

weighted seizure number (i.e., the ratio of the sum of seizure severity scores after AED injection relative to the sum of seizure severity scores after vehicle injection) during the first 6-h epoch. In addition, the single injections of 30 mg/kg topiramate significantly ($p=0.0005$) suppressed relative intensity-weighted seizure number by 0.53 (±0.18) over a 24-h period. Therefore, topiramate at 30 mg/kg caused a long-lasting reduction in seizure severity, and seizure intensity was reduced in a dose-dependent manner (0.3-100 mg/kg). Another analysis involved the ranking of intensity-weighted seizure numbers and a repeated-

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measures ANOVA. This type of analysis is normally performed on median values, but a repeated-measures, cross-over paradigm does not provide median values. However, the analysis yielded similar p-values and statistical significance was not altered with this strategy, which was used to account for heterogeneous variance and asymmetric distribution.

Discussion

Main findings of combined analyses.

In the recovery-from-treatment analysis, the effect of 30 mg/kg topiramate lasted 12-43 h (see below), and therefore had recovered prior to the next saline injection. When evaluated during the first 6-h epoch following intraperitoneal injection for the concentrations of 0.3, 1, 3, 10, and 100 kg, topiramate showed a dose-dependent effect. Topiramate was found to cause a significant reduction in seizure frequency at 10 ($p=0.02$), 30 ($p<0.0001$), and 100 mg/kg ($P<0.0001$). Seizure severity was also significantly reduced at doses of 10mg/kg ($p=0.0024$), 30 mg/kg ($p=0.0022$), and 100 mg/kg ($p=0.0053$).

The time course of recovery.

Considerable translational research activity is being focused on pharmacoresistance and epileptogenesis (1,4). If one intends to use animal models with spontaneous recurrent seizures (e.g., kainate- and pilocarpine-treated rodents) to study pharmacoresistance and to test drugs for efficacy

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against epileptogenesis, one must first know both the dose-effect relations and the time course of drug action for seizure suppression. Topiramate was expected to have a significant effect during the first 6-h epoch, with a gradual decline thereafter. In rats and mice, the anticonvulsant activity peaks within 1 to 6 h after oral administration (Shank et al., 2000). The 30 mg/kg dose had a significant effect during the first 6-h epoch, and significantly reduced seizure frequency for a second 6-h epoch (Fig. 3.3A; i.e., the drug effect was significant for at least 12 h). One concern was a persistent drug effect after the topiramate injection that would last beyond the time of the subsequent control injection, which would be a potential confound in the protocol (i.e., the effect of topiramate would still be present at the time of the saline injection). Analysis of the individual 6-h epochs during the subsequent recovery day revealed no significant difference between topiramate and saline treatments. An analysis of the relationship between time after injection of topiramate and the relative seizure frequency ($\log_{10}$ axis of Fig. 3.3B) suggested that full recovery occurred about 43 h after the topiramate treatment. Therefore, the 30 mg/kg injection of topiramate reduced the frequency of spontaneous seizures in rats with kainate-induced epilepsy for at least 12 h, and the effects could have persisted for up to 43 h.

The repeated-measures, cross-over protocol.

A cross-over protocol (where each animal serves as its own control) was used here because it accounts for differences between animals (i.e., variability in baseline seizure frequency) and the likelihood that seizures become more

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frequent over time (Hellier et al., 1998). We typically used six cross-over tests, but the protocol can be extended by using additional drug versus control tests to increase statistical power, while allowing a relatively small number of animals to be tested (as few as 6 animals were used for some doses). The experimental design allowed us to evaluate both the dose dependence of the drug effect and the time course of recovery from a single injection. The current protocol incorporated a recovery day to eliminate any possible additive effects of topiramate, and it provided pharmacokinetic information in vivo that could not be addressed in the previous protocol (Hernandez et al., 2002). However, the potential for progressive drug accumulation is an important therapeutic consideration. One potential problem with the present protocol is that it may incorrectly show a drug to be ineffective (i.e., false negative) if the drug requires long-term accumulation. The protocol used here would need to be modified to study drugs that require accumulation over multiple days. Therefore, the protocol used in the present experiments was more useful than the one employed previously (Hernandez et al., 2002) for studies of the time course of recovery from AED treatment, but not necessarily for rapid dose-effect analyses.

Pharmacoresistance.

Further experiments are needed to evaluate more rigorously whether this type of protocol will be useful for identifying drugs that are more efficacious for patients with pharmacoresistant epilepsy. The doses of 30 mg/kg and 100 mg/kg, which would be considered high doses from previous studies (Amano et

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al., 1998; Wauquier and Zhou, 1996), only decreased the relative seizure frequency to 0.44 (±0.15) and did not block all seizures. Thus, topiramate appeared to have similar effects in these experiments as on human intractable temporal lobe epilepsy. This suggests that rats with kainate-induced epilepsy are pharmacoresistant to topiramate, but other studies with prolonged treatments are needed to further test this hypothesis. In the present study, each drug-versus-control test essentially evaluated the effect of a single injection of topiramate relative to a single injection of vehicle. To test the hypothesis of pharmacoresistance, a similar repeated-measures design where the animal receives the drug for several days in each repeat (e.g., 3 days of twice daily injections followed by 3 days of recovery as a single AED versus control test) would be more appropriate. Even if motor seizures were completely blocked by higher doses of topiramate with this protocol, another important issue would be whether electrographic nonconvulsive seizures (i.e., equivalent to complex partial seizures) still persisted. Future experiments addressing pharmacoresistance also should include blood-concentration levels throughout the duration of an AED testing protocol. Differences in metabolism of AEDs between animals with kainate-induced epilepsy and age-matched control animals should be explored. Another factor possibly contributing to a reduced effect of AEDs in controlling the spontaneous seizures of rats with kainate-induced epilepsy is age-related metabolic changes, including renal insufficiency in aged rats (i.e., chronic progressive glomerulonephropathy (Couser and Stilmant, 1975). Thus, further studies are necessary to address the question of pharmacoresistance, but this

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approach should prove useful in further tests of potential AEDs. A new AED that is more effective in this protocol than topiramate (and other presently available AEDs) may be more therapeutic in humans with intractable epilepsy.

Endnote

[1]This chapter was previously published as an article in *Epilepsia* (see citation below).
Grabenstatter H.L., Ferraro D.J., Williams P.A., Chapman P.L., and Dudek F.E. (2005) Use of chronic epilepsy models in antiepileptic drug discovery: the effect of topiramate on spontaneous motor seizures in rats with kainate-induced epilepsy. *Epilepsia* 46, 8-14.

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CHAPTER 4- Anticonvulsant effects of carbamazepine on spontaneous seizures in rats with kainate-induced epilepsy: comparison of intraperitoneal injections with drug-in-food protocols

Relative contributions

Heidi Grabenstatter was responsible for formulation and design of all the experiments and all data analysis described in this chapter. She participated in data collection for the long-term AED trial evaluating the effect of CBZ administered in food three times a day for five days. The single administration trials were conducted by Dr. Suzanne Clark and Katie Kerr. Jodi Schilz and Dr. Suzanne Clark also assisted during the prolonged administration protocol. The concepts included within evolved from discussions with Dr. F. Edward Dudek, who offered suggestions and criticisms on early drafts, and the final draft was reviewed and edited by Dr. F. Edward Dudek.

Introduction

Over 30% of patients with temporal lobe epilepsy have seizures that are refractory to commonly used AEDs (Bauer and Burr, 2001). Animal models with recurrent spontaneous seizures may be useful for understanding the physiological alterations contributing to the development of intractable epilepsy and pharmacoresistance. The use of animal models with spontaneous seizures

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to define new drug targets and to determine efficacy of novel AEDs may yield more effective treatments (Stables et al., 2002), because these animals have undergone time-dependent changes that occur during epileptogenesis (e.g., alterations in receptors, ion channels, and/or synaptic circuits). Likewise, testing drugs in these models that may potentially prevent or inhibit the development of spontaneous recurrent seizures (i.e., antiepileptogenic drugs) may also produce treatments that are disease-modifying or that change the course of epileptogenesis.

Studying AEDs and antiepileptogenic drugs in chronically epileptic animals requires long-term, continuous administration. These drugs may have short half lives, thus leading to difficulties maintaining a prolonged effect. Ideally, the methods should model those used in humans, and should provide sustained, therapeutic blood levels. The effect of single IP injections and single oral feedings of 30 and 100 mg/kg CBZ, a traditionally effective but difficult-to-administer drug, were evaluated. CBZ could be administered orally in food (i.e., 100 mg/kg feedings three times per day) for several days, and anticonvulsant effects were maintained for extended periods.

Methods

Kainate treatment and rat selection criteria

In a vivarium (temperature 20-25°C, humidity 30-60%) on a 12-h light/dark cycle with lights on at 6 AM, the animals had free access to food and water before they were entered into the study. Repeated IP doses of 5 mg/kg kainic

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acid (Ocean Produce International, Shelburne, Nova Scotia, Canada) were administered hourly to male Sprague-Dawley rats (Harlan, 200 g) to induce status epilepticus (Hellier et al., 1998; Hellier and Dudek, 2005). After onset of status epilepticus, the kainate doses were titrated, based on the severity of the seizures. If the status epilepticus was mild (few class IV or V seizures), then a reduced dose of kainate (2.5 mg/kg) was given. If a rat had many class V seizures, or had been in status epilepticus for 3 h, no further doses were administered. After kainate treatment, rats were given warmed normal saline (3 ml/100 g, subcutaneous) and fresh fruit to facilitate recovery. After a few days of recovery, the rats were directly observed for 6 h/week to determine the frequency and severity of spontaneous convulsive seizures (Racine, 1972). Rats that were entered into the study had relatively regular seizures. The mean pre-study seizure rate using continuous 24-h video monitoring was 9.56 ± 1.67 seizures per day, with a range of 4.0 to 13.5 seizures per day.

Dose-specific formulated food with CBZ

CBZ was formulated into food pellets (BioServe, Chocolate Mini-Treats, Catalog # F05472, Frenchtown, NJ) at 5-mg CBZ per 1-g pellet. Control pellets were the same, but had no drug. The animals were familiarized to the drug-free food pellets before they were entered into the test-phase of a study. Rats were weighed every 3 days, and the amount of food was adjusted until the rats no longer lost weight, but also ate all of the pellets on most days. The optimal amount of food to maintain the rats' weight, but to assure that all food was eaten,

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was 60 g food/kg/day. Food intake was monitored by counting (or weighing) the remaining, uneaten pellets each day. When 100 mg/kg CBZ was administered three times per day (i.e., 300 mg/kg/day), the rats left an average of 9.95 ± 2.47 g of uneaten food. The average weight of uneaten food for individual animals varied between 0 g and 20.73 ± 4.25 g.

Repeated-measures, cross-over protocol

Four studies were conducted to assess the effects of IP-injected and orally administered CBZ (30 and 100 mg/kg), and to determine the maximally effective dose and duration of effect of that dose. Each trial involved at least three AED-versus-vehicle tests (six AED-versus-vehicle tests were completed in the IP trials) in which CBZ or vehicle were administered to chronically epileptic rats (n=8-10) every other day at 9:00 AM with a recovery day between treatment days to allow for drug clearance between treatments (Fig. 4.1). This paradigm controls for inter-animal variability in baseline seizure frequencies and time-dependent increases in seizure frequency, which is common in post-status epilepticus models (Grabenstatter et al., 2005; Hellier et al., 1998). The doses were chosen based on previous reports of oral CBZ efficacy, safety, and pharmacokinetics, which used (1) three daily treatments of 250 mg/kg CBZ as an aqueous gavage to protect against hexafluorodiethyl ether-induced seizures in rats (i.e., 750 mg/kg/day; Carl et al., 1989), and (2) 100 mg/kg CBZ oral suspension four times per day to examine the circadian effect on AED kinetics in the rat (i.e., 400 mg/kg/day; Bruguerolle et al., 1981). The results of Honack et

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RAT #	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY	SUNDAY
1	B	CBZ	R	CONTROL	R	CBZ	R
2	A	CBZ	E	CONTROL	E	CBZ	E
3	S	CBZ	C	CONTROL	C	CBZ	C
4	E	CBZ	O	CONTROL	O	CBZ	O
5	L	CONTROL	V	CBZ	V	CONTROL	V
6	I	CONTROL	E	CBZ	E	CONTROL	E
7	N	CONTROL	R	CBZ	R	CONTROL	R
8	E	CONTROL	Y	CBZ	Y	CONTROL	Y

RAT #	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY	SUNDAY
1	CONTROL	R	CBZ	R	CONTROL	R	R
2	CONTROL	E	CBZ	E	CONTROL	E	E
3	CONTROL	C	CBZ	C	CONTROL	C	C
4	CONTROL	O	CBZ	O	CONTROL	O	O
5	CBZ	V	CONTROL	V	CBZ	V	V
6	CBZ	E	CONTROL	E	CBZ	E	E
7	CBZ	R	CONTROL	R	CBZ	R	R
8	CBZ	Y	CONTROL	Y	CBZ	Y	Y

Fig. 4.1. The effect of CBZ on rats with kainate-induced epilepsy was studied with a repeated-measures, cross-over protocol. Chronically epileptic animals received IP and oral doses of CBZ in food or control at 9:00 AM on alternate days. Three AED-versus-vehicle tests (i.e., 3 cross-overs) are shown.

al.,1989 on the pharmacokinetics and efficacy of three daily systemic injections with 30 mg/kg CBZ (i.e., 90 mg/kg/day) also influenced the choice of dose and treatment schedule (i.e., 100 mg/kg three times per day or 300 mg/kg/day).

At 5:00 PM the night before an oral test dose, all food was removed from the cages, but water was freely available. Rats were fasted overnight (from 5:00 PM to 9:00 AM) to ensure they would eat all of their food within a short time-period (preferable, ~1 h) after the CBZ pellets or control pellets were administered in the morning. At approximately 9:00 AM, the rats were given all of the pellets for the day's single dose (i.e., formulated drug-containing pellets or control pellets). The pellets were placed on the floor of the cage in the clean litter. Rats had access to the pellets for 8 h, and at 5:00 PM, any remaining food pellets were counted and the rats were given ad-lib access to regular rat chow.

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During the two trials testing single administration of oral CBZ, the rats lost an average of 0.03 ± 0.60% body weight (range of 2.06 ± 1.86% gain and 3.44 ± 6.45% loss) with 30-mg/kg CBZ treatment and 3.77 ± 0.63% body weight (range of 1.02 ± 2.69% and 7.07 ±9.81%) after 100-mg/kg CBZ administration.

Three-times-per-day administrations of CBZ for 5 days

The repeated-measures, cross-over protocol was modified for chronic drug administration. A 2-day recovery period was used to ensure that no persistent drug effects occurred at the initiation of the control crossover period. This study was similar to the single-feeding experiment, except that the rats received all of their food as 1-g pellets (either with or without 5 mg/kg CBZ), and their total caloric intake was 60 g/kg/day, divided into three 20-g/kg feedings. Thus, the number of pellets administered to a rat in the test arm of the study was equal to the number of pellets given in the control period. Each morning, the animals received a clean cage, and the 9 AM dose of pellets was administered. During this phase of the study, the rats were weighed every other day; the animals lost an average of 0.23 ± 0.18% of their body weight, and changes in body weight ranged between a 0.96 ± 1.12% loss and a 0.63 ± 0.84% gain.

Video monitoring

Rats were video-monitored 24 h/day during the baseline and test periods. Three 8-h videotapes were recorded each day. A Panasonic WV-BP334 black-and-white camera (G&G Technologies, Secaucus, NJ, USA) was linked to three

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video recorders to record sequential 8-h time-periods. For recording during the "dark" phase of the day, a Kodak 1A filter over a safelight was used to illuminate the cages. An observer blinded to treatment scored the seizures (Racine, 1972).

Statistical analyses

Spontaneous seizure frequencies for rats with kainate-induced epilepsy showed heterogeneous variance between groups and were asymmetrically distributed (i.e., non-Gaussian) following both control and CBZ treatments. Therefore, seizure frequencies were log-transformed [i.e., analyzed on a $\log_{10}(y+0.1)$ scale] using a parametric test, a repeated-measures ANOVA (Grabenstatter et al., 2005). Seizure frequencies during the first 6-h epoch after single IP injections or single feedings of CBZ were compared for each AED treatment and subsequent control treatment for all AED-versus-vehicle tests. The necessary addition of 0.1 to all seizure frequencies to allow the log transformation of a data set that was bounded by zero at the lower end caused a "floor effect" that masked highly effective seizure reduction (i.e., many animals showed complete cessation of motor seizures). Therefore, the linear data (i.e., sum of seizures following every individual treatment per number of treatments) for all control and AED treatments were also compared using a non-parametric analysis, a Wilcoxon signed-rank test. Potential significant differences between different administration routes were tested using an unpaired t-test with a Welch correction.

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Results

IP versus administration in food

Single daily feedings of CBZ at 30 mg/kg and 100 mg/kg reduced seizures for a 6-h epoch when the peak drug effect would be expected to occur (Fig. 4.2). The effects of administration of 30 and 100 mg/kg CBZ in food on relative seizure frequency were similar to single IP injections. CBZ completely suppressed all seizures during the 6-h epoch in a similar percentage of animals with IP and oral administration methods. Thus, administration of CBZ in food was as effective as IP injections.

The analysis illustrated above, and our previous work on topiramate (Grabenstatter et al., 2005), used a log transformation [i.e., $\log_{10}(y+0.1)$] because the raw data were not normally distributed (i.e., not a Gaussian distribution). Parametric tests, such as the repeated-measures ANOVA, assume the data set is described by a Gaussian, bell-shaped distribution; however, the raw data on seizure occurrence during the 6-h epochs were not normally distributed primarily because of the presence of seizure clusters. Therefore, it was necessary to transform the data to allow the use of parametric statistical tests. An alternative approach that does not assume that the data have a Gaussian distribution involves non-parametric tests (e.g., Wilcoxon signed-rank test). A "floor effect" (or minimum obtainable relative seizure frequency) caused by analysis of data on the $\log_{10}(y + 0.1)$ scale is clear when parametric and non-parametric analyses of IP and oral administration of CBZ are compared (see Fig. 4.2B versus 4.2C). Nearly all seizures were blocked 6 h after 100 mg/kg CBZ given orally (i.e., 89%

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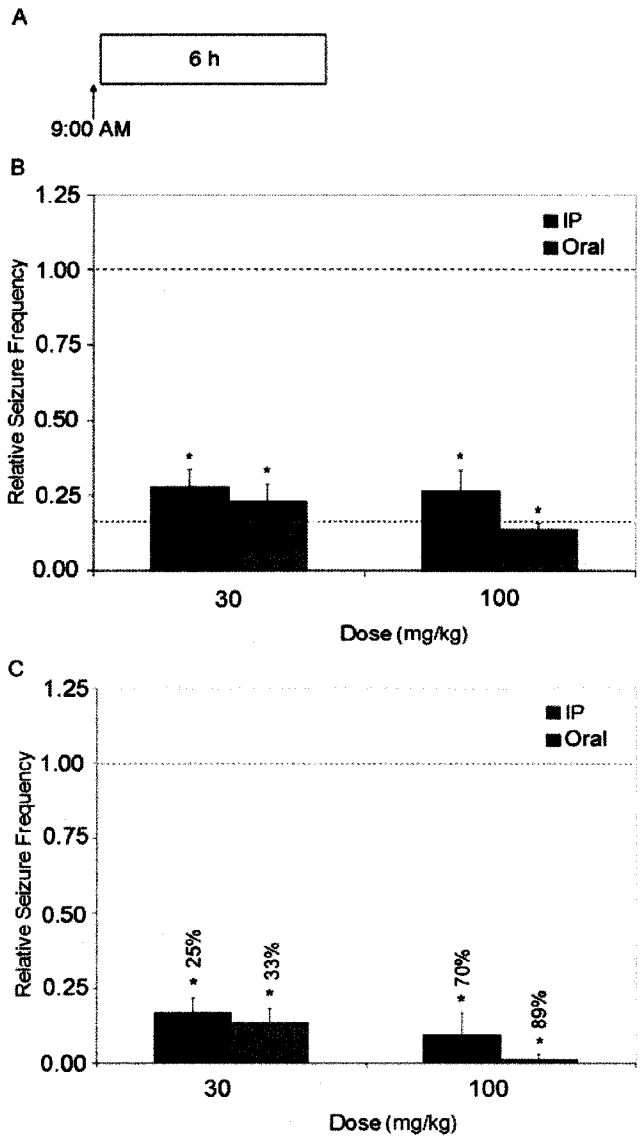

Fig. 4.2. Oral administration of CBZ was as effective as IP injections. **(A)** The effect of the two administration routes was compared for the 6-h epoch after the 9:00 AM IP injection or feeding. **(B)** Single IP injections and oral feedings of CBZ were compared to control using a parametric test (i.e., a repeated-measures ANOVA) to analyze log-transformed data. **(C)** These data were also compared with a non-parametric test (i.e., Wilcoxon signed-rank test). The percentage of animals with complete seizure cessation is shown above the bars. Upper dashed horizontal line in B and C shows baseline (i.e., no effect); lower dashed line in B represents estimated mean "floor effect" of log transformation when seizures were completely blocked. *Vertical bars*, ±SEM. Asterisks represent significant differences

complete seizure cessation), but this was only apparent when linear data were analyzed using a non-parametric statistical analysis (i.e., Wilcoxon signed-rank test).

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Duration of anticonvulsant effect

One objective of the single-feeding experiment was to determine a maximum interval at which to administer doses in a long-term, periodic-treatment protocol. An analysis of the 48 h of data after the oral CBZ treatments, binned into 4-h epochs, showed that single doses of 100 mg/kg CBZ in food pellets significantly reduced relative seizure frequency for the first five 4-h epochs (i.e., 20 h after the 9:00 AM feeding, Fig. 4.3). Thus, CBZ at 100 mg/kg had a long-lasting effect when administered in food. However, relative seizure frequency was significantly increased above control levels in the seventh epoch (i.e., 24 h to 28 h after CBZ feedings).

The data were reanalyzed without the log transformation (i.e., on the linear scale) using a non-parametric analysis (i.e., the Wilcoxon signed-rank analysis) comparing seizures per AED test to seizures per control test (Fig. 4.3). The single feeding of 100 mg/kg CBZ showed significant anticonvulsant effects for 16 h (i.e., four 4-h epochs) using this linear, non-parametric analysis. Three epochs (i.e., 20-24 h, 24-28 h, and 32-36 h after CBZ feedings) showed increased seizure frequency compared to control levels. Extrapolation of "best-fit" sigmoidal curves through linear and log-transformed data supported the interpretation that relative seizure frequency returned to control levels 20-23 h after the 9:00 AM feeding of 100 mg/kg CBZ (Fig. 4.3). Sigmoidal curves closely fit both the linear and log-transformed time-course-of-recovery data, but log transformation of the data to account for cluster-induced variance led to a right-shifted time-course of recovery. Thus, it is possible to analyze the time course of

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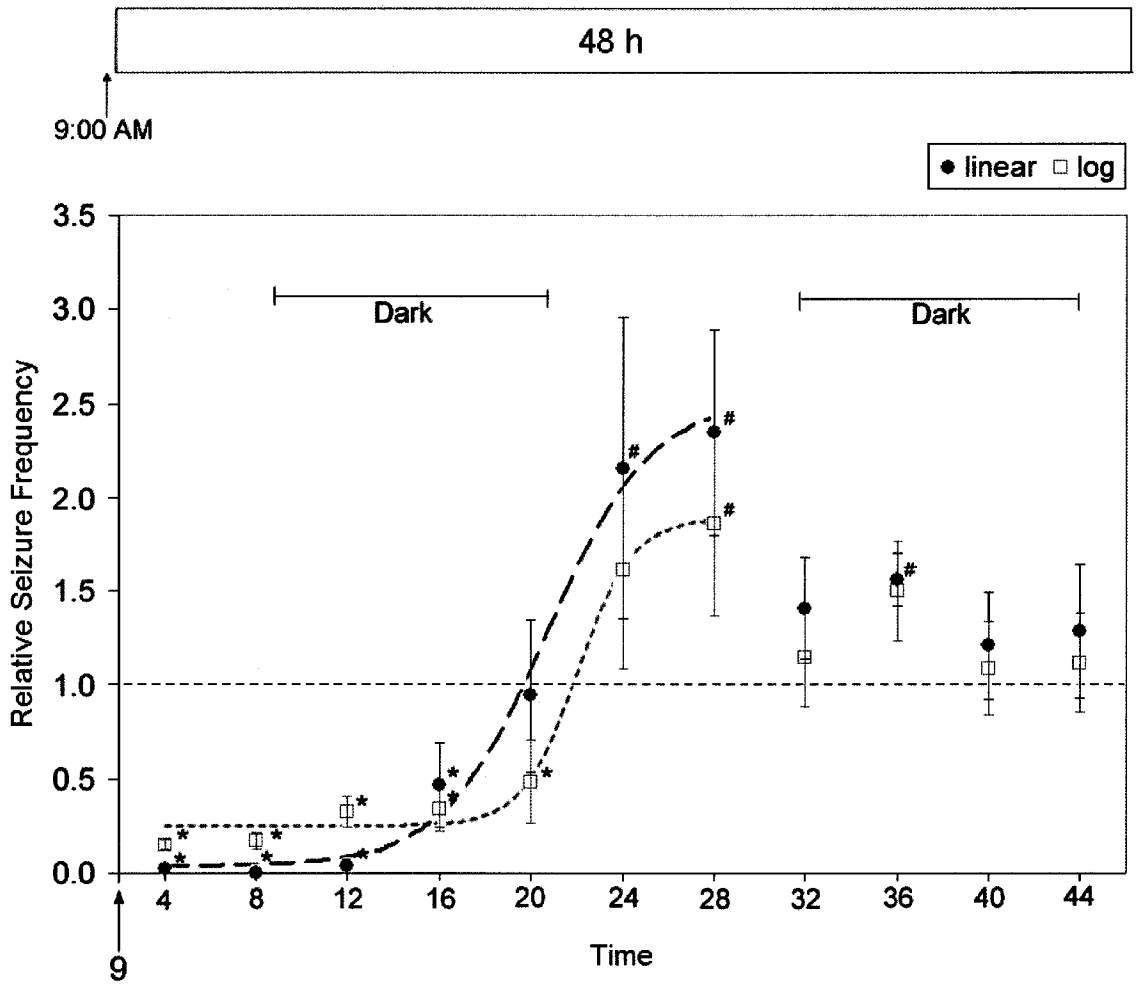

Fig. 4.3. A single 100 mg/kg dose of CBZ in food greatly reduced spontaneous seizures for nearly a day. (A) Time-course-of-recovery from CBZ was analyzed for 48 h starting 1 h after the 9:00 AM feeding. **(B)** Log-transformed data (repeated-measures ANOVA, open squares) and linear data (Wilcoxon signed-rank analysis, black circles) were analyzed. The best-fit sigmoidal curves are shown through mean values of relative seizure frequency. Dashed horizontal line shows no drug effect. *Vertical bars*, ±SEM. Asterisks show significant differences ($p<0.05$). The symbol (#) represents a significant increase in seizure frequency.

recovery from single food administrations with a repeated-measures, cross-over design, and both analyses suggest CBZ would have to be administered at least every 12-16 h to maintain a high level of seizure reduction.

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Multiple daily administrations

Single feedings of CBZ in the repeated-measures cross-over protocol suggested that the 100 mg/kg dose would provide better seizure control than the 30 mg/kg dose, so the higher dose was chosen for a study using continuous drug administration. The goal of this study was to assess the efficacy and tolerability of a 100 mg/kg dose of CBZ administered in food three times per day for 5 days (i.e., 300 mg/kg/day, Fig. 4.4B) to simulate repeated administrations during AED therapy. These experiments aimed to develop a potential method for chronic, repeated drug administration with minimal handling and stress (i.e., a method to replace repeated IP injections or gavage) while sustaining substantial anticonvulsant effects. CBZ administered three times per day (100 mg/kg in food) significantly reduced seizures over a 24-h period (i.e., 10:00 AM to 10:00 AM) and throughout the 5-day treatment protocol (Fig. 4.4C). The full anticonvulsant effect was not observed on the first day of CBZ treatment, and residual effects of CBZ were seen on the first recovery day, suggesting time was required to achieve an optimal CBZ effect and for complete recovery. Thus, CBZ administration in food three times per day may be a useful for chronic treatment with AEDs and other drugs.

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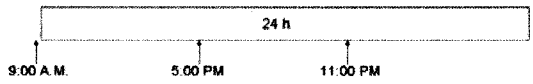
A



B

WEEK 1							
RAT #	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY	SUNDAY
1	CBZ	CBZ	CBZ	CBZ	CBZ	R	R
2	CBZ	CBZ	CBZ	CBZ	CBZ	E	E
3	CBZ	CBZ	CBZ	CBZ	CBZ	C	C
4	CBZ	CBZ	CBZ	CBZ	CBZ	O	O
5	CONTROL	CONTROL	CONTROL	CONTROL	CONTROL	V	V
6	CONTROL	CONTROL	CONTROL	CONTROL	CONTROL	E	E
7	CONTROL	CONTROL	CONTROL	CONTROL	CONTROL	R	R
8	CONTROL	CONTROL	CONTROL	CONTROL	CONTROL	Y	Y
WEEK 2							
RAT #	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY	SUNDAY
1	CONTROL	CONTROL	CONTROL	CONTROL	CONTROL	R	R
2	CONTROL	CONTROL	CONTROL	CONTROL	CONTROL	E	E
3	CONTROL	CONTROL	CONTROL	CONTROL	CONTROL	C	C
4	CONTROL	CONTROL	CONTROL	CONTROL	CONTROL	O	O
5	CBZ	CBZ	CBZ	CBZ	CBZ	V	V
6	CBZ	CBZ	CBZ	CBZ	CBZ	E	E
7	CBZ	CBZ	CBZ	CBZ	CBZ	R	R
8	CBZ	CBZ	CBZ	CBZ	CBZ	Y	Y

C

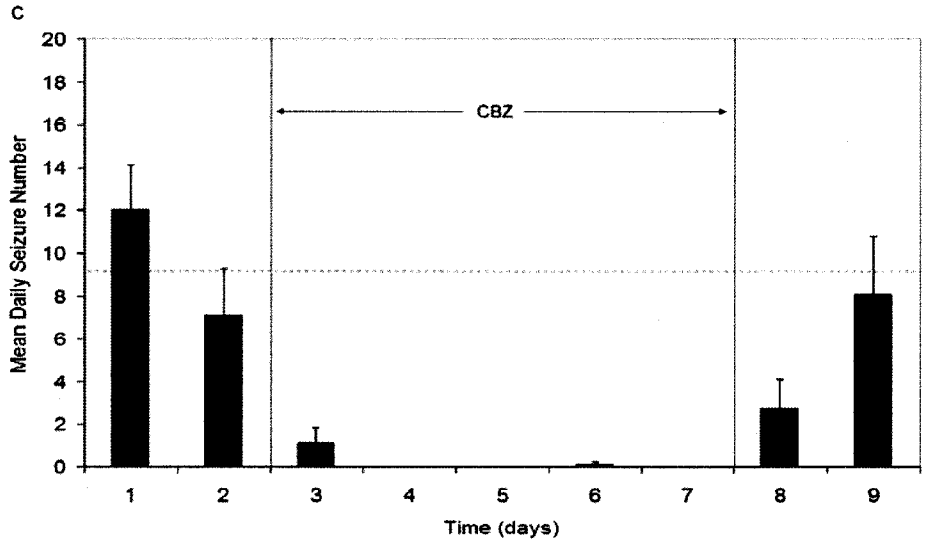


Fig. 4.4. Three-times-per-day feedings of 100-mg/kg CBZ significantly reduced seizures for the duration of the 5-day trial. **(A)** The effect of CBZ was analyzed for the 24-h epoch starting 1 h after the 9:00 AM feeding (i.e., 10:00 AM to 10:00 AM). Doses were also administered at 5:00 PM and 11:00 PM. **(B)** Experimental protocol. **(C)** Mean seizure number (i.e., total seizures per day) is illustrated 2 days prior to, during, and for 2 recovery days after three-times-per-day treatment with CBZ, which significantly reduced seizure frequency. Dashed horizontal line shows mean baseline seizure frequency during vehicle treatments. *Vertical bars*, ±SEM.

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Discussion

These data further support our earlier studies (Grabenstatter et al., 2005) suggesting that animal models of temporal lobe epilepsy with spontaneous seizures can be used to test AEDs, and the repeated-measures, cross-over protocol is amenable to both dose-effect and time-course-of-recovery studies for the direct comparison of AEDs. Single oral administration of CBZ (30 and 100 mg/kg) in dose-specific formulated food was as effective as IP injections, and this method avoided unnecessary handling of the animals. A single feeding of 100 mg/kg CBZ exerted a long-lasting anticonvulsant effect (i.e., nearly 1 day). Additionally, oral CBZ (100 mg/kg) administered three times per day in food significantly reduced seizure frequency throughout the day and for the entire 5-day protocol, thus suggesting the possibility for long-term, continuous administration of drugs in rats with spontaneous seizures.

Choice of statistical analysis

Two different methods were utilized to account for the non-Gaussian distribution of the data due to seizure clusters and data bounded below by zero. The choice of statistical analysis, however, rarely led to a change of statistical significance. Parametric tests are more powerful than non-parametric tests, but make more assumptions about the distribution of the data. The p-values tend to be higher in non-parametric tests, and it is more difficult to detect actual differences as being statistically different, especially with small samples (Ott, 2001). For a repeated-measures, cross-over paradigm based on single drug

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administrations to be valid, recovery from the AED must have occurred before the subsequent control administration; thus, the most conservative approach would be to overestimate the duration of the drug effect. The parametric test on log-transformed data would therefore be the more appropriate test for estimating the persistent effects of an AED. Conversely, an experiment designed to test the duration of the effect of such AED administrations during a chronic protocol involving repeated administrations would need to avoid overestimating the duration of the drug effect, and thus the non-parametric test on linear data would be more appropriate. Thus, both log-transformed (parametric) and linear (non-parametric) analyses have advantages and disadvantages for these studies.

IP injections versus oral administration of AED in food

The most common method for administering AEDs in experimental epilepsy research is through IP injections, which are stressful to the animals and can lead to peritonitis when they are performed repeatedly. Administering AEDs in food not only mimics a typical human dosing schedule, it also reduced stress and other problems associated with multiple daily injections. CBZ, a drug with a relatively short elimination half-life (Carl et al., 1989; Honack et al., 1989), was used in these experiments because it is widely prescribed. It has been suggested that administration of rapidly metabolized drugs via food or drinking water is inadvisable because the rats tend to eat during the night, so there is a risk that drug levels will drop during daytime hours (Loscher and Schmidt, 1988). CBZ also exhibits diurnal variations in the absorption or binding of the drug

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(Bruguerolle et al., 1981), which suggests lower peak levels may occur after the 11:00 PM dose compared to the 9:00 AM feeding. The rats usually ate the food administered within 1 hour of each feeding, and a sustained anticonvulsant effect was observed for 16–20 h following a single administration of 100 mg/kg CBZ. Three-times-daily administration of CBZ in food completely blocked seizures in half of the animals tested, and all but one of the seizures in the animals occurred within 8 h after the first CBZ administration (i.e., 4 out of 8 animals had 1-4 seizures (9 of 10 total seizures) between 10:00 AM and 6:00 PM after the first 9:00 AM drug administration). Therefore, because virtually all of the seizures were blocked relatively soon after onset of CBZ administration during the 5-day test period, no time-of-day effects were apparent; additional studies with lower doses may however reveal a potential influence of time-of-day on eating patterns or CBZ pharmacokinetics.

Abrupt withdrawal of CBZ

In the single-daily-feeding trials (Fig. 4.3), seizure frequency was analyzed for 48 h after AED administration to determine the time course of recovery for a single feeding of 100 mg/kg CBZ. The rather prolonged effect of CBZ on relative seizure frequency was not expected, nor was the apparent overshoot at 24 h (i.e., increased seizure frequency over control levels). Additional studies are needed to determine if the prolonged pharmacodynamic effects are due to the pharmacokinetics of CBZ after this form of oral administration or to some other mechanism. Because the apparent rebound effect occurred after a single

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administration of CBZ, it seems unlikely that it reflects the classical "rebound" that has been observed when patients are withdrawn from CBZ after it has been administered for prolonged periods (Schmidt and Loscher, 2005; Malow et al., 1993; Duncan et al,. 1990), but additional experiments on this phenomenon are warranted.

Metabolism of AEDs in rodent models and need for chronic, sustained drug levels

CBZ showed a sustained anticonvulsant effect on spontaneous, unprovoked seizures using a three-times-per-day dosing schedule, but several caveats exist regarding chronic administration of AEDs that need to be addressed in future experiments. Anticonvulsant effects of an AED can extend beyond the time that therapeutic drug levels are measured in plasma and brain (Carl et al., 1989). The plasma half-life after IP injections and administrations of CBZ via gastric gavage are ~3 h and ~6 h, respectively (Carl et al., 1989; Honack et al., 1989). Variability exists for the maintenance of CBZ levels related to the hour of administration (i.e., chronopharmacokinetics) and the subsequent measured half-life (Bruguerolle et al., 1981). Previous reports suggested that CBZ (100 mg/kg) would need to be administered via oral gavage at least every 8 h during the day (Carl et al., 1989) and more frequently at night (Bruguerolle et al., 1981) to sustain therapeutic blood concentrations. Chronic, repeated administration (i.e., weeks or months) of CBZ leads to auto-induction enzyme metabolism and its elimination half-life is shortened by 50% (Eichelbaum et al.,

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1975; Frey et al., 1980). Extending the 5-day treatment protocol to weeks or months may result in more substantial seizure control or a time-dependent decrease in anticonvulsant efficacy due to development of functional tolerance (Loscher and Schmidt, 1988; Frey et al,. 1987), depending on the specific profile of the AED being evaluated. CBZ administered three times per day in food substantially reduced motor seizures, but these experiments did not determine (1) the exact concentration of CBZ ingested by the rats; (2) the therapeutic blood and brain concentration of oral CBZ; (3) the effect of time of day on the concentration of CBZ; or (4) the concentration of CBZ in blood during discrete time intervals during drug introduction, potential maintenance, and recovery from AED effects. Nonetheless, AEDs like CBZ are thought to preferentially block behavioral versus electrographic seizures (Loscher and Schmidt, 1988), and in spite of these limitations, the dramatic and persistent effects of CBZ on the frequency of spontaneous motor seizures is a measure of the pharmacodynamic effect of CBZ in food.

Current methodology for chronic administration of drugs

Several methods have been employed to administer drugs chronically to epileptic animals. For example, the drug can be given in repeated systemic injections (Honack et al., 1989), subcutaneously implanted osmotic pumps (Glien et al., 2002), an implanted catheter with an external pump or delivery system (Bertram et al., 2005), or gastric oral gavage treatments (Carl et al., 1989). All of these methods have advantages and disadvantages related to practical

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prolonged use and potential harm to the animals. For repeated injections, the exact dose is known and times of administration can be strictly determined, but the animals are subjected to stressful handling and the repeated injections have the associated risk of peritonitis. Implantable pumps provide sustained levels and involve less daily handling of the rats, but osmotic pumps must be re-implanted after about 4 weeks. Most AEDs are lipophilic, and the stability of the drug will determine whether an automatic delivery system is a viable option (e.g., high-dose CBZ could not successfully be delivered in this fashion unless a new vehicle which maintains the drug in solution for days or weeks is developed). External systems utilize a tethered catheter that is implanted in the rat (e.g., femoral, jugular, intracerebral administration) and runs from the rat to the outside of the cage to a solution pump. The pumps have the advantage that they do not require re-implantation, but the catheter may restrict the rat's movement or provide a site for infections. Experiments with catheters may also have a time limitation due to tissue necrosis and the potential for the pumps to clog. Oral gavage administration avoids the risks of injections and surgically implanted devices, but is quite stressful to animals, especially for long-term administration. Thus, delivery of drugs via specially formulated food pellets may be advantageous for many types of studies, because this method involves minimal stress to the animals, is not laborious, and was shown to be effective in the present study.

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Conclusions

These results suggest it is possible to use chronically epileptic animals with spontaneous seizures to analyze the efficacy of different AEDs relative to both dose and time. Comparison of the effect of single, IP injections of 30 and 100 mg/kg CBZ with previous results obtained for topiramate at comparable doses using the repeated-measures, cross-over protocol (Grabenstatter et al., 2005) suggest that CBZ is significantly more effective at reducing spontaneous motor seizures in rats with kainate-induced epilepsy. Similar experiments can be conducted using specially formulated food to simplify an experiment and to decrease unnecessary stress of the animals. High-throughput, long-term trials administering AEDs in food continuously for months may be possible. However, continuous video-EEG data are needed to assess the effect of the AED on both convulsive and nonconvulsive seizures. Blood-concentration data demonstrating that therapeutic blood levels are maintained throughout a 24-h period, and potentially for days, are required to support the anticonvulsant effects found in these studies. While nearly all seizures were blocked during three-times-per-day feedings of 100 mg/kg oral CBZ for 5 days, toxicity tests should be conducted to determine if this is a clinically relevant dose. Despite the future work that is required, these results suggest that administering AEDs in food to animals with chronic epilepsy may be advantageous for pre-clinical drug development. They also show that CBZ, a classical use-dependent AED, could be highly effective at suppressing motor seizures in experiments on animal models of chronic epilepsy.

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CHAPTER 5: A new potential AED, RWJ-333369, substantially reduces motor seizures in rats with kainate-induced epilepsy

Relative contributions

Heidi Grabenstatter was responsible for conducting all the AED trials and data analysis described in this chapter. Heidi Grabenstatter and Dr. Suzanne Clark collected serum from saline- and kainate-treated rats for the dose and time-related analyses of RWJ-333369, and the samples were shipped to Johnson and Johnson R&D, LLC. Drs. Boyu Zhao, Norman Huebert, Bill Hageman, Daksha Desai-Krieger and Mr. Edward Kaczynsk for performed mass spectroscopy analysis on the provided samples. The concepts included within evolved from discussions with Drs. R.E. Twyman, H.S. White, and F.E. Dudek, who offered comments and suggestions on early drafts. The final draft was reviewed and edited by Dr. Dudek.

Introduction

About two dozen compounds are marketed as antiepileptic drugs (AEDs) worldwide, nine of which were developed since 1993. Nonetheless, 30 to 40% of patients with epilepsy remain ineffectively treated with the presently available AEDs (Bauer and Burr, 2001; Cole, 2004; Elger, 2003; Pitkanen and Sutula,

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2002; Rogawski and Loscher, 2004; Sisodiya, 2003; Weiser, 2004). New methods of AED testing and development may identify treatments for this large population of pharmacoresistant patients. An important issue to consider while testing an AED is whether the animal model mimics the condition for which the drug is a potential therapy (i.e., clinical pathology and associated seizure type). Little research has been performed testing AEDs on animal models of acquired epilepsy. Rapid screens using acute seizure models such as pentylenetetrazol or maximal electroshock (i.e., chemically or electrically induced seizures in an otherwise normal animal) have been the preferred choice for animal testing and development of new AEDs. Chronically epileptic rats with spontaneous seizures, although more labor-intensive to use, share similarities with human patients with temporal lobe epilepsy. These include: 1) the presence of spontaneous seizures, 2) histopathologic changes similar to hippocampal sclerosis (Margerison and Corsellis, 1966), and 3) molecular changes that may occur as a result of time-dependent epileptogenesis. Hypothetically, chronically epileptic rats will more successfully predict a potential AED's clinical efficacy (Heinemann et al., 1994; Kupferberg, 2001; Leite et al., 2002; Levy et al., 2002; Loscher and Schmidt, 1988; Schmidt and Rogawski, 2002; Stables et al., 2002, 2003; White, 1997, 2003).

RWJ-333369, an investigational AED with a unique anticonvulsant profile, has demonstrated broad-spectrum activity at non-toxic doses in rodent models of generalized and partial epilepsy (White et al., 2006). The main objectives of the present study were to evaluate the effect of RWJ-333369 on the frequency of

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spontaneous motor seizures, and whether RWJ-333369 completely blocks the seizures. The dose-dependent effects of RWJ-333369 were assessed in relation to the measured concentration of RWJ-333369 in the blood and were compared to those of topiramate and carbamazepine. Ultimately, the aim of these studies was to provide "proof-of-principle" for the use of animal models of chronic epilepsy in the testing of new AEDs.

Methods

Kainate treatment and status epilepticus.

Kainate (Ocean Produce International, Shelburne, Nova Scotia, Canada) was administered via repeated, low-dose, intraperitoneal injections (5 mg/kg) every hour until each adult Sprague-Dawley rat (180-200 g, n=8 per treatment series, 7 separate kainate treatments) experienced convulsive status epilepticus for >3 h (Hellier et al., 1998). Motor (i.e., convulsive) seizures were scored on a scale from III to V using the Racine scale (Racine, 1972). A class III seizure was characterized by forelimb clonus, an erect tail, and lordotic posturing, which could progress into a class IV seizure with continued forelimb clonus and rearing on hindlimbs. Animals that showed all of these behaviors in combination with a fall were defined as having a class V seizure, although class V seizures could include a fall without some of the other precipitating behaviors.

Following status epilepticus, rats were housed in an environmentally controlled vivarium (humidity 31-55%, temperature 21 to 22°C, 12-h light-dark cycle, lights on at 6:00 AM, food and water ad-libitum). All animals were directly

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observed for the occurrence of spontaneous seizures for 6 h/week, and animals with low seizure rates were not used in the experiments. The animals were used for the doses of 0.3, 1, 3, 10, and 30 mg/kg RWJ-333369 at 212.63 ± 42.14, 176.63 ± 42.14, 297.89 ± 64.048, 315 ± 38.26, and 321.89 ± 42.77 days respectively after kainate-induced status epilepticus.

Repeated-measures, cross-over protocol and RWJ-333369 administration.

Five 1-month trials (n=8-10 rats) were conducted to assess the effects of 0.3, 1, 3, 10, and 30 mg/kg RWJ-333369 on spontaneous convulsive seizures. Each trial involved six pairs of RWJ-333369 and vehicle treatments administered as intraperitoneal injections on alternate days with a recovery day between each treatment day (Fig. 5.1). This paradigm controlled for changes in seizure frequency with time, and each animal served as its own control. RWJ-333369 was dissolved in 10% Solutol-HS-15 (BASF, Ludwigshafen, Germany) with vigorous stirring in a warm water bath (40-60°C).

Continuous video-monitoring of spontaneous seizures.

Each rat was continuously video-monitored for the entire experiment on 8-h videotapes by a Panasonic WV-BP334 black-and-white camera (G&G Technologies, Secaucus, NJ, USA). A trained technician, blinded to the treatments and dates, analyzed videotapes for any possible seizures. The Racine scale (Racine, 1972) was implemented to score seizure occurrence and severity during the first 6-h epoch (i.e., 10:00 a.m. to 4:00 p.m.) and a second 8-h epoch approximately 24 h after drug administration (12:00 a.m. to 8:00 a.m.).

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RAT #	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY	SUNDAY
1		RWJ-333369		VEHICLE		RWJ-333369	
2	B	RWJ-333369	R	VEHICLE	R	RWJ-333369	R
3	A	RWJ-333369	E	VEHICLE	E	RWJ-333369	E
4	S	RWJ-333369	C	VEHICLE	C	RWJ-333369	C
5	E	RWJ-333369	O	VEHICLE	O	RWJ-333369	O
6	L	VEHICLE	V	RWJ-333369	V	VEHICLE	V
7	I	VEHICLE	E	RWJ-333369	E	VEHICLE	E
8	N	VEHICLE	R	RWJ-333369	R	VEHICLE	R
9	E	VEHICLE	Y	RWJ-333369	Y	VEHICLE	Y
10		VEHICLE		RWJ-333369		VEHICLE	

RAT #	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY	SUNDAY
1	VEHICLE		RWJ-333369		VEHICLE		
2	VEHICLE	R	RWJ-333369	R	VEHICLE	R	
3	VEHICLE	E	RWJ-333369	E	VEHICLE	E	
4	VEHICLE	C	RWJ-333369	C	VEHICLE	C	
5	VEHICLE	O	RWJ-333369	O	VEHICLE	O	
6	RWJ-333369	V	VEHICLE	V	RWJ-333369	V	
7	RWJ-333369	E	VEHICLE	E	RWJ-333369	E	
8	RWJ-333369	R	VEHICLE	R	RWJ-333369	R	
9	RWJ-333369	Y	VEHICLE	Y	RWJ-333369	Y	
10	RWJ-333369		VEHICLE		RWJ-333369		

Fig. 5.1. Experimental protocol. RWJ-333369 or 10% HS-Solutol-15 was administered every other day for 24 days (e.g., Tuesday, Thursday, Saturday, Monday, Wednesday, and Friday). The protocol shown above was doubled in length to include six AED versus vehicle-control tests. Daily 24-h video recordings were made for the duration of the protocol. The tapes that contained the actual injection began 1 h before and ended 7 h after the injection (i.e., start tape at 8:00, inject at 9:00 A.M.) For the dose-response data, 6 h of data from each tape were analyzed to determine the maximal effect of each dose, starting 1 h after injection (i.e., 10:00 A.M – 4:00 P.M).

Seizure frequency and severity were not analyzed for the first hour after intraperitoneal injection of AED to eliminate possible seizure-inducing effects of animal handling from the analysis.

Blood concentration analysis.

In a separate series of experiments, the blood concentration of RWJ-333369 was measured 4 h after injections of vehicle (i.e.,10% Solutol-HS-15), 3

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mg/kg RWJ-333369, 10 mg/kg RWJ-333369, and 30 mg/kg of RWJ-333369 in a saline-control group (n=8) and in a group of rats with kainate-induced epilepsy (n=8). Therefore, each rat in the saline group and in the kainate group was given the four treatments, and the blood was tested 4 h later. In a second series of experiments, the blood concentration of RWJ-333369 was measured 24 h after injections of vehicle (10% Solutol-HS-15) and 30 mg/kg of RWJ-333369 in saline-treated control rats (n=8) and in rats with kainate-induced epilepsy (n=8). In separator tubes with lithium heparin (Becton Dickinson and Company, Franklin Lakes, NJ, USA), 400 µl of whole blood was collected via tail vein. The tubes were inverted several times and iced immediately. From these samples, plasma (200 µl) was collected into Eppendorf tubes after centrifugation (3000 rpm at 4°C for 5 min). The plasma samples were frozen and stored at −80°C until shipment on dry ice for mass spectral analysis.

Statistical analyses.

Due to asymmetrical distribution (i.e., non-Gaussian distribution) and heterogeneous variance, seizure frequency data were originally analyzed in the $\log_{10}$ (y+0.1) scale. The inter- and intra-animal variability in seizure frequencies is likely explained by the occurrence of both seizure clusters and seizure rates equivalent to zero (Grabenstatter et al., 2005). AED and vehicle treatments were compared using a repeated-measures ANOVA (Grabenstatter et al., 2005). Relative seizure frequencies (i.e., a ratio of seizure frequency following AED injection per seizure frequency following vehicle injection) in the log scale were

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back-transformed to the original linear scale for presentation of results. The necessary addition of 0.1 to all seizure frequency values due to the high occurrence of zeros in the data set permitted a log transformation and a parametric analysis of the data (i.e., repeated-measures ANOVA). However, the effects of highly potent AED treatments (i.e., nearly complete seizure cessation) were masked due to the "floor effect" created by the addition of 0.1 to all values. The data were reanalyzed on the linear scale (i.e., sum of all seizures after individual treatments per total number of AED tests conducted) using a non-parametric test, the Wilcoxon signed-rank test, which does not assume Gaussian distribution of the data (Grabenstatter et al., in prep). Potential significant differences between similar doses of AEDs were tested using an unpaired t-test with a Welch correction.

To evaluate possible alterations in seizure severity, an analysis of mean severity scores (Racine, 1972) was conducted during 6-h epochs following AED and vehicle treatments. The severity score was calculated as the ratio of the sum of severity scores per number of seizures, excluding epochs with no seizures. Significant differences in these severity scores were assessed using a Student's t-test. In another analysis, the frequency of seizures in each severity class (Racine, 1972) during AED treatments was compared to the frequency of that severity class during the vehicle treatments (i.e., the frequency of class III seizures after a particular dose of RWJ-333369 was compared to the frequency of class III seizures after vehicle). Significant differences in the frequency of class III, IV, or V seizures during AED treatment compared to vehicle treatment were

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determined using a Student's t-test with an a priori Bonferroni adjustment for multiple comparisons. For blood analyses, the concentration of RWJ-333369 was measured at 4 h after injections of 3 mg/kg, 10 mg/kg, and 30 mg/kg of RWJ-333369 in a saline-control group and in a group of rats with kainate-induced epilepsy, and the differences were assessed with a Student's t-test using an a priori Bonferroni adjustment. For a dose of 30 mg/kg RWJ-333369, a similar Student's t-test was used to compare blood concentrations of vehicle- and kainate-treated animals at 4 h and 24 h.

Results

Seizure frequency

The first issue addressed in these experiments was the effect of RWJ-333369 on the frequency of spontaneous convulsive seizures. Figure 5.2 shows the effect of different doses of RWJ-333369 on the number of spontaneous seizures during the 6-h epoch after drug injection. For comparison, Figure 5.2 also shows similar results obtained previously for topiramate (Grabenstatter et al., 2005) and carbamazepine (CBZ, [Grabenstatter et al., in prep]). Single intraperitoneal injections of 30 mg/kg RWJ-333369 reduced relative seizure frequency by 74% during the first 6-h epoch, producing a relative seizure frequency of 0.26 (±0.06, $p<0.0001$). Using the same method and dosage, topiramate (30 mg/kg) only reduced convulsive seizures by about half (i.e., relative seizure frequency = 0.51 ±0.20, $p<0.0001$). CBZ (30 mg/kg) had a

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substantial effect on convulsive seizures reducing relative frequency by 72% (i.e., relative seizure frequency = 0.28 ±0.06, $p<0.0001$). While each drug significantly reduced relative seizure frequency when compared to vehicle at high doses, RWJ-333369 exerted a more robust effect at 10 mg/kg than topiramate ($p = 0.03$) and CBZ ($p=0.01$), and 30 mg/kg RWJ-333369 was significantly more effective than 30 mg/kg or 100 mg/kg topiramate ($p = 0.04$ and $p = 0.02$, respectively). The effects of RWJ-333369 and CBZ at 30 mg/kg were not significantly different from each other following the parametric, repeated-measures ANOVA (Fig 5.2A).

Data analysis using a non-parametric statistical analysis (i.e., the Wilcoxon signed-rank test), showed RWJ-333369 (3, 10, and 30 mg/kg) reduced seizure frequency in a dose-dependent manner regardless of the statistical test used. Using the latter non-parametric analysis on non-transformed data, I found RWJ-333369 was still more effective than topiramate and CBZ at 10 mg/kg. Although RWJ-333369 had comparable effects to CBZ at 30 mg/kg on seizure frequency (based on the repeated-measures ANOVA); using the non-parametric, Wilcoxon signed-rank test, I found 30 mg/kg RWJ-333369 was significantly more effective at reducing seizure frequency than 30 mg/kg CBZ or 30 mg/kg topiramate (Fig 5.2B). Therefore, RWJ-333369 reduced seizure frequency more effectively than both CBZ and topiramate.

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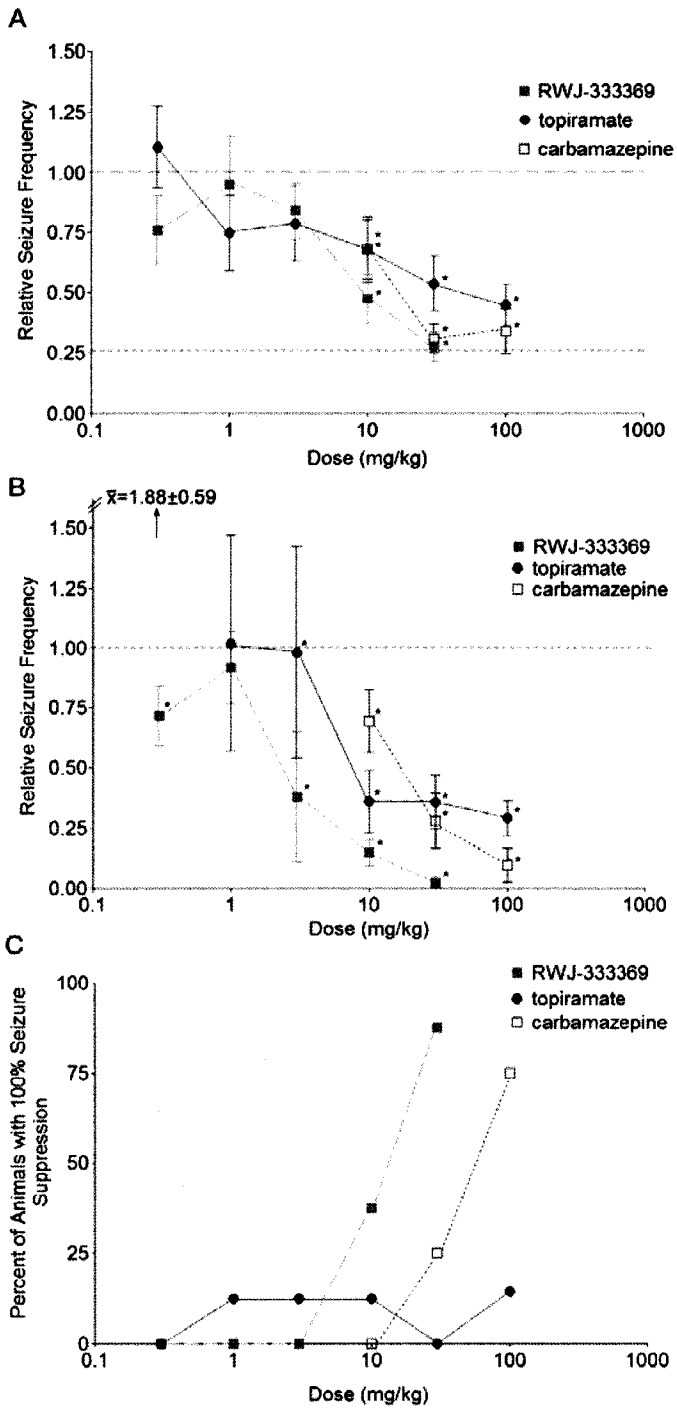

Fig. 5.2. RWJ-333369 reduced seizures more effectively than topiramate or CBZ. (A) Seizure frequencies were log-transformed [i.e., $\log_{10}(y+0.1)$] because the data set had a non-Gaussian distribution and was bound below by zeros. The transformation allowed the use of a parametric test (i.e., a repeated-measures ANOVA). RWJ-333369, topiramate (TPM), and CBZ reduced relative seizure frequency. Because the log transformation necessarily involves the addition of 0.1 to all seizure frequencies, "relative seizure frequency" would have a discrete value even with a complete block of all seizures (Grabenstatter et al., in prep). The lower dashed horizontal line represents the estimated mean "floor effect" when absolute seizure block was simulated. In this and subsequent figures, the upper dashed horizontal line shows the baseline (i.e., no effect). **(B)** The other approach for data with a non-Gaussian distribution is a non-parametric test. A Wilcoxon signed rank test also showed that RWJ-333369, TPM, and CBZ significantly reduced seizure frequency at these doses. **(C)** The percent-of-animals showing complete seizure cessation during the first 6-h epoch following a single IP injection of AED were compared for RWJ-333369, TPM, and CBZ. *Vertical bars*, ±SEM. Asterisks show significant differences ($p<0.05$).

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Complete cessation of seizures

Another measure that may be predictive of clinical success for a potential new AED is the percentage of chronically epileptic animals with complete seizure cessation during drug administration (i.e., 100% seizure suppression). RWJ-333369 completely blocked seizures in 3 of 8 animals at 10 mg/kg and 7 of 8 rats at 30 mg/kg. Topiramate only suppressed all spontaneous seizures in 1 of 8 animals tested in the 10 mg/kg trial and the 100 mg/kg trial, but did not completely block seizures in any of the animals at 30 mg/kg. CBZ did not completely block seizures in any animals at 10 mg/kg, 2 of 8 animals experienced complete seizure cessation with 30 mg/kg CBZ treatment, and 7 of 10 animals tested in the 100 mg/kg CBZ trial had complete seizure block (Fig. 5.2C). RWJ-333369 was more effective than topiramate and CBZ at blocking all seizures (i.e., complete seizure cessation in a higher percentage of animals) at 10 and 30 mg/kg (Fig 5.2C). Therefore, RWJ-333369 was much more effective than topiramate and CBZ at completely suppressing spontaneous convulsive seizures in rats with kainate-induced epilepsy.

Seizure severity

An important question is whether RWJ-333369 reduced the severity of the remaining seizures (e.g., did the AED cause a shift from more-severe to less-severe seizures or did it preferentially block the more severe seizures?). Our previous analysis of the possible effect of topiramate on seizure severity (Grabenstatter et al., 2005) utilized intensity-weighted seizure number (i.e., the

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sum of severity scores) as a measure, and yielded apparent dose-dependent reductions in seizure severity. The use of intensity-weighted seizure number for analysis of possible drug effects on seizure severity, however, included drug-induced alterations in seizure frequency, which may have dominated the analysis and led to an apparent reduction in seizure severity when the primary effect was actually on seizure frequency. Using mean seizure severity (i.e., the ratio of the sum of severity scores per number of seizures, excluding epochs with no seizures) as a measure, RWJ-333369 only reduced seizure severity at a dose of 10 mg/kg ($p=0.0205$). A possible effect of 30 mg/kg RWJ-333369 on seizure severity could not be determined because this dose had such a large effect on seizure frequency (i.e., 7 of 8 animals had no seizures during the 6-h epoch after 30 mg/kg RWJ-333369). A re-analysis of the previous topiramate data (Grabenstatter et al., 2005) using mean severity score showed no effect on seizure severity at doses of 10 mg/kg ($p=0.1056$), 30 mg/kg ($p=0.5860$) and 100 mg/kg ($p=0.2368$). Therefore, although a small but statistically significant effect of seizure severity was found with 10 mg/kg RWJ-333369, topiramate and RWJ-333369 at other doses had no detectable effect on the severity of behavioral seizures (i.e., independent of the overall drug effects on seizure frequency).

To assess further possible drug effects on seizure severity, the frequency of seizures of each severity class (Racine, 1972) during drug treatment (relative to vehicle treatment) was compared across doses. Figure 4.3A shows that RWJ-333369 significantly reduced class III at 10 mg/kg ($p<0.05$) and class III and class V seizures at 30 mg/kg ($p<0.05$), blocking all seizures of the latter type (i.e.,

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tonic-clonic seizures). Topiramate significantly reduced class III seizures at 100 mg/kg ($p<0.05$), but did not reduce class IV and class V seizures at 30 mg/kg or 100 mg/kg (Fig. 5.3B). Although the two drugs did appear to block some severity classes, the main effect appeared to be at high doses when the drugs had large effects on seizure frequency.

Blood concentration

The corresponding blood concentration of three doses of RWJ-333369 was measured in order to analyze the relationship between blood concentration and anticonvulsant activity. Blood concentration was measured in both control rats and in rats with kainate-induced epilepsy. The effect of the dose of RWJ-333369 on blood concentration was determined in the middle of the first 6-h time epoch after drug administration (i.e., 4 h after the injection of RWJ-333369, or at 1:00 p.m.). The doses of 3 mg/kg, 10 mg/kg, and 30 mg/kg of RWJ-333369 were chosen for blood-level determinations, because they ranged from ineffective (3 mg/kg) to highly effective (30 mg/kg) at suppressing seizures (Fig. 5.4). At 4 h after a single intraperitoneal injection, the measured blood concentrations for the doses of 3 mg/kg, 10 mg/kg, and 30 mg/kg paralleled the effect of RWJ-333369 on percent seizure suppression. No significant difference was observed in the concentration of RWJ-333369 between animals with kainate-induced epilepsy and aged-matched control animals.

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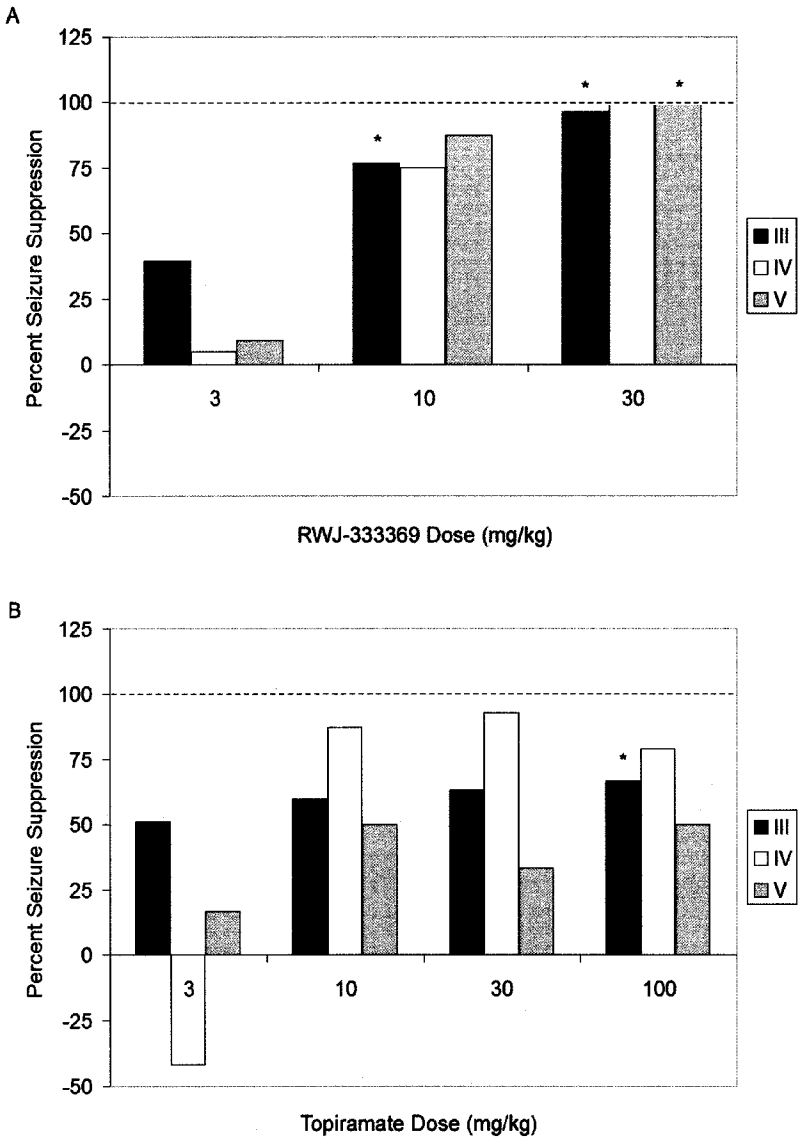

Fig. 5.3. Effect of RWJ-333369 and topiramate on seizures of different Racine classes. A categorical analysis showing the distribution of seizure occurrence according to seizure severity (Racine, 1972). The magnitude of suppression for different classes of seizures is shown using black bars to represent reduction in Class III seizures, white bars to show the percent reduction of Class IV seizures, and grey bars to show the effects of the two AEDs on Class V seizures. A) RWJ-333369 significantly ($p<0.05$) reduced the frequency of class III seizures at a dose of 10 mg/kg, and class III and class V seizures were significantly reduced at 30 mg/kg RWJ-333369. B) Topiramate (100 mg/kg) significantly ($p<0.05$) decreased class III seizures, and was not significantly effective in reducing class IV or V seizures. The dashed horizontal line marks the 100% suppression level.

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Using blood concentration measurements of RWJ-333369, the issues of time-dependent recovery from single intraperitoneal injections and the potential for drug accumulation at the highest dose were addressed. Seizure frequency was not significantly different from control in the 8-h epoch between 15 h and 23 h after administration of 30 mg/kg RWJ-333369 (Fig. 5.5); therefore, the concentration of RWJ-333369 was measured at 24 h after treatment for comparison.

Measurement of blood levels of RWJ-333369 indicated virtually complete drug clearance had occurred by 24 h in the repeated-measures, cross-over protocol (i.e., before the subsequent vehicle-control injection at 48 h). A significant difference ($p<0.05$) in blood concentration of RWJ-333369 was detected between the two time points (4 h versus 24 h). No significant difference in blood concentration of RWJ-333369 was observed between animals with kainate-induced epilepsy and control rats. Therefore, the availability of the drug in the blood after a high-dose injection (as measured by blood concentration values) was directly linked to seizure suppression by RWJ-333369.

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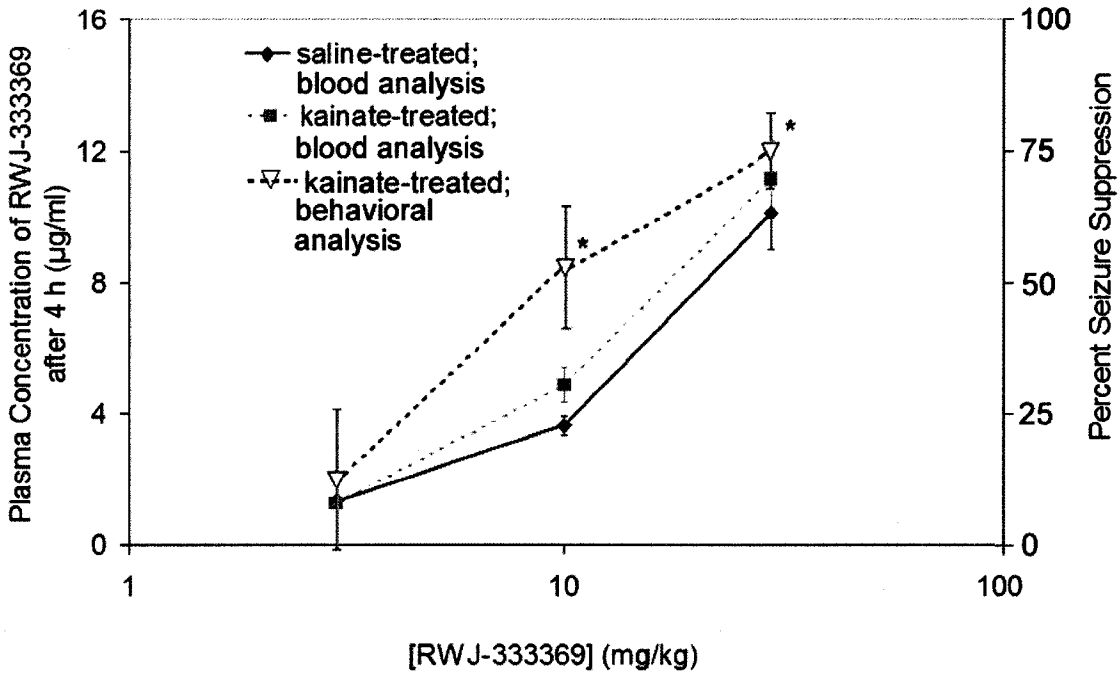

Fig. 5.4. Effect of dose of RWJ-333369 on plasma concentration of RWJ-333369 4 h after drug injection. The blood concentration for each dose (3, 10, and 30 mg/kg) was measured 4 h after systemic injection of RWJ-333369. Each dose is compared using black diamonds and solid lines to represent blood concentration results for saline-treated animals; grey squares with grey dashed lines correspond to plasma concentrations of RWJ-333369 for kainate-treated animals; and open triangles with black dashed lines display seizure data for each concentration of RWJ-333369 measured. No significant difference was found between epileptic and control animals. Vertical bars, ±SEM.

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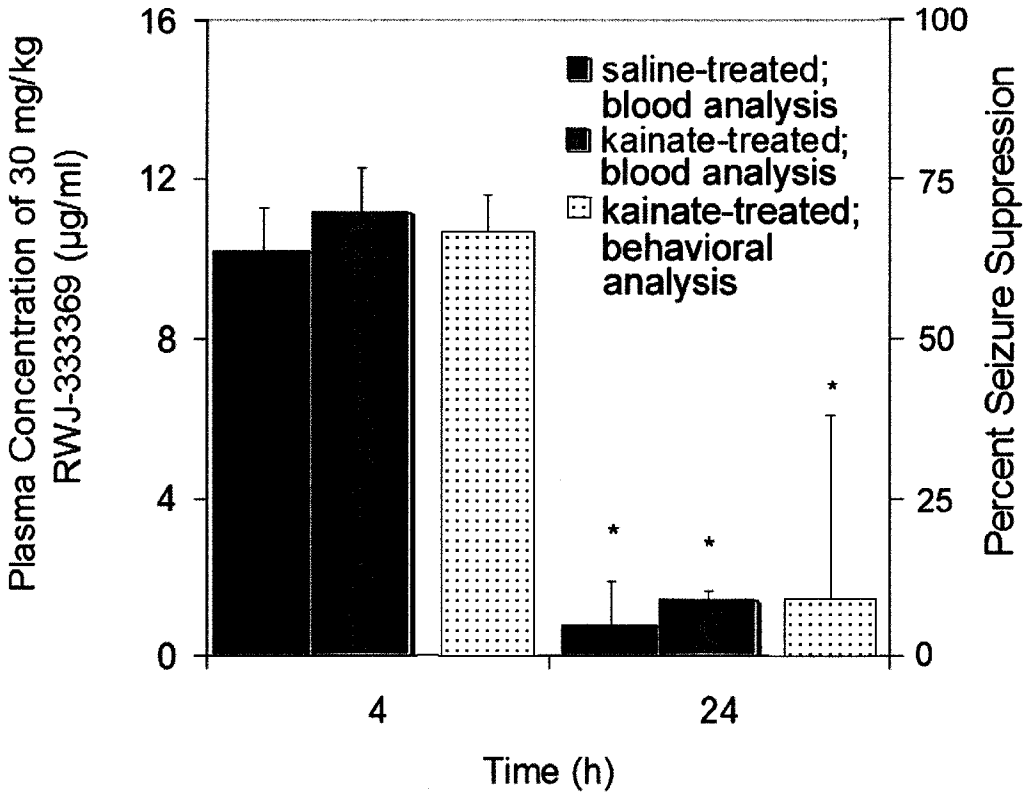

Fig. 5.5. Effect of 30 mg/kg RWJ-333369 plasma concentration at 4 and 24 h after drug injection. Blood concentrations of AED at 4 h and 24 h after IP injection of 30 mg/kg RWJ-333369 were significantly different ($p<0.0125$). Results were compared for saline-treated animals (black bars) and rats with kainate-induced epilepsy (grey bars). Behavioral data analyzed to determine percent seizure suppression is displayed for kainate-treated animals (dot-filled bars) for both time points. The high concentration of RWJ-333369 at 4 h was associated with high seizure suppression, which was not present at 24 h when RWJ-333369 was at low levels in the blood. No significant difference was detected in the concentration of RWJ-333369 in control and epileptic animals. Vertical bars, ±SEM.

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Discussion

Summary of comparative analysis of RWJ-333369, topiramate, and CBZ

The repeated-measures, cross-over protocol in rats with kainate-induced epilepsy previously showed that topiramate significantly reduced the frequency of spontaneous seizures, but did not completely block the seizures (Grabenstatter et al., 2005). The same protocol was used in the present study to assess the effects of RWJ-333369, a new AED. RWJ-333369 was more effective than topiramate and CBZ at reducing seizure frequency and the percentage of animals with complete seizure cessation. Blood concentrations of RWJ-333369 1) increased with administration of higher doses of drug, which correspondingly reduced seizure frequency, and 2) decreased over time, as the frequency of convulsive seizures returned to control values. These results suggest that RWJ-333369 may potentially be an improved AED for the treatment of refractory temporal lobe epilepsy.

The effect of three different AEDs: (1) RWJ-333369, an investigational neuromodulator (White et al., 2006); (2) a new-generation, broad-spectrum AED (i.e., topiramate); and (3) a traditional use-dependent sodium channel blocker (i.e., CBZ), administered via single IP injections were directly compared. RWJ-333369 more effectively reduced seizure frequency and increased percentage of seizure freedom at 30 mg/kg than topiramate and CBZ.

A high percentage of animals had complete seizure block after single-daily IP injections of RWJ-33369 and CBZ, thus demonstrating the potential "floor

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effect" that occurs when results are analyzed on the $\log_{10}(y+0.1)$ scale. When these data were reanalyzed on the linear scale with a non-parametric statistical analysis, it was possible to ascertain the differences between drugs at doses where seizures were completely blocked or nearly completely blocked. In the direct comparison of RWJ-333369, topiramate, and CBZ, reanalysis with the Wilcoxon signed-rank test showed that the observed dose-response curves of the three drugs in that (1) CBZ was no longer significantly more effective than topiramate at 30 mg/kg; (2) RWJ-333369 was now statistically more effective than CBZ at 30 mg/kg; and (3) the previously reported dose dependence of topiramate was not present, but CBZ appeared to reduce seizures in a dose-dependent manner. Interpreting relative frequency measures using a parametric test of log transformed data (i.e., a repeated-measures ANOVA) is a rigorous method to detect low-levels of antiepileptic effect (Grabenstatter et al., in prep). A non-parametric statistical analysis of linear data (i.e., the Wilcoxon signed-rank test) is the most appropriate method for assessing highly effective AEDs that block nearly all spontaneous seizures (Grabenstatter et al., in prep).

Measures of drug efficacy

Relative seizure frequency versus percent seizure suppression

The effect of a drug on the frequency of seizures is typically normalized to seizure frequency without drug. Our previous experiments on topiramate (Grabenstatter et al., 2005) used *relative seizure frequency*, which is the ratio of the number of seizures observed in a defined time period (i.e., seizure frequency

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during a 6-h epoch) after an AED injection relative to the seizure frequency after a control injection. Relative seizure frequency essentially yields an expression of the reduction in seizure frequency induced by the drug. *Percent seizure suppression* (100 x [1- relative seizure frequency]) is a measure of the degree to which a drug blocks seizures during a specific time period; therefore, *percent seizure suppression* and *relative seizure frequency* are similar but converse measures. For the purposes of this study, percent seizure suppression is a useful measure of efficacy because it provides a direct comparison to the percentage of animals with total seizure cessation (i.e., 100% seizure suppression, see below) and to the blood concentration of RWJ-333369.

Percentage of rats with complete seizure suppression.

An ideal AED would suppress all seizures, and the percentage of animals that experience complete seizure cessation after AED treatment is another measure of efficacy. *Complete seizure suppression* in any particular animal was defined as occurring when no seizures were observed during the first 6-h epoch after any of the AED treatments. Although inclusion of data from animals that were not tested in all six AED-versus-vehicle tests per dose is a potential confound in the interpretation of the data on *complete seizure cessation*, the likelihood of an animal being eliminated from the study was equal for RWJ-333369, topiramate, and CBZ. RWJ-333369 completely suppressed seizures in nearly all rats at 30 mg/kg *for 6 h*, but additional work is needed to test whether

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prolonged treatment with RWJ-333369 (e.g., chronic administration of 30 mg/kg) completely eliminates seizures in rats with kainate-induced epilepsy.

Seizure severity

The level of behavioral involvement is an important measure of seizure severity. Two interrelated analyses did not consistently detect an effect of either RWJ-333369 or topiramate on seizure severity, when performed in a manner to account for alterations in seizure frequency. This lack of effect could be due at least in part to a lack of statistical power, because our analyses essentially divided all seizures into three categories (i.e., class III, IV, and V). Another possibility is that the distinction between class III, IV, and V seizures is artificial in this context. Future studies concerning the effect of RWJ-333369 on seizure severity need to investigate non-convulsive electrographic seizures and seizure duration.

Blood concentration analysis

These experiments showed that higher doses of RWJ-333369 led to increased blood concentrations, which caused greater seizure suppression. The repeated-measures, cross-over protocol requires complete recovery from drug within 48 h before the subsequent vehicle treatment, and the present studies showed that the concentration of RWJ-333369 had returned to baseline levels within 24 h. Therefore, the effect of RWJ-333369 on spontaneous seizures was dose-dependent.

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Use of rats with chronic epilepsy in AED discovery

The repeated-measures, cross-over protocol for testing potential AEDs has the advantage that one can use an animal as its own control, and the design has a relatively high level of statistical power, considering the use of a comparatively small number of animals. At a significance level of α=0.05, 8 animals and six AED-versus-vehicle tests yields a power of 0.9 (i.e., a total of 48 AED-versus-vehicle tests per trial). In its present form, approximately 1 month was required to complete the analysis of each dose, but fewer cross-overs (i.e., AED-versus-vehicle tests) and less time would be needed if more animals were used. Thus, the same power would be achieved with each dose if an investigator performed the same total number of tests using more animals (e.g., 24 animals and two cross-overs). The kainate-treated rats were selected to ensure that they had high seizure rates, and so one question is whether the results apply as well to animals with low seizure rates. Finally, the present studies only analyzed behavioral seizures, and so non-convulsive electrographic seizure activity was not assessed. Therefore, the approach of using chronically epileptic animals has several possible variants for future study, and it has the advantage of potentially allowing detection of AEDs that preferentially act on "epileptic" versus normal brain.

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CHAPTER 6: Effects of carbamamazepine on spontaneous electrographic seizures studied with video-EEG in rats with kainate-induced epilepsy

Relative contributions

Heidi Grabenstatter was responsible for implanting animals and collecting fieldpotential recordings from epileptic and control rats. Heidi Grabenstatter also conducted the AED trials during which animals were monitored via video and with field-potential recordings from the dentate gyrus. Dr. Phil Williams trained her in the surgery techniques used in these experiments. Dr. Jennifer Hellier was responsible for collecting the evoked field potential data set. Heidi Grabenstatter performed all the data analysis described in this chapter. The concepts included within evolved from discussions with Dr. Dudek, who offered suggestions and criticisms on early drafts, and reviewed and edited the final draft.

Introduction

Traditional AED testing has utilized acute seizures (e.g., maximal electroshock and pentylenetetrazol-induced seizures) where the effect of an AED on seizure threshold is assessed in a normal animal. New AEDs with minimal side effects that would be effective in pharmacoresistant epilepsy may be

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discovered by testing them in animal models with chronic spontaneous epileptic seizures. An advantage of chronic epilepsy models is that they have recurrent seizures and develop histopathological changes qualitatively similar to the mesial temporal sclerosis observed in human temporal lobe epilepsy (Margerison and Corsellis, 1966). The use of animals that have experienced epileptogenesis (and any changes that occur during this process) may detect more selective drugs that act on specific components of epileptic seizures (Heinemann et al., 1994; Kupferberg, 2001; Leite et al., 2002; Levy et al., 2002; Loscher and Schmidt, 1988; Schmidt and Rogawski, 2002; Stables et al., 2002, 2003; White, 1997, 2003). An important goal is to understand how different AEDs act on spontaneous epileptic seizures.

In vitro experiments in cell cultures and brain slice preparations have been used to better understand the cellular mechanisms of action of AEDs (Rogawski and Loscher, 2004), but little research has been aimed at the electrophysiological mechanisms of action of AEDs on electrographic seizures in freely behaving animals with chronic epilepsy. Chronic field-potential recordings from rats with chronic epilepsy may reveal how AEDs alter the electrophysiological structure of a seizure, and thus how these drugs suppress epileptic seizures. The dentate gyrus is highly suitable for field-potential analysis of synchronous postsynaptic potentials and action potentials. Unlike the neocortex, the hippocampus contains compact layers where the somata and dendrites of granule and pyramidal cells are arranged in parallel, which leads to the generation of large field potentials that can be analyzed as synchronous synaptic potentials and action potentials.

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The dentate gyrus may play a role in seizure generation and/or propagation (Stringer and Lothman, 1989; Stringer et al., 1991; Lothman et al., 1992; Heinemann et al., 1992; Buckmaster and Dudek, 1997; Behr et al., 1998), and thus it is a useful site to study the effects of AEDs on isolated components of seizure activity (i.e., synchronous synaptic potentials and action potentials). The main objective of these experiments was to develop a method for testing how AEDs suppress electrographic seizures in freely behaving rats with chronic epilepsy using in vivo electrophysiological recordings of hippocampal field potentials in the dentate gyrus.

CBZ is thought to preferentially suppress convulsive tonic-clonic seizures (Albright et al., 1980; Schmidt, 1986) and to attenuate ictal activity. CBZ reduces high-frequency repetitive firing by interacting with sodium channels to slow the rate which these channels recover from inactivation (Ambrosio et al., 2002; Macdonald,1989; Soderpalm, 2002; Rogawsi and Loscher, 2004). Thus, CBZ hypothetically reduces the maximal rate of occurrence of population spikes, in addition to reducing the frequency of seizures. With bandpass filtering, fPSPs and population spikes could be isolated from each another, which allowed analysis of them during an electrographic seizure. Thus, we aimed to determine if CBZ preferentially altered (1) convulsive versus nonconvulsive seizures, (2) the severity of electrographic seizures, and/or (3) the mean or maximum frequency of fPSPs and populations spikes.

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Methods

Kainate treatment and status epilepticus insult.

Convulsive status epilepticus was induced for >3 h in adult, Sprague-Dawley rats (180-200g) with low dose (5 mg/kg) intraperitoneal (IP) administrations of kainic acid (Ocean Produce International, Shelburne, Nova Scotia, Canada) repeated hourly. Equivalent volumes of normal saline were administered hourly to age-matched controls. Motor seizures were scored on a scale from III to V (Racine, 1972). Behaviors during a class III seizure included an erect tail, lordotic posturing, and forelimb clonus; a class IV seizure included the above mentioned behaviors, rearing on hindlimbs, and continued forelimb clonus; and finally, a class V seizure was characterized by the behaviors ascribed to a class IV followed by a fall.

Surgical implantation of electrodes.

Naïve or chronically epileptic rats with kainate-induced epilepsy were implanted with electroencephalograph (EEG) and hippocampal recording electrodes. Surgeries were performed in naïve or chronically epileptic rats. The head of the rat was placed in a stereotaxic apparatus and the surgical site was clipped and washed with betadine scrub and solution. The rat was anesthetized with 2% isoflurane and pretreated with 0.8 ml subcutaneous atropine (0.54 mg/kg), 0.2 ml subcutaneous dexamethasone (2 mg/ml), and 0.2 ml subcutaneous penicillin (300,000 IU). After skin incision and retraction, small holes were drilled through the skull, and the dura was removed for implantation

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of support screws and electrodes. A monopolar, Teflon-insulated stainless-steel stimulating electrode was lowered into the right entorhinal cortex or the angular bundle projecting to the hippocampus. The indifferent stimulating electrode was placed on the dura of the left hemisphere. The depth-recording electrode was positioned in the granule cell layer of the right dentate gyrus, the EEG recording electrode (i.e., a watch screw) was positioned below the dura of the left hemisphere over the temperolimbic cortex, and two ground electrodes were placed in the skull over both hemispheres (for exact electrode coordinates see Fig. 6.1A). The precise location of the electrode in the dentate gyrus with respect to the granule cells was determined using electrophysiological criteria (e.g., positive extracellular synaptic potentials and negative population spikes). Dentate acrylic was used to fasten the electrodes in place and to cover and seal all exposed skull surfaces. The wire leads were gathered into a plastic connector that was also cemented to the skull of the rat. The incision was closed with absorbable 4-0 Dermalon sutures up to the level of the dental cement covering the skull.

Perforant path stimulation and evoked potentials in vivo.

Perforant path stimulation was used in some rats to evoke field potentials from the granule-cell layer of the dentate gyrus (n= 5 kainate- and n=3 saline-treated rats). Single stimuli (0.05 Hz) were delivered to evoke fPSPs with superimposed population spikes, and only those population spikes with distinct fast components (i.e., 1-2 msec) were used for analysis. In preparation for later

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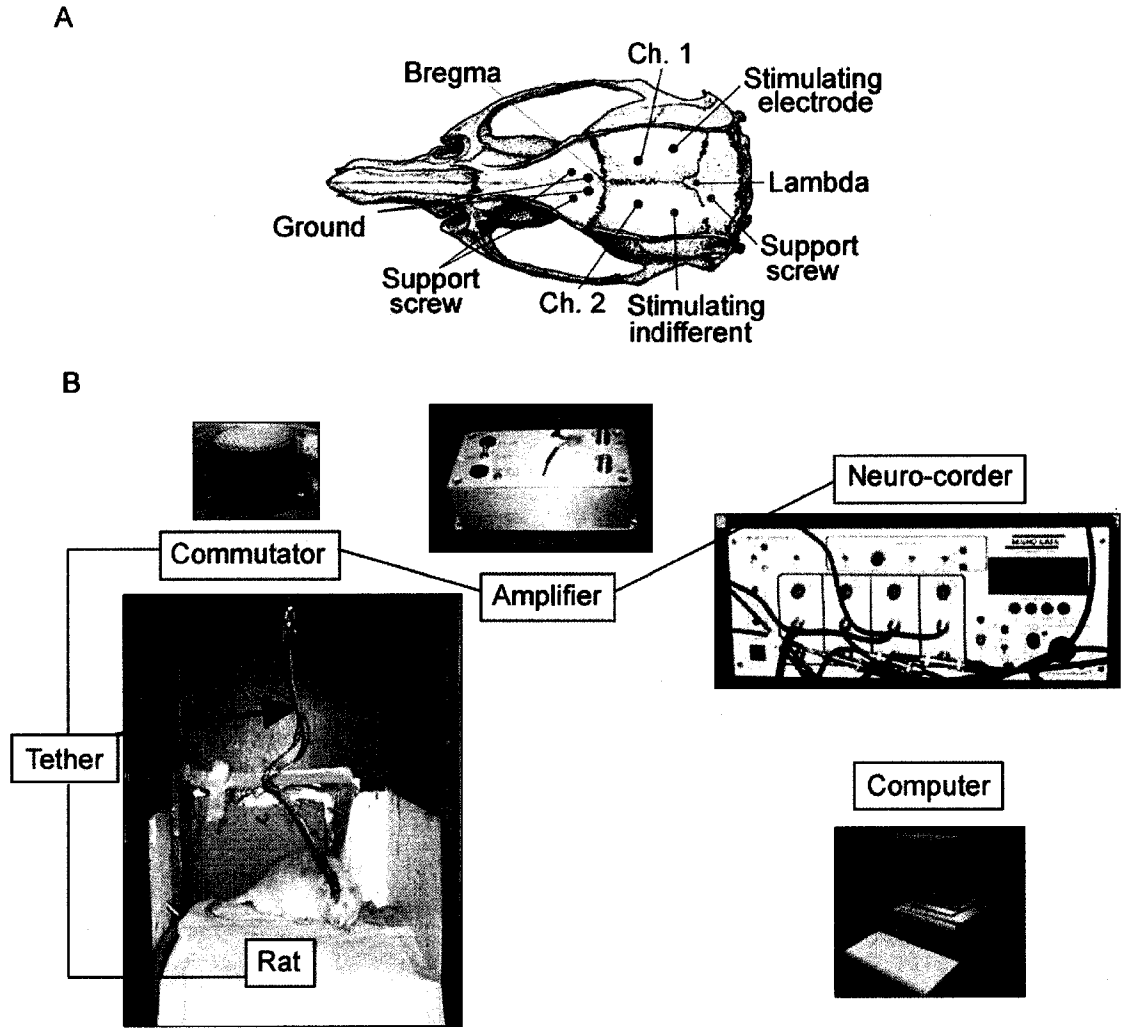

Fig 6.1. Data acquisition. (A) Location of electrode coordinates in reference to bregma. The stimulating electrode was stereotaxically placed in the right entorhinal cortex (4.0 mm lateral, 8.0 mm caudal, 2.3-3.5 mm depth; 0.0045" diameter). The indifferent electrode was positioned on the dura of the left hemisphere (4.4 mm lateral, 8.0 mm caudal; 0.007" diameter). The hippocampal recording electrode (Ch. 1) was placed in the granule cell layer of the dentate gyrus (2.5 mm lateral, 4.0 mm caudal, 2.7-2.9 mm depth; 0.003" diameter). The EEG recording electrode (Ch. 2) was positioned on the dura of the left hemisphere (2.5 mm lateral, 4.0 mm caudal, 0.007" diameter). Ground electrodes were placed on both hemispheres (2.0 mm lateral, 3.5 mm rostral, 0.013" diameter). **(B)** Implanted rats were connected to a commutator and custom-built amplifier. Field-potential recordings were amplified X100 and acquired on videotape via a Neuro-Corder for off-line analysis.

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experiments, individual fast Fourier analyses (using a Hamming window, padded with endpoints) of isolated fPSPs (n= 38 events from kainate-treated rats and 23 events from saline-treated rats) and fPSPs with superimposed population spikes (~20 per recording, 272 total responses from kainate-treated rats and 147 from saline-treated rats) were conducted to isolate the bandwidths in which fPSPs and population spikes occurred.

Repeated-measures, cross-over protocol for AED testing.

The rats were connected to the commutator for recording sessions to test the effect of CBZ versus vehicle-control on spontaneous electrographic seizures. The repeated-measures, cross-over protocol outlined in Figure 6.2 was performed with 10, 30, and 100 mg/kg CBZ. Each trial involved six treatments of AED and six vehicle treatments administered on alternated days (e.g., Sun., Tues., Thurs., Sat.) with a recovery day between the treatment days (Fig. 6.2). IP injections of AED or vehicle were administered at 9:00 AM on treatment days to rats with kainate-induced epilepsy and relatively regular seizures (Table 6.1). Analysis of 8-h field-potential recordings (9:00 AM to 5:00 PM) were conducted to determine the peak effect of CBZ on electrographic seizures. Each trial spanned about 4 weeks for 6 AED-versus-vehicle tests. Two rats could be recorded simultaneously, so a 1-month-long trial tested one dose of AED relative to vehicle in 4 rats. Each trial was conducted twice to achieve 8 replicates for a single dose of AED (i.e., 2 months).

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Video-monitoring of spontaneous seizures.

Each rat was video-monitored for the entirety of the protocol on 8-h videotapes by a Panasonic WV-BP334 black and white camera to associate the motor manifestations with electrographic seizure activity. Seizure severity was scored as follows: a resting rat with no behavioral manifestations during electrographic seizure activity was described as having a class zero seizure, a class I seizure was defined by behavioral arrest; a class II seizure included facial muscle contractions, head bobbing, staring, and chewing behaviors; and class III-V seizures are described above (Racine, 1972). Class zero, class I and class II were considered nonconvulsive seizures (i.e., roughly equivalent to complex partial seizures). Convulsive seizures (i.e., tonic-clonic generalized seizures) were defined as class III and above. A trained technician blinded to the treatment and date of the recordings viewed and scored the behavior.

Chronic in vivo recordings and data analysis of electrophysiology.

Electrographic recordings were conducted during vehicle and CBZ treatments. Rats were placed in the recording chamber and the tether was attached to a commutator system that allowed the animal to move freely. Rats were monitored both behaviorally and electrographically for 8 h (9:00 AM to 5:00 PM) for each recording session. Seizures were quantified and analyzed based on seizure number, duration, and type (i.e., convulsive or nonconvulsive, as described above). Seizure onset was determined as a decrease in the interval between interictal spikes, sometimes marked by a sentinel spike. The amplitude

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A

Sunday	Monday	Tuesday	Wednesday
AED	recovery		recovery
	recovery	AED	recovery
recovery	AED	recovery	
recovery		recovery	AED

B

Week 1							
Rat #	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
1							
2							
3							
4							
Week 2							
Rat #	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
1							
2							
3							
4							
Week 3							
Rat #	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
1							
2							
3							
4							
Week 4							
Rat #	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
1							
2							
3							
4							

Fig 6.2. Experimental Protocol. (A) One AED-versus-vehicle test (i.e., a single cross-over) in which CBZ or vehicle (20% (2-Hydroxypropyl)-ß-cyclodextrin) was administered on alternate days to each animal. **(B)** The AED-versus-vehicle test shown in A was repeated to ensure each animal received approximately six AED-versus-vehicle tests. Two rats could be recorded simultaneously, so a 1-month-long trial tested one dose of CBZ in 4 rats. The protocol shown in B was conducted twice to achieve at least six AED-versus-vehicle tests in 8 animals (i.e., ~2 months per dose).

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Table 6.1. Summary of animals used to evaluate the effect of CBZ on electrographic seizures

rat	Time since SE (days [months]) start-end	Median (days [months])	Dose	Est. sz/day (vehicle) Non.	Conv.	Sz block	Ephys.
1	147-252 [4.9-8.4]	200 [6.7]	30	2	12	N*	N
2	148-293 [4.9-9.8]	221 [7.4]	30	3	13	N>	Y
	298-427 [9.9-14.2]	363 [12.1]	100	2	14	Y	N
3	132-152 [4.4-5.1]	142 [4.7]	30	65	9	Y	N
4	117-257 [3.9-8.6]	187 [6.2]	30	18	10	N>	Y
	281-302 [9.4-10.1]	292 [9.7]	100	18	3	N>	Y
5	118-175 [3.9-5.8]	147 [4.9]	30	6	15	N*	N
6	208-261 [6.9-8.7]	235 [7.8]	30	3	21	Y	N
	279-372 [9.3-12.4]	326 [10.9]	100	1	27	N*	N
7	191-223 [6.4-7.4]	207 [6.9]	30	3	9	N>	Y
8	180-223 [6.0-7.4]	202 [6.8]	30	11	8	N>	Y
	244-362 [8.1-12.1]	303 [10.1]	100	6	6	Y	N
9	205-240 [6.8-8.0]	223 [7.4]	100	6	10	Y	N
10	210-233 [7.0-7.8]	222 [7.4]	100	9	16	N>	Y
11	324-357 [10.8-11.9]	341 [11.4]	10	5	8	N	-
	199-299 [6.6-10.0]	249 [8.3]	100	5	18	N*	N
12	216-242 [7.2-8.1]	229 [11.3]	100	12	0	N>	Y
13	216-242 [7.2-8.1]	229 [11.3]	100	0	7	Y	N
14	304-398 [3.8-13.3]	351 [11.7]	10	5	10	N	-
	163-273 [5.4-9.1]	218 [7.3]	100	9	21	Y	N
15	378-480 [12.6-16.0]	429 [14.3]	10	8	9	N	-
16	145-195 [4.8-6.5]	170 [5.7]	10	52	4	N	-
17	146-239 [4.9-8.0]	193 [6.4]	10	11	5	N	-
18	149-240 [5.0-8.0]	195 [6.5]	10	16	5	N	-
19	150-241 [5.0-8.0]	196 [6.5]	10	1	8	N	-
20	134-208 [4.5-6.9]	171 [5.7]	10	14	14	N	-

SE, status epilepticus
[ ], signifies estimated number of months assuming I month equals 30 days
sz, seizure
Est., estimated
Non., nonconvulsive
Conv., convulsive
N*, animals with poor dentate recordings
N>, animals used in electrophysiological analysis
Ephys., electrophysiological analysis
-, not applicable

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of recorded activity needed to exceed three times baseline noise in order to be defined as a spontaneous seizure. The EEG data were amplified (100x), digitized (Neuro-Corder, Neuro Data Instruments), and stored on videotapes for off-line analysis (Fig. 6.1B) of field-potential recordings using Biopac software (Biopac Systems, Inc., Goleta, CA). Field-potential recordings were digitized at 2 kHz (MP150, Biopac Systems, Inc.), and then low-pass and band-pass filtered to independently isolate both the fPSP band (<100 Hz) and the population spike band (200-500 Hz).

Statistical Analyses

Frequency of convulsive and nonconvulsive seizures during CBZ treatment relative to vehicle treatments was compared using a parametric statistical test (i.e., a repeated-measures ANOVA) of log-transformed data [i.e., data was analyzed on the $\log_{10}(y+0.1)$ scale]. Potential differences in drug effect on different seizure types were compared using a paired student's t test. A non-parametric statistical test (i.e., a Wilcoxon signed-rank test), which does not assume a Gaussian distribution of the data, was also used to compare the frequency of convulsive and nonconvulsive seizures (i.e., number of seizures per treatment) during CBZ and control treatments on a linear scale (Grabenstatter et al., in prep). The effect of CBZ on convulsive and nonconvulsive seizures was compared to determine significant differences ($p<0.05$) using a paired t test. A Chi-squared test on contingency tables was performed to determine if the difference in the proportion of nonconvulsive versus convulsive seizures during

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CBZ treatment was significant. Mean seizure durations per recording period for animals with remaining seizures after both drug and control treatments were also compared using a Wilcoxon signed rank test. A repeated-measures ANOVA with a Bonferroni's multiple comparison test was used to compare maximum and mean frequency of fPSPs and population spikes occurring during seizures after drug or control treatment.

Results

CBZ preferentially reduced convulsive seizures compared to nonconvulsive seizures.

Both convulsive and nonconvulsive seizures recorded electrographically in the dentate gyrus typically began with positive-going events that appeared to be fPSPs; population spikes usually began to occur later in the seizure; and, the seizure ended with slowing of these events, followed by a silent period (Fig. 6.3). CBZ (10, 30, and 100 mg/kg) significantly reduced relative seizure frequency, and completely blocked seizures in 25% and 50% of animals tested in the 30 mg/kg and 100 mg/kg trials, respectively (i.e., 2 out of 8 animals and 5 out 10 animals had complete seizure cessation with 30 and 100 mg/kg, respectively). One measure of severity is whether the seizure is convulsive or nonconvulsive; therefore, the effect of CBZ was determined on convulsive and nonconvulsive seizure types. Two statistical analyses were conducted to control for the variance present in the data: (1) a parametric test on the $\log_{10}(y)$ scale (i.e., a

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repeated-measures ANOVA), and (2) a non-parametric test on the linear scale (i.e., a Wilcoxon signed-rank test). Using a parametric test on log-transformed data, CBZ significantly reduced convulsive seizures relative to vehicle at 10, 30, and 100 mg/kg (Fig. 6.4A1). Single IP injections of 100 mg/kg CBZ, in particular, were significantly more effective at reducing convulsive seizures than nonconvulsive seizures. Reanalysis of the data set using a non-parametric test (i.e., a Wilcoxon signed-rank test) also showed CBZ to be more effective at reducing convulsive seizures than nonconvulsive seizures, but the effect at 100 mg/kg was not significantly different ($p<0.05$) using this test (Fig. 6.4 A2). Increases in the dose of CBZ led to greater reductions in the occurrence of convulsive seizures, but did not progressively reduce the frequency of nonconvulsive seizures. A summary analysis showed that the ratio of nonconvulsive to convulsive seizures increased from 10 to 30 to 100 mg/kg CBZ (Fig 6.4B).

Effect of CBZ on seizure duration.

Another measure of seizure severity is duration, which is best measured with electrographic recordings. Seizure duration varied between animals and within individual animals. CBZ did not significantly reduce mean seizure duration per animal relative to control treatment in that animal at any of the doses (Fig. 6.5A). Seizure durations (i.e., analyzed across all animals) were distributed similarly for control and 30 mg/kg CBZ (Fig. 6.5B), but at 100 mg/kg CBZ, the proportion of seizures <20 s was increased and >60 s was decreased compared

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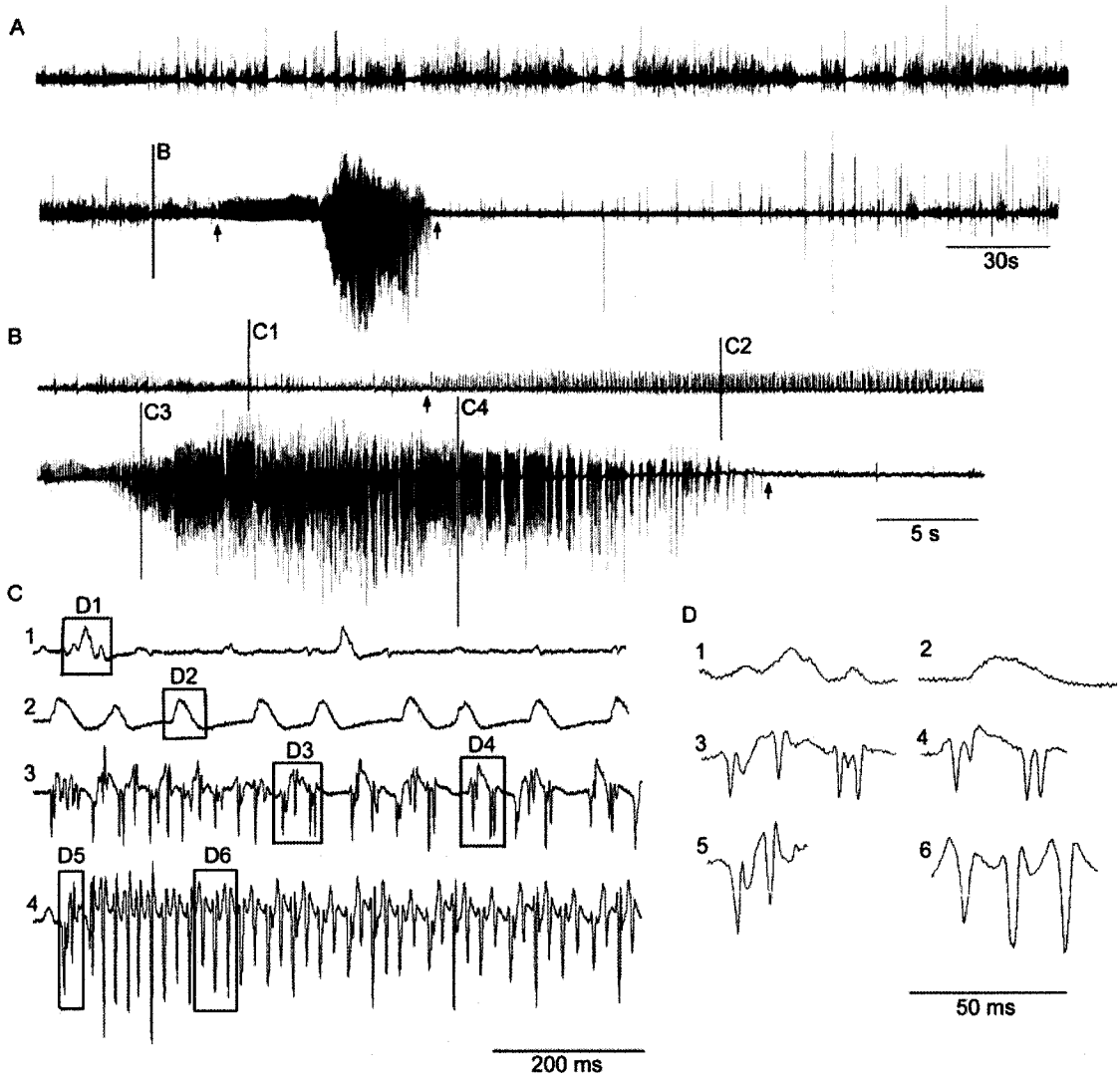

Fig 6.3. Electrographic seizure. (A) A class V, convulsive seizure recorded from the dentate granule cell layer of the hippocampus 190 days after kainate treatment. The seizure was followed by a relatively silent period. **(B)** The seizure shown in A was expanded to illustrate the gradual increase in amplitude and frequency of positive-going field potentials. These events were ~20-50 ms and interpreted to be fPSPs with superimposed population spikes (i.e., synchronous action potentials) preceding seizure onset and during the ictal event. Arrows show the estimated onset and offset of the seizure event. **(C)** Further temporal expansion of electrophysiological events shown in B. Segments of activity occurring before the seizure (1), during seizure onset (2), during tonic activity (3) and during clonic activity (4) are shown. **(D)** Additional temporal expansion of events in C that contain clear fPSPs (1 and 2) or population spikes (3-6) are shown on a faster time scale.

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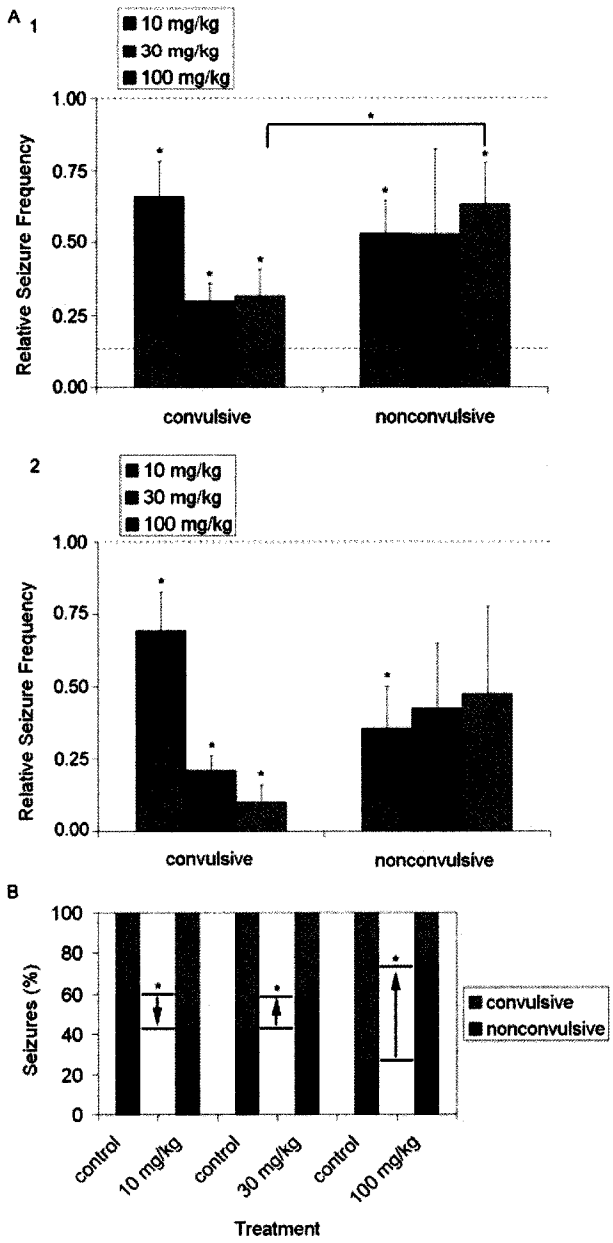

Fig 6.4. Differential effect of CBZ on convulsive and nonconvulsive seizures. (A) The antiepileptic effects of the different doses of CBZ on both seizure types are compared. (1) Following a parametric test on log-transformed data (i.e., a repeated-measures ANOVA), CBZ had significant effects on seizure frequency relative to vehicle at the concentrations of 10, 30 and 100 mg/kg for convulsive seizures, and CBZ (100 mg/kg) was significantly more effective at reducing convulsive seizures relative to nonconvulsive seizures. For this and subsequent figures, the upper dashed horizontal line shows the baseline (i.e., no effect). The lower dashed line represents the estimated "floor effect" that occurs using this analysis method when complete seizure block is simulated. Vertical bars, ±SEM. Asterisks represent significance differences of $p<0.05$ (*). (2) The data were reanalyzed on the linear scale using a non-parametric test (i.e., the Wilcoxon signed-rank test). CBZ 30 and 100 mg/kg reduced the relative frequency of convulsive seizures more effectively than nonconvulsive seizures. **(B)** The percent of seizures that were convulsive versus nonconvulsive after 10, 30, or 100 mg/kg CBZ treatments compared to their respective controls. A greater proportion of the seizures were nonconvulsive than convulsive after higher doses of CBZ. Asterisks represent a significant difference, $p<0.05$.

to vehicle (Fig. 6.5C). This effect on seizure duration was mostly represented in 2 of 5 rats. In particular, 100 mg/kg CBZ shortened 80% of the seizures to a length less than half the mean seizure duration during vehicle treatment. Therefore, although CBZ did not reduce the mean seizure duration analyzed by animal at any dose, the overall distribution of seizure durations remaining in 100 mg/kg CBZ was shifted from longer seizures to shorter seizures (i.e., <20 s).

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Both convulsive and nonconvulsive seizures occurred with short and long durations and a range of interseizure intervals during vehicle and drug treatment (Fig. 6.6). However, a high proportion of nonconvulsive seizures present in 100 mg/kg not only had a relatively short duration, but these seizures were typically preceded by a short interseizure interval (see B2 in Fig. 6.6). Therefore, the high proportion of short-duration nonconvulsive seizures remaining at 100 mg/kg CBZ likely occurred as subsequent or later seizures in a cluster (i.e., occurred within 1 h after a preceding seizure).

Isolation of field postsynaptic potentials and population spikes to specific frequency bandwidths.

Fast Fourier transforms of the field potentials (i.e., fPSP with superimposed population spike) evoked in saline (n=147 events in 3 rats, Fig. 6.7A) and kainate-treated rats (n=272 in 5 rats, Fig. 6.7B) in response to single stimuli of the perforant path at relatively low (trace 1 in Fig. 6.7A and B) and high intensity (trace 2 in Fig. 6.7A and B) were conducted in order to separate the fPSP and population spike in the evoked response based on the frequency range for these events (Fig. 6.7). Power was consistently detected in two separate physiological ranges in both kainate- and saline-treated rats representative of fPSPs (i.e., <200Hz) and population spikes (i.e., 200-500 Hz). Fast Fourier analysis of evoked fPSPs at low-intensity stimulation (i.e., with no associated population spike) in saline (n=23 events in 3 rats) and kainate-treated rats (n=38 events in 5 rats) confirmed that the frequency range of fPSPs was <200 Hz, and

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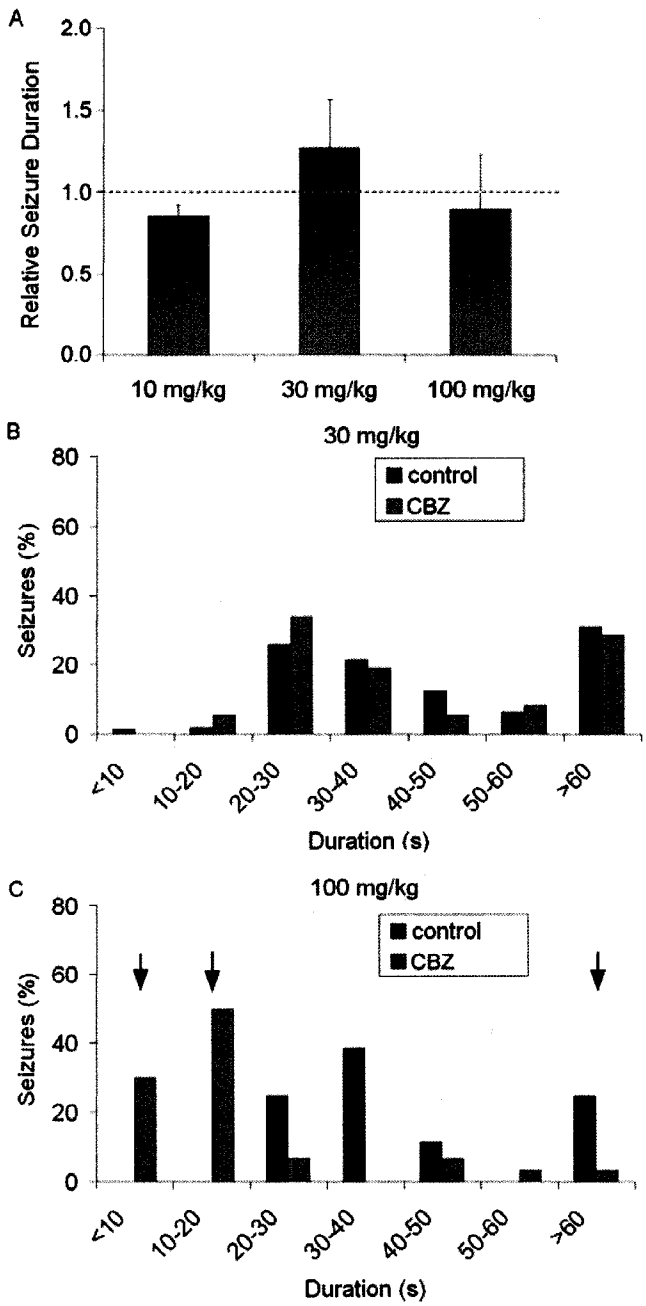

Fig 6.5. Effect of CBZ on duration of spontaneous seizures. (A) Following a non-parametric, Wilcoxon signed-rank analysis on linear data, 10, 30 and 100 mg/kg CBZ did not significantly reduce the duration of total spontaneous seizures relative to vehicle. Vertical bars, ±SEM. **(B)** The percentage of seizures that were short versus long was similar for 30 mg/kg CBZ and control treatments. **(C)** Many more seizures were short duration (i.e., <20 s) and fewer were long duration (i.e., >60 s) at 100 mg/kg CBZ. Arrows show the shift in seizure duration after 100 mg/kg CBZ.

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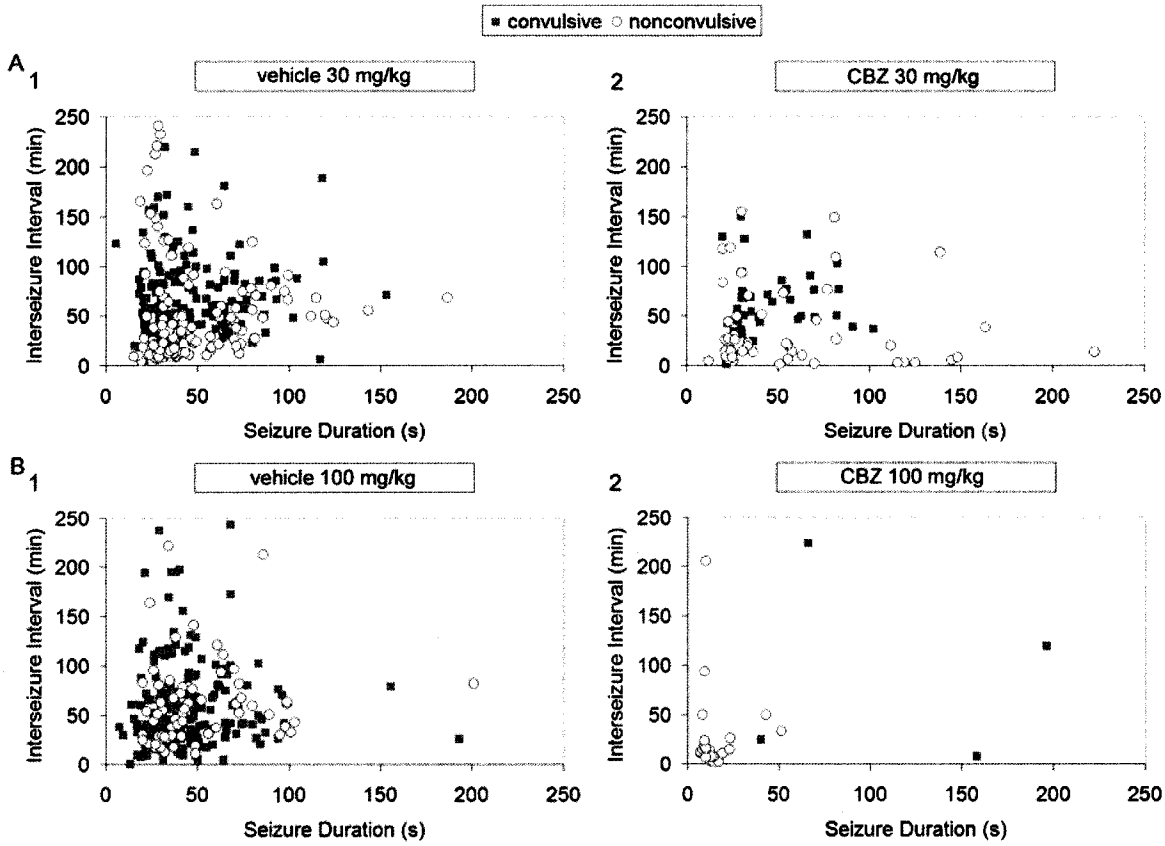

Fig 6.6. Relationship between seizure duration and interseizure intervals for convulsive and nonconvulsive seizures. (A) The interseizure interval before the second and subsequent seizures observed during all 8-h recording periods after 30 mg/kg CBZ was plotted as a function of seizure duration. After CBZ treatment, most of the remaining nonconvulsive seizures had short interseizure intervals and short durations. **(B)** This pattern is more apparent when interseizure interval is plotted as a function of seizure duration for the second and subsequent seizures occurring after vehicle and compared to 100 mg/kg CBZ.

the peak values were roughly (15-70 Hz, mean of about 51 Hz). In both control and kainate-treated rats, fast Fourier analysis of the responses to high-intensity stimulation that included a population spike revealed a frequency band of 200-500 Hz, with a peak at 281 and 313 for control and kainate-treated rats, respectively (i.e., mean of about 297 Hz). Thus, the frequency bands were separate and the peak frequency ranges differed by nearly five-fold, providing a

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physiological basis for separating fPSPs from population spikes with band-pass filtering (see mean frequency ranges for all events in Table 6.2A).

In a second experiment, fast Fourier transforms of short segments of fPSPs and population spikes as different electrophysiological components of spontaneous activity in saline-treated rats (n=62 30-s segments in 4 rats) versus interictal (n=112 30-s segments, no less than 10 min before or after a seizure in 5 rats) and seizure activity (n=189 seizures in 5 rats) in kainate-treated rats. Power was detected at varying magnitudes in two physiological frequency ranges (i.e., <100 Hz and 200-500 Hz) in these three groups (see mean frequency ranges for all events in Table 6.2B, Fig. 6.8). The magnitude of the power detected in both low- and high-frequency bands was greater in interictal periods of recordings from kainate-treated rats relative to normal controls, and greater still in seizures versus interictal periods in epileptic rats (Fig. 6.8). Although the larger population spikes usually occurred during the latter portions of the seizure (Fig. 6.9), activity in the 200-500 Hz range could be detected during the initial events of a seizure (e.g., see lowest trace in Fig. 6.9 B1). Therefore, these cumulative results support previous work (Bragin et al., 1999a; 1999b; 2004; Staba, 2002) suggesting that power representative of fPSPs and population spikes is measurable below 100 Hz and within a frequency range of 200–500 Hz, respectively. Field-potential recordings were subsequently low-pass filtered below 100 Hz and band-pass filtered between 200 and 500 Hz to isolate fPSPs from population spikes (Fig. 6.9).

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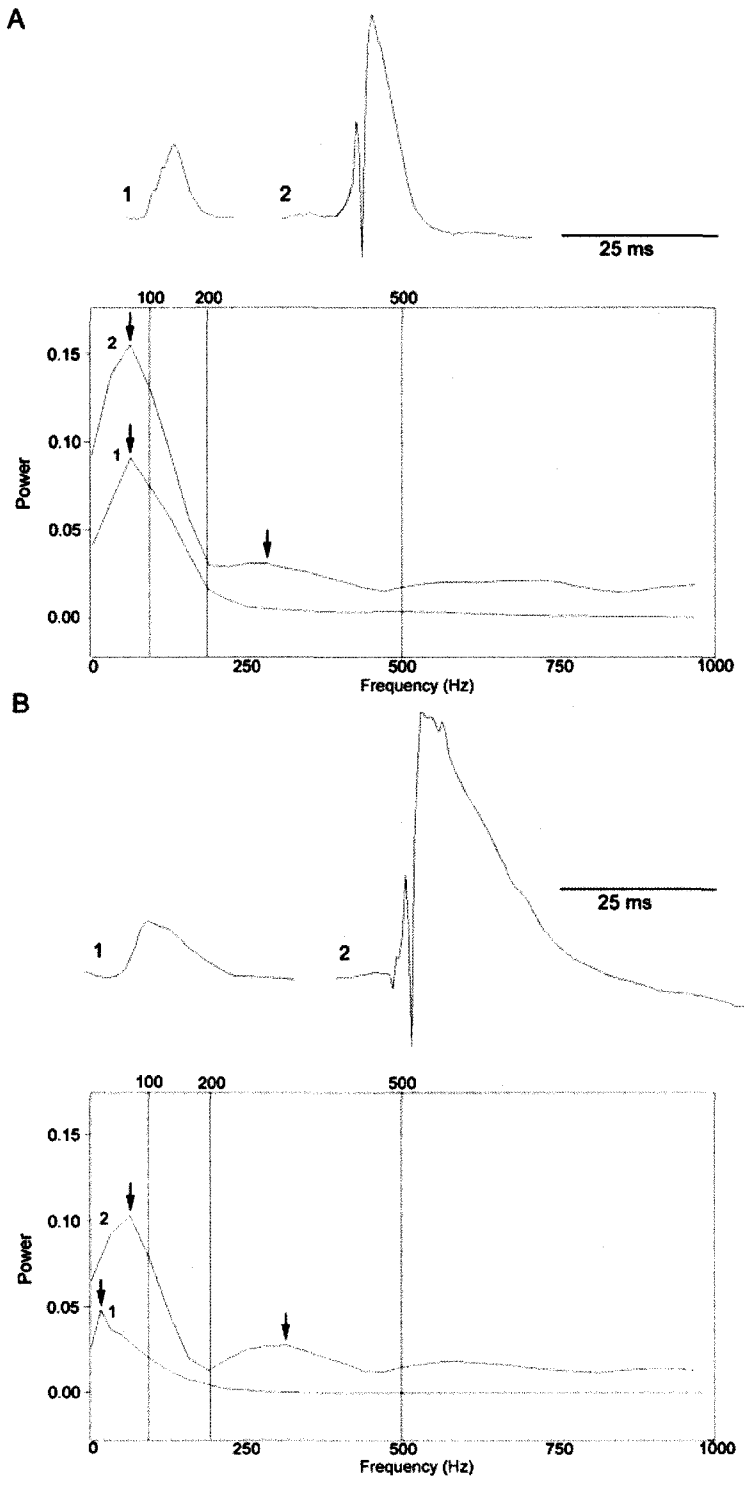

Fig 6.7. Fast Fourier analysis of evoked potentials in the granule cell layer from normal and kainate-treated rats. (A) Responses to low- and high-intensity stimuli, respectively (1 and 2) in saline-treated rats. Traces are examples of randomly selected events used to generate each averaged power spectrum. The frequency ranges of isolated fPSPs and evoked fPSPs with superimposed population spikes were evaluated. The averaged power spectrum of eight randomly-selected, evoked fPSPs (1) and the averaged power spectrum of eight evoked population spikes superimposed on a fPSP in saline-treated animals are shown here (2). **(B)** In kainate-treated animals, (1) the averaged power spectrum of randomly-selected, evoked fPSPs (n=12) and (2) the averaged power spectrum of evoked population spikes superimposed on a single fPSP (n=10) are shown. Arrows show the frequency where peak power was measured for given event.

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Table 6.2. Frequency (Hz) ranges and peaks of electrophysiological components based on spectral analyses (i.e., fast Fourier transforms).

A	single fPSP (Hz)	fPSP and superimposed population spike	
		fPSP	population spike
Control	<171 ± 4 (63)	<169 ± 3 (63)	249 ± 5 to 504 ± 8 (281)
Kainate	<141 ± 6 (16)	<131 ± 2 (63)	218 ± 3 to 490 ± 5 (313)

( ), the frequency where peak power was measured

B	Spontaneous-Control	Interictal Kainate	Ictal Kainate
fPSPs	<103 ± 3	<116 ± 3	<110 ± 2
Population spikes	201 ± 6 to 448 ± 12	219 ± 4 to 473 ± 8	204 ± 4 to 444 ± 9

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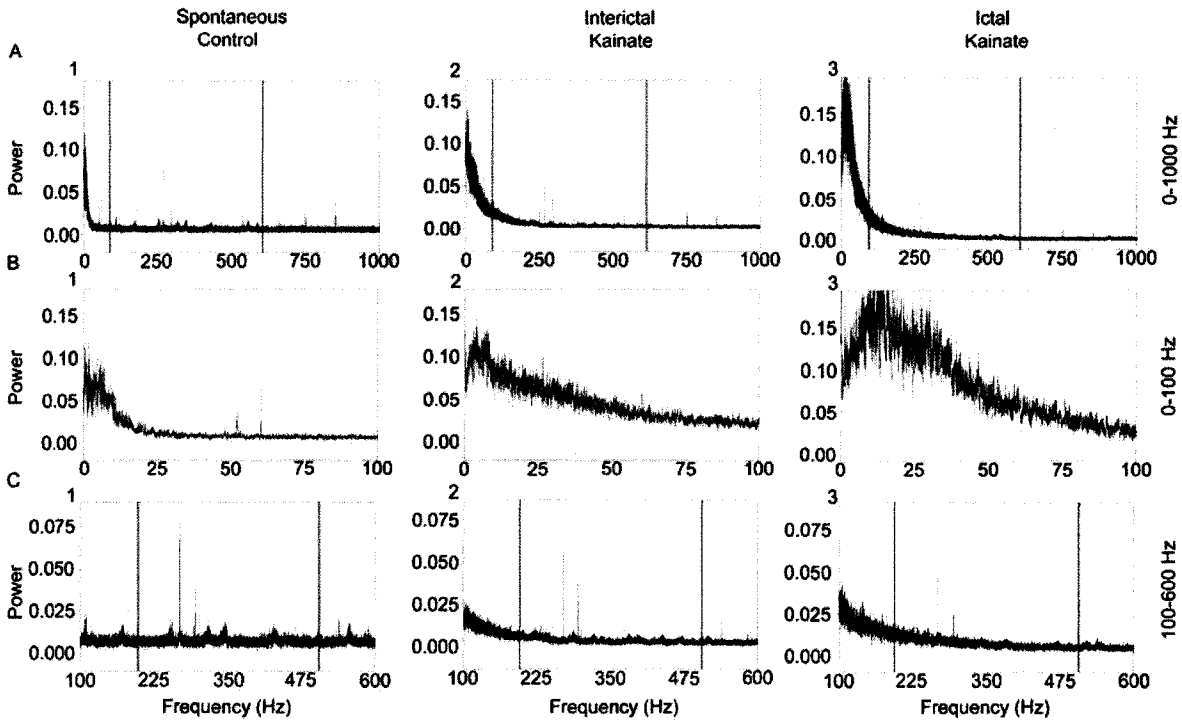

Fig 6.8. Averaged power spectrum of spontaneous activity from the dentate gyrus of control and kainate-treated rats. (A) Averaged power spectrum of randomly-selected, 30-s segments of spontaneous activity recorded from the dentate granule cell layer of saline-treated animals (n=6; 1) and interictal activity (n=14; 2) and spontaneous seizures (n=12; 3) randomly selected from kainate-treated rats. **(B)** The power in the low-frequency range of <100 Hz (i.e., fPSP activity) is expanded and shown for spontaneous activity from saline-treated animals (1) and during interictal periods (2) and spontaneous seizures from kainate-treated animals (3). **(C)** The power in the high-frequency band (i.e., activity representative of population spikes) between 100-600 Hz is shown at higher gain for periods of spontaneous activity from saline-treated animals (1) and interictal activity (2) and spontaneous seizures from kainate-treated animals (3). The magnitude of power in low- and high-frequency bands was greater during the interictal period in kainate-treated animals than during spontaneous activity in control rats. Both frequency bands had more power during spontaneous seizures than during the interictal period in rats with kainate-induced epilepsy.

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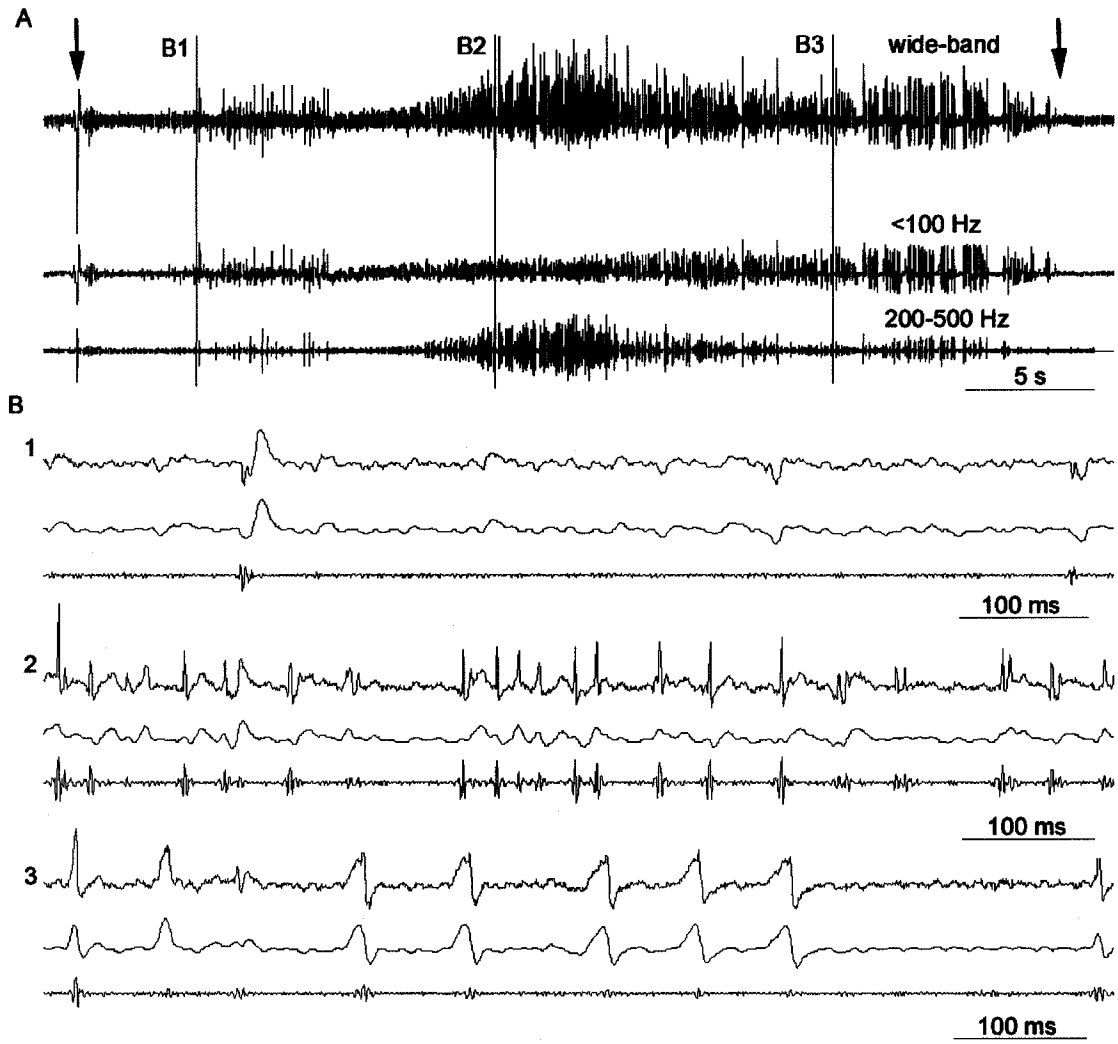

Fig 6.9. Bandpass separation of fPSPs and population spikes in field-potential recordings from the dentate gyrus during an electrographic seizure. (A) A field-potential recording of a seizure from onset to termination, showing positively-going fPSPs with superimposed negative-going population spikes. Second trace, isolated fPSPs (low-pass filtered, <100 Hz). Third trace, isolated population spikes (band-pass filtered, 200-500 Hz). Arrows indicate onset and offset of seizure. **(B)** Expanded segments from the (1) beginning, (2) middle, and (3) end of the seizure in A show isolated fPSPs in the second trace (low-pass filtered, <100 Hz) and isolated population spikes (band-pass filtered, 200-500 Hz) in the third trace of each section.

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Effect of CBZ on the frequency of fPSPs or population spikes.

CBZ is known to reduce maximum firing frequency, and the effect of CBZ on mean and maximum frequency of fPSPs and population spikes was analyzed. At 30 mg/kg and 100 mg/kg, CBZ had no detectable effect on either mean or maximum frequency of the fPSPs (Fig. 6.10). The maximum frequency of the population spikes (but not the mean frequency), however, was significantly reduced at 30 mg/kg, but not 100 mg/kg CBZ. At 30 mg/kg, CBZ significantly reduced the maximum frequency of population spikes during spontaneous seizures by 22% (Fig. 6.10A). The lack of effect on maximum frequency of population spikes at 100 mg/kg (Fig. 6.10B) could have been due to higher variance and a smaller number of remaining seizures at this dose. This finding is consistent with the hypothesis that the shortest inter-event intervals of population spikes would be longer during CBZ treatment, due to the known effect of CBZ on high-frequency firing. Further analysis of the relationship between seizure duration and maximum frequency of fPSPs and population spikes suggested that the highest maximum frequency of population spikes was often associated with the longest seizures (Fig. 6.11 B1), and the seizures remaining after 100 mg/kg CBZ tended to have a lower maximum population spike frequency and lower seizure durations. Therefore, the overall effect of CBZ on the structure of the remaining seizures was not only to increase the proportion of nonconvulsive seizures and increase the proportion of short (<20 s) vs. long (>60 s) seizures in clusters, but also to lower the maximum frequency of population spikes, particularly during seizures of short duration.

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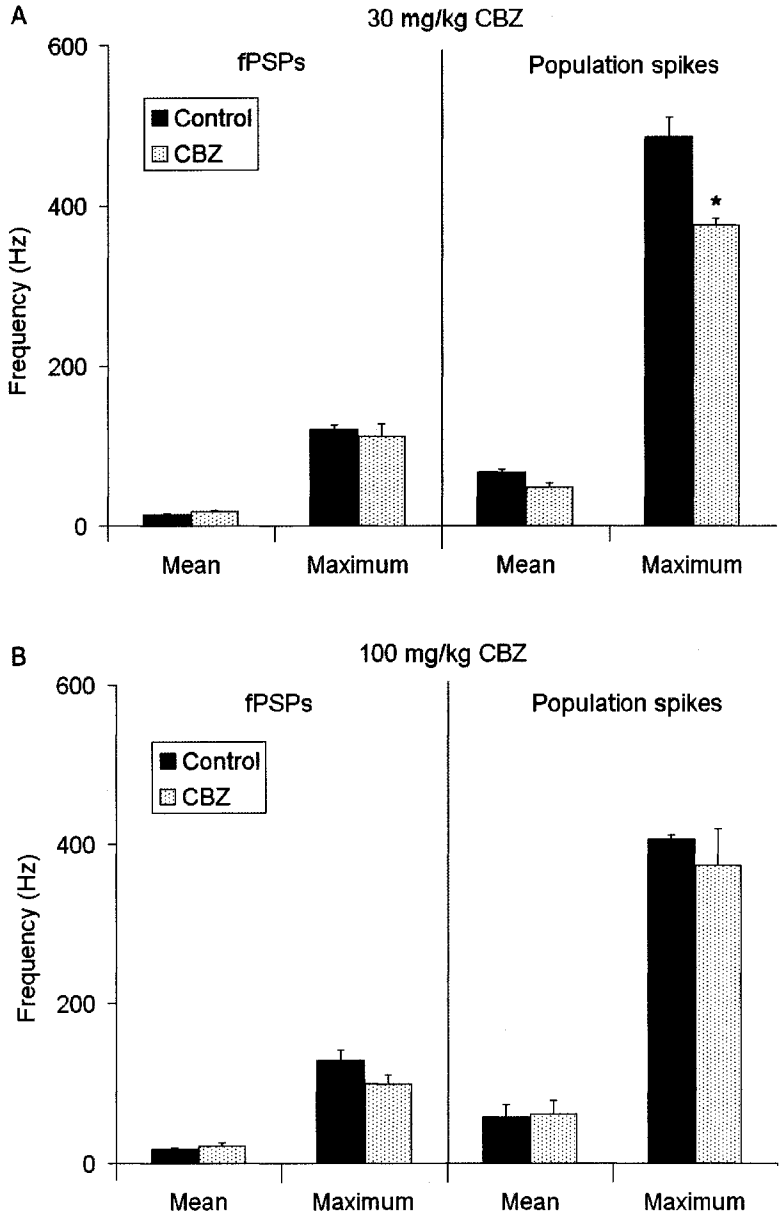

Fig 6.10. Effect of CBZ on electrophysiological characteristics of spontaneous seizures in vivo. The effect of 30 mg/kg CBZ **(A)** and 100 mg/kg CBZ **(B)** on mean and maximum frequency of fPSPs (<100 Hz) and population spikes (200-500 Hz) during spontaneous seizures relative to vehicle treatment. Black bars and dotted bars show the length of interevent intervals during control and CBZ, respectively. Vertical bars, ±SEM. Asterisk (*), significant difference, $p<0.05$.

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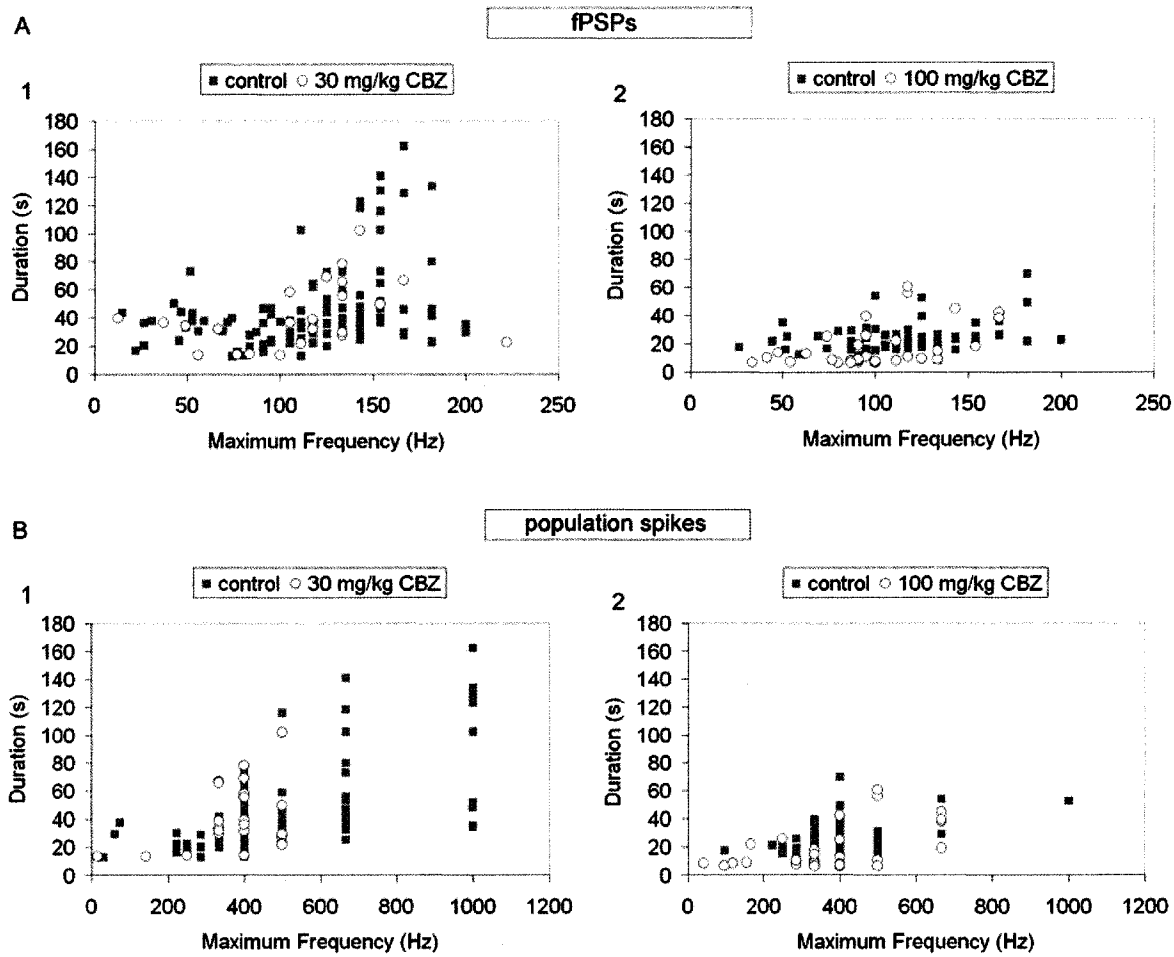

Fig 6.11. Relationship between maximum frequency of fPSPs and population spikes as a function of seizure duration. (A) Seizure duration and the maximum frequency of the fPSPs were measured during the seizures after control and CBZ injections at (1) 30 mg/kg and (2) 100 mg/kg. **(B)** Likewise, the shortest measured interval between population spikes (i.e., maximum frequency) as a function of seizure duration after control and CBZ treatments at (1) 30 mg/kg and (2) 100 mg/kg.

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Discussion

These experiments used chronic field-potential recordings from the dentate gyrus of rats with kainate-induced epilepsy to extend our previous work assessing the effects of AEDs on convulsive, motor seizures to analyses of electrographic seizures (Grabenstatter et al., 2005). As before, a repeated-measures, cross-over design was used to control for differences in seizure frequency between kainate-treated rats, to partially mitigate against seizure clusters, and to optimize statistical power (i.e., enhance the number of replications) while minimizing the number of experimental subjects. Also, as before (Grabenstatter et al., 2005), the rats were studied many months after kainate-induced status epilepticus, and were shown to have relatively high seizure frequencies before inclusion in the studies (see Table 6.1).

The data suggest that CBZ preferentially blocks convulsive versus nonconvulsive seizures, and although significant differences were not detected in mean seizure duration when normalized by animal, the proportion of all seizures across all animals <20 s was increased and >60 s was decreased at 100 mg/kg (in 2 out of 5 animals that still had seizures at 100 mg/kg CBZ). An analysis of perforant path stimulation in control and kainate-treated animals allowed evaluation of the frequency bands that represent the fPSPs and population spikes, which in turn revealed that both bands showed increases in power during seizures versus interictal periods in rats with kainate-induced epilepsy, and these values were higher than controls. CBZ reduced the maximum frequency of population spikes at 30 mg/kg CBZ, but significant differences for population

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spikes were not detected at 100 mg/kg or at either dose for fPSPs. Thus, electrographic recordings revealed a CBZ-mediated reduction of seizure frequency and seizure severity, and also a reduction in the maximum frequency of population spikes at one dose of CBZ. These studies also highlight some of the difficulties involved in analyzing the effects of AEDs on the components of electrographic seizures.

Frequency of seizures

CBZ is a highly effective and widely prescribed AED, and with increasing doses, CBZ more effectively reduced the frequency of spontaneous seizures, especially convulsive seizures. Analysis of linear data using a non-parametric test showed dose-dependent effects by CBZ on the frequency of convulsive seizures. This effect was not seen in the log-transformed data, which may relate to the presence of the "floor effect" that arises inherently from this type of analysis. The strong anticonvulsant effect of CBZ, particularly at 100 mg/kg, leads to an important underlying problem because the smaller number of seizures that occur as the dose is increased lowers the statistical power of the experiments that involve analyses of the remaining seizures during CBZ treatment. Although CBZ may have substantial effects on the properties of some seizures (see below), variance in the properties of the seizures between animals is not reduced. This is a major caveat in analyzing the effects of AEDs on the severity and electrophysiologic components of spontaneous electrographic seizures in freely behaving animals.

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CBZ preferentially reduced convulsive versus nonconvulsive seizures

CBZ often effectively blocks convulsive seizures, but is ineffective on nonconvulsive seizures in patients with temporal lobe epilepsy (Schmidt, 1986). The differential effects of CBZ on convulsive relative to nonconvulsive seizures in rats with kainate-induced epilepsy were similar to those observed in patients with refractory temporal lobe epilepsy. These data suggest either nonconvulsive seizures were relatively resistant to CBZ treatment and/or CBZ effectively reduced convulsive seizures to a less severe, nonconvulsive seizure. For each dose, the simplest analysis was conducted to evaluate the percent reduction of relative seizure frequency for each seizure type (i.e., convulsive versus nonconvulsive). Because statistical power decreases with increasing dose due to the problem of fewer available seizures, the relative proportion of nonconvulsive seizures versus convulsive seizures during drug and control treatment was the best summary analysis of the preferential effect of CBZ on convulsive seizures (Fig. 6.4B). In rats with kainate-induced epilepsy, it is likely that a high proportion of convulsive seizures are reduced to nonconvusive seizures, and fewer nonconvulsive seizures are blocked during CBZ treatment, but further experiments are necessary to evaluate whether CBZ reduces convulsive seizures to nonconvulsive seizures.

Seizure Duration

Two methods of analysis were used to assess the effects of CBZ on seizure duration, a second measure of severity. First, the animals were used as

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their own controls, and mean seizure duration of each recording session during which CBZ was administered was analyzed relative to the subsequent vehicle administration. Those animals with complete seizure block were excluded from the analysis. Controlling for inter-animal variance, CBZ had no significant effects on mean seizure duration at any dose. In a second analysis, all seizure durations recorded during vehicle and CBZ treatments were analyzed, which revealed that some animals had a decrease in long seizures and an increase in short seizures. The animals with shorter seizures during CBZ were the animals with the most seizures during CBZ treatment, and these seizures often occurred in clusters. Interestingly, clustered seizures also manifest clinically as particularly difficult-to-treat seizures (Bauer et al., 1992; Haut et al., 2002). Seizure clustering may have contributed to the results from the analysis of seizure duration. When CBZ was effective at reducing seizures, the remaining nonconvulsive seizures often occurred in clusters, and seizures were shorter when the inter-seizure interval was short. The persistence of short, nonconvulsive seizures during 100 mg/kg may indicate that these seizures are particularly resistant to pharmacological treatment.

Effect of CBZ on components of electrographic seizures

A relatively simple method was used to isolate the two main electrophysiological components (i.e., fPSPs and action potentials) of seizures to specific frequency bands. An fPSP is usually >10 ms, and action potentials are approximately 1-3 ms. Fast Fourier analyses of evoked and spontaneous fPSPs

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and action potentials in normal and kainate-treated rats showed that power representing fPSPs and action potentials occurred <100 Hz and between 200 and 500 Hz, respectively. This an expected result based on the known duration of these electrophysiological events. Although this method is a simple approach to define the bounds of the events in question, it is not perfect and the frequency ranges may vary during different levels of activity. Low-pass and band-pass filtering was used to separate fPSPs from population spikes (i.e., synchronous action potentials), and based on these parameters, the effect of CBZ on these electrophysiological components of seizure activity was evaluated.

CBZ a use-dependent sodium-channel antagonist, should decrease repetitive firing of action potentials, specifically high-frequency firing. Synchronous fPSPs should be driven by synchronous action potentials, but CBZ had no effect on mean or maximum frequency of fPSPs at either dose tested. Hypothetically, the mean inter-event interval of fPSPs was not affected by CBZ treatment, because the measure of "mean inter-event interval" includes both the short and the long intervals during the seizure, which presumably obscures an effect from being detected. The shortest detected inter-event interval of fPSPs during the analysis of maximum frequency was about 10 ms (Fig. 6.10), and CBZ may not affect this frequency, plus fPSPs with shorter inter-event intervals likely undergo summation and would not have been detected in our analysis.

CBZ also did not reduce the mean frequency of population spikes, presumably for the reasons mentioned above. Population spikes occurred at varying frequencies across the length of a seizure, and using "mean frequency'

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as a measure masks the effects on high-frequency firing. CBZ reduced the maximum frequency of population spikes at 30 mg/kg, but not at 100 mg/kg. The lack of effect at 100 mg/kg may be directly related to the experimental caveat that increasing doses lead to fewer seizures and reduced statistical power. Additionally, other analyses showed that the remaining seizures were potentially different in structure (i.e., convulsive versus nonconvulsive). A small but significant difference was detected in maximum population spike frequency during CBZ treatment, and different AEDs could have different effects on seizures.

Conclusions

Using EEG and hippocampal field-potental recordings to record spontaneous seizures from freely moving rats, CBZ has preferential effects on convulsive versus nonconvulsive seizures (similar to the effects of CBZ documented in humans with temporal lobe epilepsy). Although CBZ had no effects on seizure duration when normalized by animal, an increase in the proportion of short seizures occurs in some rats with kainate-induced epilepsy at 100 mg/kg CBZ. Thus, electrographic analyses suggest that CBZ not only decreases seizure frequency, but it also reduces seizure severity (i.e., less convulsive seizures and a greater proportion of shorter seizures), but the effect is not robust. Isolation of population spikes from fPSPs during seizures allowed the analysis of CBZ on these specific components of seizure activity, and although this analysis was difficult to conduct, 30 mg/kg reduced the maximum firing

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frequency of population spikes. Therefore, electrographic analysis shows evidence for a decrease in severity and a reduction in high-frequency firing during CBZ treatment.

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CHAPTER 7: Discussion and Conclusions

Summary

Effect of topiramate on spontaneous motor seizures

By expanding on previous work from our lab (Hernandez et al., 2002), where AEDs were tested on rats with pilocarpine-induced epilepsy, we developed a repeated-measures cross-over protocol in which chronically epileptic rats served as their own controls (Grabenstatter et al., 2005). This protocol was used to assess the effects of topiramate via intraperitoneal injection on spontaneous seizures in rats with kainate-induced epilepsy. With this approach, each rat received the AED or the vehicle-control on alternate days (i.e., was crossed over to the other treatment). This design aimed to control for differences between animals in baseline seizure frequency and also controlled for increases in seizure frequency over time. Because each animal is repeatedly given the AED and control (i.e., approximately six times), the design incorporates repeated-measures that contribute to a high degree of statistical power. In this study, topiramate decreased the number of seizures that occurred during the first 6-h observation period. Topiramate, a new generation, broad-spectrum AED, exerted significant effects at 10, 30, and 100 mg/kg and reduced spontaneous recurrent motor seizures in a dose-dependent manner. Another analysis

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examined recovery from single 30-mg/kg injections of topiramate. A significant effect of topiramate was observed for 12 h after the 30-mg/kg injection, and full recovery from the drug effect was complete within 24 h. The anticonvulsant effects of topiramate parallel those observed in humans with intractable temporal lobe epilepsy (i.e., topiramate reduces seizure frequency but does not block all seizures). Therefore, this experimental design allowed us to evaluate the overall effect on spontaneous seizures, the time-course of recovery from a single injection of AED, and the dose-dependence of drug effect.

Effect of CBZ on spontaneous motor seizures: comparison of IP injections with drug-in-food protocols

To test new AEDs in these chronic models, one also needs to establish different routes of administration and administer the AED over long periods in a convenient manner. Therefore, we administered specially formulated food pellets containing 5 mg carbamazepine per 1-g pellet in both a single-treatment and a prolonged-treatment protocol to mimic a clinical treatment regime (i.e., three times per day for 5 days). CBZ administered in food was as effective at reducing seizure frequency as IP injections 6 h after administration, and CBZ in food (100 mg/kg) had prolonged significant effects on motor seizure frequency after a single feeding (i.e., 20 h). CBZ administered three times a day in food nearly blocked all seizures during the 5-day prolonged-protocol. This design allowed us to routinely administer AEDs in food to animals, evaluate the effects of a drug throughout the day (versus 6 h) and over many days. The drug-in-food

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protocol also minimized the adverse effects (e.g., stress) of the repeated intraperitoneal injections, which is important because stress can induce seizures in these rats.

Effect of RWJ-333369 on spontaneous motor seizures

After proof-of-principle was established for the repeated-measures, cross-over protocol, the effect of an investigational AED, RWJ-333369, on spontaneous seizures in rats with kainate-induced epilepsy was evaluated. RWJ-333369 reduced the frequency of spontaneous motor seizures in a dose-dependent manner, and was more effective than topiramate and carbamazepine at reducing seizures in rats with kainate-induced epilepsy 6 h after IP injection. Measured AED blood concentration levels of RWJ-333369 demonstrated a dose and time-dependent effect that directly paralleled seizure reduction. RWJ-333369 fits the profile of drugs the field would like to develop in the future (i.e., therapeutic doses block all seizures).

Variance and choice of statistical analysis

The methods of statistical analyses and measures of efficacy were modified with experienced gained from continued use of the repeated-measures, cross-over protocol and epileptic animals with spontaneous seizures occurring at variable rates. Cluster-induced variance in seizure frequency necessitated that a log-transformation be performed on data prior to parametric analyses. In early experiments, the effect of AED relative to vehicle was determined using a

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parametric test on log transformed data (i.e., $\log_{10}[y+0.1]$ scale), but experiments with highly effective doses of AEDs and during prolonged treatment when nearly 100% seizure block occurred indicated that the addition of 0.1 to all measures of seizure number per unit (i.e., short-term measures of frequency) time to allow the log transformation masked robust anticonvulsant effects causing a "floor effect". A non-parametric test of linear data (i.e., the Wilcoxon, signed-rank test) was a more appropriate analysis of highly potent AED effects. The original parametric test (i.e., the repeated-measures ANOVA) that was used to analyze log-transformed seizure frequencies was more appropriate for assessing whether an AED is potentially effective versus ineffective at reducing (versus blocking) seizure frequency following single IP injections.

Effect of CBZ on electrographic seizures

While motor seizure frequency is a direct measure of AED effect, it can not illustrate potential AED effects based on mechanisms of action as electrographic data measures do. Information related to seizure type (i.e., convulsive vs. nonconvulsive seizures), region of onset, seizure duration, and frequency and amplitude of synchronous synaptic potentials and action potentials can be obtained using focal field potentials from specific brain sites, such as the dentate gyrus and implanting brain electrodes into rats with chronic epilepsy. Thus, in a final experiment, a method was developed for testing the pharmacosensitivity of AEDs, specifically how they suppress electrographic seizures in freely behaving rats with chronic epilepsy using in vivo

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electrophysiological recordings of hippocampal field potentials. The effects of CBZ on (1) seizure severity (i.e., potential reduction in convulsive seizures and/or seizure duration) and (2) high frequency firing during spontaneous electrographic seizures were evaluated using a repeated-measures, cross-over protocol. CBZ preferentially reduced convulsive seizures versus nonconvulsive seizures, which is consistent with the effects of carbamazepine in human patients with TLE. CBZ did not significantly reduce mean seizure duration, but the proportion of short seizures increased and the proportion of long seizures decreased in some animals after 100 mg/kg CBZ. Band-pass filtering was used to isolate fPSPs from population spikes during spontaneous seizures, and we asked how carbamazepine affects the synchronous synaptic events and action potentials that occur during spontaneous seizures in rats with kainate-induced epilepsy. CBZ did not affect fPSPs, but significantly reduced maximum population spike frequency at 30 mg/kg, but not at 100 mg/kg. A potential caveat in these experiments is the reduced statistical power that occurs with increasing dose and fewer remaining seizures. Additionally, despite the robust AED effects at high doses, inter-animal variance persists. A subtle mechanistic change was detected in high frequency firing during CBZ treatment, but testing the effects of AEDs on electrophysiological components of seizures to determine mechanism of actions was laborious and relatively inefficient due to the lack of power and variance across animals. Hypothetically, different AEDs would have different effects on fPSPs and population spikes based on the known mechanism of action (i.e., effective AEDs have a mechanistic fingerprint). Conversely, all effective AEDs

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may reduce the duration of spontaneous seizures (regardless of mechanism of action, and therefore, may reduce the maximum frequency of population spikes during spontaneous seizures in rats with kainate-induced epilepsy.

Challenges in AED development

Although chronic epilepsy models with spontaneous seizures may be the best suited for the development of AEDs, experiments are hampered by the biologic limitations and lack of high throughput associated with screening drugs in chronically epileptic models. Acquired epilepsy involves a time-dependent change in the brain following traumatic and seizure-induced insults in animal models. The evolving alterations that take place during epileptogenesis in these animals occur over weeks or months finally resulting in a stable seizure rate. This limits the rate at which an investigator can prepare animals and test drug-induced changes in seizure frequency. Additionally, problems with long-term drug administration and documentation of unpredictable seizures are present despite the fact that these models may parallel human epilepsy more closely than other models used for AED screening. These biological constraints ultimately determine the "throughput" and impose limitations on experimental design for preclinical evaluation of AEDs and drugs that may modify postinjury epileptogenesis. The primary goal of this research was to develop possible strategies to treat epilepsy that is refractory to presently available antiepileptic AEDs. These experiments have set the groundwork for those goals. One future aim is to modify the repeated-measures, crossover design used in these

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experiments to achieve a higher degree of power in a shorter period of time in order to utilize the chronically epileptic animals in a high-throughput manner.

The advantage of the repeated-measured, cross-over protocol is that a high degree of power is achieved with only 8 animals. Six AED-versus-vehicle tests in 8 animals generate 48 separate tests, but approximately 24 days (i.e., six repetitions, each requiring 4 days = 24 days) are required to test one dose of one compound. Therefore, in the future experiments, to achieve a high-throughput rate of AED screening, the repeated-measures component of the protocol should be reduced. For example, two crossover tests can be conducted in 24 animals (versus 8) to maintain statistical power. With this modification, rats with kainate-induced epilepsy may be used to rapidly screen AEDs with similar statistical power. Additionally, a well-controlled, daily, drug-in-food protocol will allow the analysis of drug effects over single day-long periods, thus greatly accelerating the pace of AED screening/testing with the kainate model, and reducing adverse effects to rats chronically receiving an experimental AED.

Rats with kainate-induced epilepsy: role in preclinical drug development

Additional work is necessary to complete the pharmalogical profile of rats with kainate-induced epilepsy and to determine whether they are pharmacoresistant. However, experiments testing a traditional AED and a new-generation AED validate that these drugs act similarly on spontaneous seizures in kainate-treated rats as on seizures in patients with temporal lobe epilepsy. Although more AEDs need to be tested, this model (1) is pharmacosensitive to

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clinically successful drugs, (2) does not discriminate between clinical types of AEDs, and (3) detected both effective and ineffective doses of all the AEDs tested. While this model is useful for determining the antiepileptic effect of potential AEDs, studies of toxicity are required in these animals but preliminary data should be gathered in normal animals. If an AED has adverse behavioral effects in normal animals, it should be eliminated from any further screening. This model can be incorporated into current preclinical screening protocols used by the NIH-sponsored Anticonvulsant Drug Development program as a secondary screen of potential AEDs that demonstrate efficacy and minimal toxicity during early testing and in acute seizure tests (i.e., in a similar manner to the way the kindling model is used). Furthermore, rats with kainate-induced epilepsy may potentially be a model of pharmacoresistant epilepsy (i.e., AEDs do not block all seizures in all rats), but additional studies are required to understand the mechanisms underlying the refractory nature of spontaneous seizures (especially nonconvulsive seizures) in this model.

Conclusions

Using the repeated-measures protocol, spontaneous seizures in kainate-treated rats can be used to assess the effects of single drug administrations or prolonged AED effects; and dose-dependent effects of AEDs with different mechanisms of action can be directly compared. Administering AED-in-food is as effective as IP injections at reducing spontaneous seizure frequency and provides a convenient way to conduct and analyze prolonged effects of AEDs

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(i.e., for weeks or months) without adverse effects to the animals. Focal field-potential recordings and EEG can be used to evaluate the effect of an AED on electrographic seizures in rats with kainate-induced epilepsy, specifically (1) reductions in seizure severity, such as reduced seizure duration and preferential reduction of convulsive seizures to nonconvulsive seizures and (2) modulation of electrophysiological components contributing to seizures (i.e., high-frequency firing). Collectively these experiments show that testing potential AEDs in rats with kainate-induced epilepsy is an effective method to detect short and long-term effects of drugs on spontaneous seizures. The techniques developed in these experiments to test AEDs in chronically epileptic rats can be directly used for preclinical AED development, and subsequently may help overcome existing obstacles in epilepsy treatment.

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