Characterization of pharmacoresistance to benzodiazepines in the rat Li-pilocarpine model of status epilepticus

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Abstract

Status epilepticus is usually initially treated with a benzodiazepine such as diazepam. During prolonged seizures, however, patients often lose their sensitivity to benzodiazepines, thus developing pharmacoresistant seizures. In rats, administration of LiCl followed 20–24 h later by pilocarpine induces a continuous, self-sustained, and reproducible form of status epilepticus that can be terminated with diazepam when it is administered soon after the pilocarpine injection. However, when administered after a 45 min delay, diazepam is less effective. Previous findings have suggested that the development of pharmacoresistance is related to the stage of status epilepticus. In the present study, we characterized the seizure stage-dependence of diazepam pharmacoresistance. Following administration of different doses of diazepam at varying time intervals after specific behaviorally- and electrographically-defined seizure stages, stage-, time-, and dose-dependent pharmacoresistance to diazepam developed. We also studied two other antiepileptic drugs commonly used in the treatment of status epilepticus, phenobarbital and phenytoin. Consistent with previous studies, our results indicated a similar relationship between stage, time and dose for phenobarbital, but not for phenytoin. Our data are consistent with rapid modulation of $GABA_A$ receptors during status epilepticus that may result in pharmacoresistance to antiepileptic drugs that enhance $GABA_A$ receptor-mediated inhibition. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Status epilepticus is defined as a condition that occurs "whenever a seizure persists for a sufficient length of time or is repeated frequently enough that recovery between attacks does not occur

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(Commission on Classification and Terminology of the International League Against Epilepsy, 1981)''. Status epilepticus is usually operationally defined as a single or intermittent series of seizures that last at least 30 min, during which the patient does not regain consciousness. Limbic status epilepticus has been suggested to result from increased glutamatergic excitatory synaptic transmission, increased activation of the hippocampal circuit, and/or a loss of $GABA_A$ receptor-mediated hippocampal inhibition (Macdonald and Kapur, 1999). Initial treatment of status epilepticus generally employs administration of benzodiazepines, such as diazepam, lorazepam, or midolazam, which interact with $GABA_A$ receptors to increase inhibition (Macdonald, 1999). Late treatment of refractory status epilepticus often includes barbiturates, such as phenobarbital and pentobarbital, which also act on $GABA_A$ receptors to increase inhibition (Macdonald, 1999). Alternative treatment options involve antiepileptic drugs that act on other receptors or channels; for example, phenytoin acts on voltage-gated sodium channels to reduce high frequency repetitive firing of action potentials (Macdonald, 1999). Status epilepticus can be induced in rats by the administration of a small dose of LiCl followed 20–24 h by the administration of pilocarpine hydrochloride. Prolonged limbic seizures induced by Li-pilocarpine, however, are less responsive to diazepam (Walton and Treiman, 1988; Kapur and Macdonald, 1997) and appear to decrease $GABA_A$ receptor-mediated inhibition and its enhancement by benzodiazepines (Kapur et al., 1989; Kapur and Macdonald, 1997). The cellular mechanisms underlying the loss of benzodiazepine efficacy have yet to be elucidated.

$GABA_A$ receptors are the primary mediators of fast inhibitory synaptic transmission in the central nervous system and are formed by assembly of multiple subunit subtypes into a pentamer. Once assembled, $GABA_A$ receptors form chloride ion channels, and $GABA_A$ receptor currents can be modulated by a number of positive and negative allosteric regulators, including barbiturates, benzodiazepines, and neurosteroids, as well as bicuculline, picrotoxin, and zinc. However, sensitivity to these modulators is dependent on $GABA_A$ receptor subunit subtype composition (Olsen and Macdonald, 2002). For example, sensitivity to benzodiazepines requires the presence of a γ subunit, whereas sensitivity to zinc is reduced by the presence of a γ subunit. It is possible, therefore, that functional changes in $GABA_A$ receptors caused by prolonged seizures may underlie the pharmacoresistance to benzodiazepines seen after status epilepticus. The aim of the present study was to investigate the time course of development of pharmacoresistance to diazepam in the Li-pilocarpine rat model of status epilepticus and to determine whether the same pharmacoresistance develops to two other antiepileptic drugs that are commonly used to treat status epilepticus, phenobarbital and phenytoin.

2. Methods

2.1. Experimental animals

The subjects were 27–40 day old male Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN). The rats were individually housed in standard Plexiglas cages on a 14:10 h light–dark cycle (lights on at 07:00 h), provided with free access to food and tap water, and handled daily. Studies were carried out in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The experimental protocol was approved by the University of Michigan University Committee on the Use and Care of Animals.

2.2. Drugs

All drugs were administered via intraperitoneal (i.p.) injection. Lithium chloride (LiCl; Sigma, St. Louis, MO) was prepared as a stock solution of 1 mEq/ml in distilled water. Pilocarpine hydrochloride (Sigma, St. Louis, MO) was prepared as a stock solution of 20 mg/ml in distilled water.

Diazepam (Sigma, St. Louis, MO) was prepared as a stock solution of 40, 60, 80 or 120 mg/ml in 100% ethanol. When 100% ethanol was used as a vehicle, potential vehicle effects were minimized by using smaller injection volumes. For diazepam

injections; all animals received injections of < 0.1 cc for low dose experiments (0–20 mg/kg) and of < 0.2 cc for high dose experiments (40–120 mg/kg). The vehicle dose for all animals was normalized to that of the experimental animal receiving the highest dose, ensuring that all animals received equal amounts of vehicle. All vehicles were tested independent of drug administration and were determined to have no anticonvulsant efficacy at the doses used. Vehicle-alone controls did not show impaired motor activity or increased sedation, when compared to untreated controls, in standard open-field locomotive tests.

Phenobarbital (sodium salt; Sigma, St. Louis, MO) was prepared as a stock solution of 100 mg/ml in distilled water. The vehicle dose normalization procedure was the same as diazepam, except distilled water was used as the vehicle.

Phenytoin (sodium; Parke–Davis, Detroit, MI) was prepared as a stock solution of 100 mg/ml in a mixture of 40% propylene glycol, 10% ethyl alcohol, and 1.5% benzyl alcohol (pH 6.68). The vehicle dose normalization procedure was the same procedure used for diazepam, except that a propylene glycol mixture was used as the vehicle.

2.3. Li-pilocarpine-induced status epilepticus in rats

Status epilepticus was induced in 27–33 day old male Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN) by i.p. injection of 3 mEq/kg LiCl (Sigma, St. Louis, MO) followed 20–24 h (± 1 h) later by 50 mg/kg pilocarpine hydrochloride (Sigma, St. Louis, MO), taken from the method by Honchar et al. (1983). Due to size constraints for surgical implantation of epidural recording electrodes, rats undergoing electroencephalogram (EEG) recordings during behavioral seizures were 35–40 days old at the time of seizure induction. Following injection of pilocarpine, the rats were observed continuously for behavioral seizures using the five-stage classification of Racine (1972). Entry into each seizure stage was noted and recorded.

2.4. Treatment of prolonged Li-pilocarpine seizures with diazepam, phenobarbital, and phenytoin

In the first set of experiments, diazepam was administered in increasing doses of 0, 2.5, 5, 10, 20, 40, 80, and 100 mg/kg either 10, 20, 30, or 45 min after pilocarpine administration. In the second and third set of experiments, increasing doses of 0, 0.625, 1.25, 2.5, 5, 10, and 20 mg/kg of diazepam were also given at the onset of stage 3 (S3) seizures, 10 min after the onset of S3 seizures, at the onset of ictal spike activity, at the onset of spike and wave activity, or 10 min after the onset of spike and wave activity. Phenobarbital (sodium salt) was administered in increasing doses of 0, 12.5, 25, 50, 100, and 150 mg/kg either 10 min after pilocarpine administration, at the onset of S3 seizures, or 10 min after the onset of S3 seizures. Phenytoin (sodium salt) was administered in increasing doses of 0, 12.5, 25, 50, 100, 150, and 200 mg/kg at the onset of S3 seizures or at a dose of 100 mg/kg 10 min after pilocarpine administration.

Animals were observed for up to 3 h following antiepileptic drug administration to verify behavioral and electrographic recovery from status epilepticus. Seizure termination (recovery) was defined as absence of: forelimb clonus or falling, facial twitching, and stop and stare activity and resumption of normal behavior. To assess recovery of normal behavior, animals were examined to determine the presence of a righting reflex, motor coordination in an open field, continuous locomotion and eating and drinking. Any cessation in normal activity was considered a post-ictal event (non-convulsive status), and the animal was recorded as 'not recovered'.

2.5. Surgical implantation of bipolar recording electrodes for EEGs

EEGs were obtained from 35 to 40 day old male Sprague–Dawley rats. Surgery was performed 3 days prior to experimentation. Prior to surgery, the rats were treated with atropine methyl nitrate (0.4 mg/kg body weight), anesthetized with an i.p. injection of Nembutal (sodium pentobarbital, 40–65 mg/kg body weight), and mounted in a stereotaxic apparatus (David Kopf Instruments, Tu-

junga, CA). The scalp was incised and retracted, and head position was adjusted to place bregma and lambda in the same horizontal plane. Small burr holes (1 mm diameter) were drilled in the skull for bilateral placement of epidural recording electrodes, which were fabricated from stainless steel screws (size 0–80, 3/16 in.). One pair of electrodes was placed over the frontal cortex (2.0 mm anterior to bregma and 3.0 mm lateral to the midline) and another pair of electrodes was implanted over parietal cortex (4.0 mm posterior to bregma and 3.0 mm lateral to the midline). This yielded right frontal (FR), left frontal (FL), right parietal (PR), and left parietal (PL) placements. A ground screw was placed over the frontal sinus. The electrodes and connector (FR-12S-4, Microtech, Inc., Boothwynn, PA) were secured to the skull with dental acrylic, and the rats were allowed to recover on a heating pad before returning to their home cage.

2.6. EEG recording

A modified rodent conditioning chamber (30 × 24 × 21 cm; MED-Associates Inc., Burlington, VT, USA) was used for all behavioral and electrophysiological testing. The chamber was situated in a sound-attenuating cabinet, which was located in an isolated room. A 1% acetic acid solution was used to clean the chamber between subjects.

On the fourth post-operative day, the rats were transported to the conditioning chamber for a 30–180 min recording session. Before each recording session, a cable was connected to the electrode connector on each rat's head; the cable was connected to a mercury commutator, which permitted the rats to move freely within the conditioning chamber. Four channels of EEG data were amplified (1000 ×) and filtered (0.1–200 Hz) (Neuralynx, Tuscon, AZ, USA) and continuously acquired and digitized (500 Hz/channel) with Experimenter's Workbench software (DataWave Technologies, Longmont, CO, USA) during the recording session. The channels were referenced in the following manner to generate a bipolar montage: FR-PR (right frontal to parietal), PR-PL

(right to left parietal), PL-FL (left parietal to frontal), and FL-FR (left to right frontal).

3. Results

3.1. Progression of Li-pilocarpine-induced seizures

We characterized the behavioral and electrographic features of the Li-pilocarpine model of status epilepticus prior to administration of antiepileptic drugs to ensure a reproducible and reliable method of seizure induction. The Li-pilocarpine seizures followed a general progression through the five stages (S1–S5) (classes) of seizures classified by Racine (1972), specifically, mouth and facial movements (S1), head nodding (S2), forelimb clonus (S3), rearing with forelimb clonus (S4), and rearing and falling with forelimb clonus (S5), which correlated with electrographic activity (Fig.
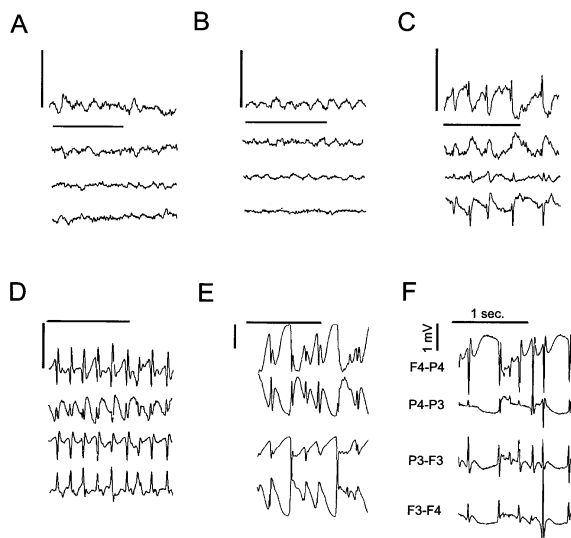

Fig. 1. EEG recordings obtained after Li-pilocarpine administration. (A) Administration of Li-pilocarpine produced electrographic activity that was correlated with distinct ictal events. Spikes or spike–waves were not observed prior to pilocarpine injection; (B) similarly, paroxysmal activity was not observed at the onset of facial automatisms; (C) the onset of spikes was correlated with head nodding (S2); (D) S3 forelimb clonus was correlated with the onset of spike–wave activity; and (E, F) stages S4 and S5 also exhibited a correlation with spike–wave activity, with the dominant spike and wave pattern progressively decreasing in frequency, and increasing in amplitude.

1). Two novel behaviors, bilateral hindlimb grooming and stop and stare activity were also noted in this model. The first behavioral manifestation of seizures was bilateral hindlimb grooming, which primarily occurred within the first 6 min following pilocarpine administration, during which the animal exhibited a rhythmic and highly repetitive grooming motion with its hindlimbs without making contact with its body. This behavior was followed by stop and stare activity, occurring primarily in the first 15 min following pilocarpine administration, during which the animal exhibited a brief pause in motor activity. The progression continued with S1–S5 activity (mouth and facial automatisms, head nodding, forelimb clonus, rearing, and rearing with falling, respectively). In animals that progressed to S4 or S5 seizures, a series of cyclical repetitive seizures occurred. Animals would progress from S2 to S3 and then rapidly progress to S4 or to S4 followed by S5; this was then followed by a return to S2. After a variable period of time, the sequence of S3 and S4 or S3, S4 and S5 seizures would recur multiple times. S1 (mouth and facial automatisms) and S2 (head nodding) seizures plateaued at a moderate frequency (about 25%) throughout the seizures within the population, whereas stage S3, S4 and S5 seizures (forelimb clonus and rearing with and without falling) exhibited a steady increase in frequency over the duration of the seizures within the population, plateauing at a high frequency (mean $\geq 45\%$, data not shown).

3.2. EEG correlates of seizure stage

The lowest stage (S1) seizures did not exhibit an electrographic correlate (Fig. 1A and B); however, the higher seizure stages (S2–S5) had distinct electrographic correlates (Fig. 1C–F). Initial spiking correlated with S2 behavioral seizures (Fig. 1C) while 3–4 Hz spike–wave activity correlated with S3 behavioral seizures (Fig. 1D). The electrographic onset of spike activity did not correlate with the increased motor activity associated with behavioral S2 head nodding. S3, S4 and S5 seizures were characterized by increased spike amplitude and decreased frequency of spiking as the behavioral seizures progressed (Fig. 1D–F).

However, not all electrographic activity was accompanied by overt motor seizures, emphasizing the necessity of using electrographic time points, rather than behavioral time points (data not shown).

3.3. Treatment of Li-pilocarpine-induced seizures with diazepam

Diazepam was first administered 10 min after pilocarpine injection. The animals recovered in a
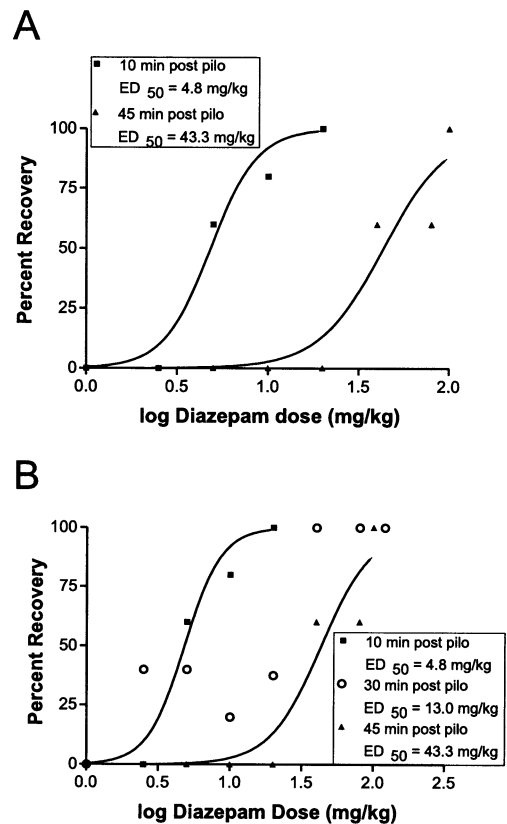

Fig. 2. Diazepam dose–response curves obtained 10, 30, and 45 min after pilocarpine administration. Pilocarpine injection was followed either 10, 30, or 45 min later with increasing doses of diazepam (0, 2.5, 5, 10, 20, 40, 80, 100, 120 mg/kg). (A) Administration of diazepam 10 min after pilocarpine injection induced dose-dependent recovery, while at 45 min after pilocarpine injection, responsiveness to diazepam was reduced. (B) Recovery at the 30 min time point did not appear to be dose-dependent.

dose-dependent manner, with an ED_{50} of 4.8 mg/kg (Fig. 2A, filled squares), consistent with previously published results (Walton and Treiman, 1988; Kapur and Macdonald, 1997). When diazepam was administered 45 min after pilocarpine injection, the animals exhibited a reduced sensitivity to diazepam, with an ED_{50} of 43.3 mg/kg (Fig. 2A, filled triangles), consistent with previously published results (Walton and Treiman, 1988; Kapur and Macdonald, 1997). Full recovery from status epilepticus at 45 min after pilocarpine treatment only occurred following administration of a high dose (100 mg/kg) of diazepam.

To determine the time course over which pharmacoresistance to diazepam developed, animals were treated at an intermediate time point, 30 min following pilocarpine administration. At this intermediate time point, a clear dose–response relationship was not obtained (Fig. 2B, open circles). Recovery at the 30 min time point alone was not significantly dependent on dose (Fig. 2B; χ^2-test, $P > 0.05$). At lower diazepam doses (2.5–20 mg/kg), 20–50% of animals recovered independent of dose, while at the higher diazepam doses (40–120 mg/kg) 100% of animals recovered. Animals treated at 20 min following pilocarpine administration also exhibited a similar lack of dose-dependence of recovery (data not shown).

3.4. Stage- and time-dependent recovery from behavioral seizures with diazepam

Due to lack of dose-dependence to recovery at intermediate time points, we explored the possibility that the variable determining outcome might be the time following the onset of specific seizure stages. Accordingly, we determined the dependence of recovery on time from the onset of each seizure type to time of diazepam treatment for each diazepam dose. When data from all experiments were included (including the 20 and 30 min time points), there was an overall diazepam dose-dependence (Fig. 3A; χ^2-test, $P < 0.001$). No animals recovered (filled squares) without diazepam administration (0/13) and a subset of animals recovered (open circles) with diazepam adminis-

tration (3/14 at 2.5 mg, 3/11 at 5 mg, 2/10 at 10 mg, 26/45 at 20 mg).

While there was no dose-dependence for the diazepam effect, post-hoc analysis of the data suggested that there was a relationship between the time of diazepam administration following the first S3 seizure and recovery (Fig. 3A). At all diazepam doses, recovery (Fig. 3A) occurred only when the diazepam was administered less than 10 min following the onset of S3 seizures.

To further characterize this observation, the percentage of animals recovering following diazepam administration (regardless of dose) was plotted as a function of the time to diazepam treatment following the onset of S3 seizures (Fig. 3B). Statistical analysis revealed that recovery for all previous experiments was significantly dependent on the amount of time elapsed after the first S3 seizure until the time of diazepam treatment (Fig. 3B; χ^2-test, $P < 0.0001$).

Based on our post-hoc findings demonstrating stage- (S3-dependent) and dose-dependent components to recovery, we administered diazepam at either the onset or 10 min following the onset of the first S3 seizure. When diazepam was administered at the onset of the first S3 seizure, the animals exhibited recovery in a dose-dependent manner, with an ED_{50} of 1.6 mg/kg (Fig. 3C, inverted triangles). However, when diazepam was administered 10 min after the first S3 seizure, the majority of animals (92% or greater) did not exhibit recovery, regardless of dose up to 20 mg/kg (Fig. 3C, open diamonds).

To more accurately determine the time course of the development of pharmacoresistance, shorter durations between the onset of S3 seizure and diazepam treatment were studied (Fig. 3D). Our initial findings were supported, as 100% of animals recovered at shorter time points (onset of S3 or 2.5 min after the initial S3 seizure), but fewer recovered at longer time points (5 min = 60% of animals recovered; 7.5 min = 43% recovered; 10 min = 17% recovered). The percentage of animals that recovered was significantly dependent on the time after the first S3 seizure until diazepam treatment (Fig. 3D; χ^2-test, $P < 0.01$).
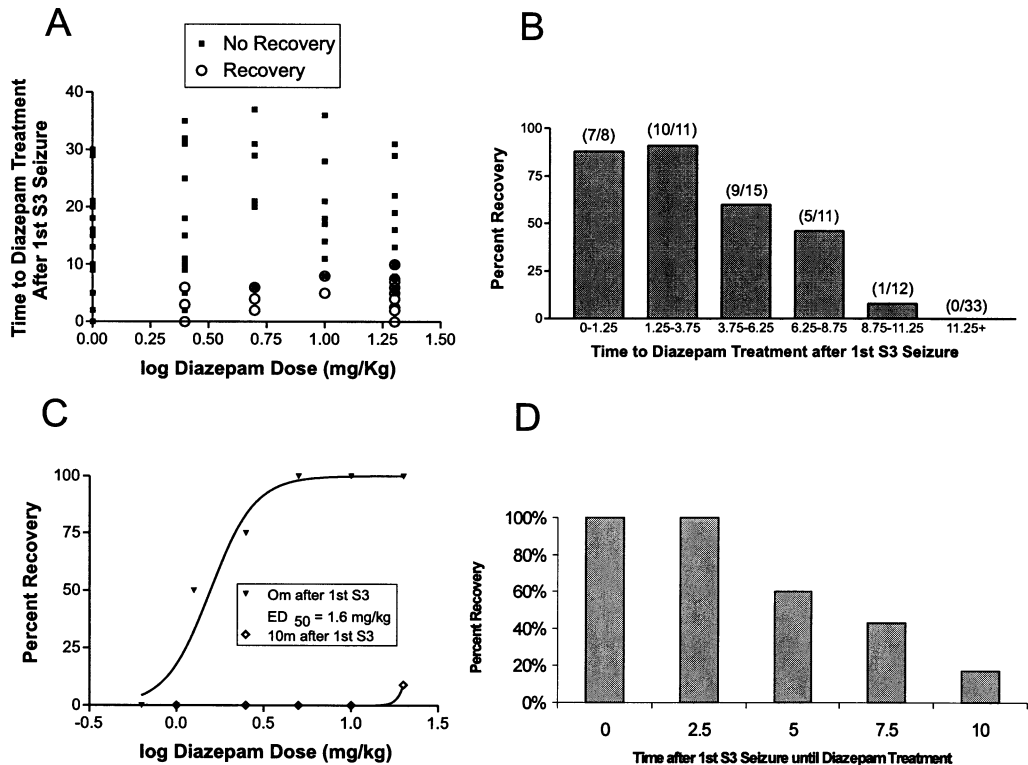
Fig. 3. (A) Retrospective analysis of recovery for all animals at diazepam doses of 0, 2.5, 5, 10, and 20 mg/kg ($n = 90$). The time to diazepam treatment after the first S3 seizure was plotted against the diazepam dose. Animals that did not recover were plotted with filled squares and those that recovered were plotted with open circles. The plot illustrated that recovery with diazepam was a function of dose and time to treatment after the initial S3 seizure. Animals recovered only if treated within the first 10 min following the onset of S3 seizures. (B) The data were replotted as a histogram of % recovery versus the time to diazepam treatment after the first S3 seizure. The % recovery decreased as the time to diazepam treatment after the initial S3 seizure increased. (C) Diazepam dose–response curves obtained at the onset or 10 min after the onset of Li-pilocarpine-induced S3 seizures. Recovery depended on the time of treatment following the onset of S3 seizures. Increasing doses (0, 0.625, 1.25, 2.5, 5, 10, 20 mg/kg) of diazepam were administered at the onset of S3 seizures (0 m) or 10 min after the onset of S3 seizures (10 m). Administration of diazepam at the onset of S3 seizures induced dose-dependent recovery, whereas 10 min after the onset of S3 activity, recovery was not observed at any dose tested. (D) Animals were given diazepam (20 mg/kg) at one of five fixed time points following the initial S3 seizure (0, 2.5, 5, 7.5, 10 min after, respectively). The % recovery decreased as the time after the onset of S3 seizures increased.

3.5. Stage- and time-dependent recovery from electrographic seizures with diazepam

Diazepam was also administered at the onset of ictal spike activity, at the onset of spike and wave activity, or 10 min following the onset of spike and wave activity to ensure that non-convulsive electrographic seizures were not present after behavioral recovery with diazepam. These electrographic administration points were further used to validate previous administration of treatment at behavioral time points.

There was no electrographic activity recorded during pre-pilocarpine baseline recording sessions (Fig. 4A, Fig. 5A). When diazepam was administered at the onset of ictal spike activity (data not shown) or at the onset of spike and wave activity (Fig. 4B), the animals exhibited recovery in response to diazepam (20 mg/kg). Electrographic recovery with diazepam was rapid, occurring within 15 s of injection (Fig. 4C) and was also prolonged, lasting for the entire time of behavioral observation (up to 3 h) (Fig. 4D). However, behavioral recovery was not immediate and gen-
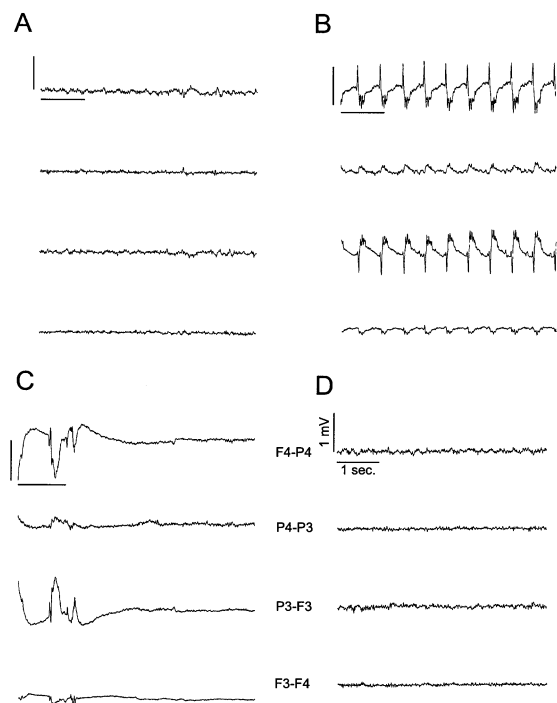
Fig. 4. Representative EEG traces from a rat administered diazepam at the onset of spike and wave (S3 correlate) activity. (A) Spikes were not observed prior to the onset of behavioral seizures; (B) the onset of spike and wave activity was correlated with the onset of S3 behavioral seizures; (C) recovery was rapid, occurring electrographically within 15 s of a 20 mg/kg diazepam injection; (D) recovery was also prolonged, occurring over the duration of behavioral observation.
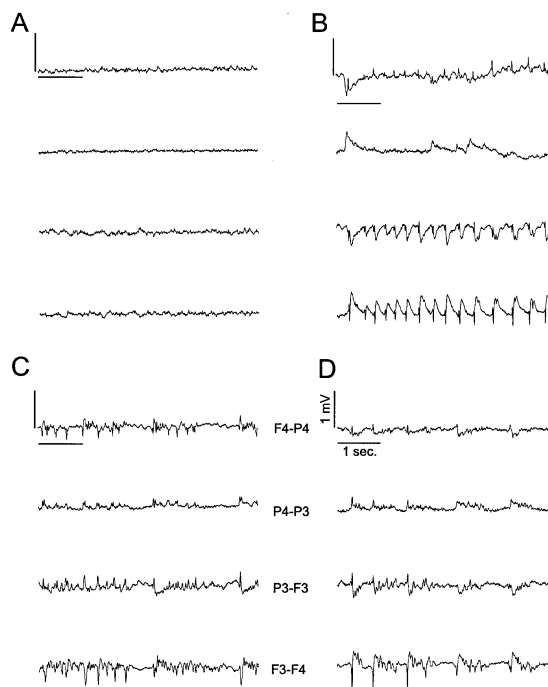
Fig. 5. Representative EEG traces from a rat administered diazepam 10 min after the onset of spike and wave (S3 correlate) activity. (A) Spikes were not observed prior to the onset of behavioral seizures; (B) the onset of spike and wave activity was correlated with the onset of S3 behavioral seizures; (C) diazepam (20 mg/kg) was administered 10 min after the onset of spike and wave activity but spikes and spike–wave activity continued; (D) recovery did not occur, either electrographically or behaviorally, over the duration of behavioral observation.

erally did not occur for at least 1 h after diazepam administration, presumably due to sedation caused by the ethanol vehicle. When the same dose of diazepam was administered 10 min after (Fig. 5C) the onset of spike and wave activity (Fig. 5B), the majority of animals (60%) did not exhibit recovery (Fig. 5C and D). Furthermore, these animals did not exhibit behavioral sedation, as did the animals that were treated at the onset of spike and wave activity. The percent recovery was significantly dependent on the time of diazepam treatment, and specifically, on the relationship of the time of treatment to the primary behavioral seizure events or their electrographic correlates (Fig. 6; χ^2-test, $P < 0.0001$).
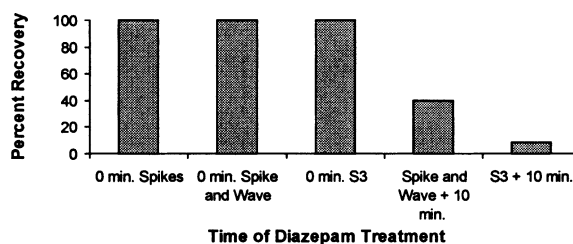

Fig. 6. Diazepam-induced recovery was time- and stage-dependent. Animals were then given 20 mg/kg diazepam at one of five fixed time/stage (at the onset of spikes, at the onset of spike and wave activity, at the onset of S3 seizures, 10 min after the onset of spike and wave activity, or 10 min after the onset of S3 seizures, respectively). Animals recovered if the diazepam was administered at the onset of spike or spike–wave activity. However, recovery was decreased if diazepam was administered 10 min following the onset of spike–wave activity.

3.6. Treatment of Li-pilocarpine-induced seizures with phenobarbital

Since our findings suggested that prolonged seizures reduced the benzodiazepine sensitivity of $GABA_A$ receptors, we studied another antiepileptic drug that also acts on $GABA_A$ receptors to determine if the changes were specific to benzodiazepine modulation. Phenobarbital, which enhances $GABA_A$ receptor current by binding to a separate site on $GABA_A$ receptors, was administered 10 min after administration of pilocarpine, at the onset of the first S3 seizure, or 10 min following the first S3 seizure. The results obtained with phenobarbital were similar to those obtained with diazepam. When phenobarbital was administered 10 min after pilocarpine injection, the animals exhibited recovery in a dose-dependent manner, with an ED_{50} of 51.6 mg/kg (Fig. 7A, open triangles). At the onset of the first S3 seizure, the animals continued to exhibit recovery in a dose-dependent manner, with an ED_{50} of 62.6 mg/kg (Fig. 7A, filled circles). However, when phenobarbital was administered 10 min after the first S3 seizure, the animals again did not exhibit recovery, regardless of dose (Fig. 7A, open squares).

3.7. Treatment of Li-pilocarpine-induced status epilepticus with phenytoin

We also determined whether the changes occurring were specific to antiepileptic drugs that interacted with $GABA_A$ receptors. Phenytoin, which acts on voltage-gated sodium channels, was administered 10 min after the injection of pilocarpine, or at the onset of the first S3 seizure. Phenytoin was ineffective as an anticonvulsant in the Li-pilocarpine rat model of status epilepticus, regardless of dose or time of delivery, consistent with previous studies (Fig. 7B) (Morrisett et al., 1987).

4. Discussion

This study supports the clinical and experimental observations that the responsiveness of status epilepticus to treatment with diazepam decreases
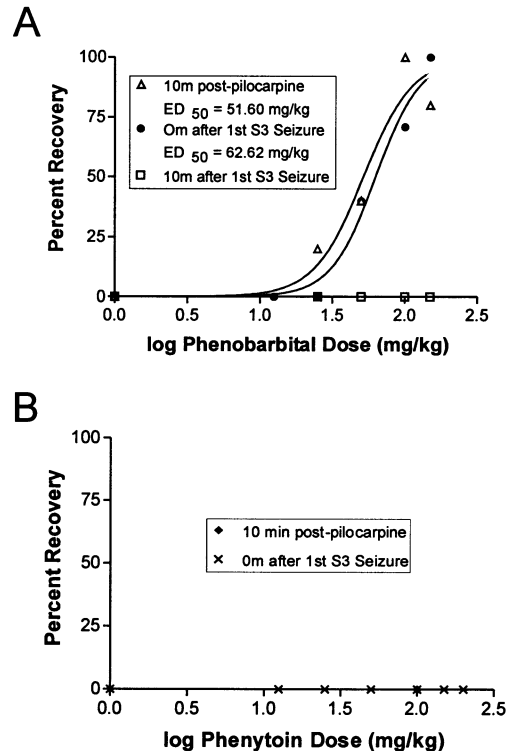

Fig. 7. Phenobarbital and phenytoin dose–response curves were obtained after Li-pilocarpine administration. Pilocarpine injection was followed 10 min later, at the onset of S3 seizures (0 m), or 10 min after the onset of S3 seizures (10 m), with increasing doses of: (A) phenobarbital (of 0, 12.5, 25, 50, 100, and 150 mg/kg; or (B) phenytoin (0, 12.5, 25, 50, 100, 150, and 200 mg/kg). Administration of phenobarbital at 10 min after pilocarpine injection, or at the onset of S3 seizures induced dose-dependent recovery, whereas at 10 min after the onset of S3 activity, recovery was not observed at any dose tested. Phenytoin was ineffective, regardless of dose.

as seizure duration increases. Our results demonstrate that the effectiveness of the antiepileptic drugs diazepam and phenobarbital, but not phenytoin, declines rapidly after limbic seizures become prolonged. Pharmacoresistance to traditional antiepileptic drugs has been previously reported following prolonged seizures in experimental animals and in humans (Morrisett et al., 1987; Walton and Treiman, 1988; Kapur and Macdonald, 1997; Lowenstein and Alldredge, 1993, 1998; Aminoff and Simon, 1980). Walton and Treiman (1988) reported that in rats, Li-pilocarpine induced status epilepticus became

more difficult to treat as electrographic seizures continued after long durations of seizure activity. Kapur and Macdonald (1997) also reported that diazepam had reduced anticonvulsant potency after prolonged seizures in rats. In humans, clinical studies also demonstrated that status epilepticus could be terminated with first line treatments such as diazepam and phenytoin in 80% of patients when treated within 30 min of seizure onset, but that responsiveness decreased to less than 40% when treatment was increased to several hours after seizure onset (Lowenstein and Alldredge, 1993, 1998). It has also been suggested that pre-hospital therapy shortens the duration of status epilepticus and allows for better patient outcome (Alldrege et al., 2001). The results reported here confirm those results and extend them by demonstrating that the development of diazepam pharmacoresistance is rapid, as well as dependent on the stage during which the drug is administered, occurring within 10 min of stage three motor seizures, reemphasizing the clinical need for rapid treatment of status epilepticus.

Several possible mechanisms have been hypothesized previously to explain the plastic changes occurring in the hippocampus during status epilepticus that may explain the development of pharmacoresistance during prolonged seizures. Increased glutamatergic excitatory synaptic drive during the evolution of the seizures could overwhelm hippocampal inhibition, and/or GABA$_A$ receptor-mediated inhibition may fail (Doherty and Dingledine, 2001), even in the presence of enhancing modulators, suggesting a functional modulation of GABA$_A$ receptor inhibitory circuits (Kapur et al., 1989; Kapur and Coulter, 1995). While the results presented here cannot distinguish between these alternative mechanisms, the observation of refractoriness to diazepam and phenobarbital after prolonged limbic seizures suggests a specific alteration in the benzodiazepine and barbiturate sensitivity of GABA$_A$ receptors. Consistent with this suggestion, Kapur and Macdonald (1997) demonstrated that GABA$_A$ receptor currents recorded from acutely dissociated dentate granule cells from rats undergoing prolonged Li-pilocarpine induced seizures were less sensitive to diazepam and zinc, but retained their sensitivity to

GABA and pentobarbital. Determination of pharmacoresistance to other antiepileptic drugs acting on other modulatory sites of the GABA$_A$ receptors, but not acting on other ion-channels or receptors, also suggests a phenomenon of rapid functional modulation that is specific to GABA$_A$ receptors over the course of the prolonged seizures. Furthermore, this finding suggests that GABAergic inhibitory circuits, in particular, are altered during a critical time period as status epilepticus progresses, and argues against an increase in excitatory activity being solely responsible for the observation of pharmacoresistance.

The decreased sensitivity to diazepam that develops during prolonged seizures, therefore, suggests a specific functional alteration in the benzodiazepine and barbiturate sensitivity of GABA$_A$ receptors. Several hypotheses may be advanced for this decreased sensitivity.

An initial hypothesis for the possible mechanism of the rapid functional modulation of the receptor is a change in the subunit composition of the receptor over the course of the seizures. Sensitivity to benzodiazepines requires the presence of a γ subunit; it is possible that the loss of the $\gamma2$ subunit and incorporation of another subunit, such as the δ subunit, might cause pharmacoresistance to diazepam. However, this would not explain the loss of sensitivity to phenobarbital noted in our study, or the reduction of zinc sensitivity of GABA$_A$ receptor currents noted in a previous study by Kapur and Macdonald (1997).

A second hypothesis is that a similar subunit swap occurs resulting in expression of a different α subunit. It has been previously shown that recombinant GABA$_A$ receptors expressing the $\alpha6\beta \times \gamma2$ subunit composition exhibit both low diazepam and moderately low zinc sensitivity (Fisher et al., 1997[L1]). Again, this hypothesis would not explain the decreased efficacy of phenobarbital following the prolonged seizures.

A third hypothesis invokes activation of non-functional 'spare' GABA$_A$ receptors to be assembled and inserted into the cell membrane. However, previous studies by Kapur and Macdonald (1997) note that there was no change in sensitivity to GABA in cells obtained from animals following prolonged seizures; the GABA$_A$ recep-

tors expressed in these animals responded to GABA. Demonstration of such rapid receptor turnover would require further pharmacological characterization of the GABA$_A$ receptors following the development of pharmacoresistence.[L2]

A fourth hypothesis is that the benzodiazepine and barbiturate binding sites are 'uncoupled' from the GABA binding sites or from the GABA$_A$ receptor transduction process. The multiple drug recognition sites on functional GABA$_A$ receptors (e.g. benzodiazepines and barbiturates) are allosterically coupled to modulate GABA$_A$ receptor channel current. It is possible that the benzodiazepine and barbiturate binding sites become uncoupled from the GABA binding site as the GABA$_A$ receptor activation associated with massive release of GABA increases during the epileptic discharge. Previous studies have shown that chronic exposure of cultured cortical neurons to GABA leads to the down regulation of benzodiazepine receptors, GABA$_A$ receptors, GABA$_A$ receptor channels, and to the uncoupling of the GABA$_A$ and benzodiazepine receptors (Mehta and Ticku, 1992; Klein et al., 1995; Lyons et al., 2000).

A fifth hypothesis is that post-translational modification of the GABA$_A$ receptor may be involved in the development of pharmacoresistance. In particular, the rapid loss of sensitivity of the benzodiazepine and barbiturate sites of the receptor may result from a change in phosphorylation state of the GABA$_A$ receptor. GABA$_A$ receptor subunits contain consensus sequences for phosphorylation by cyclic AMP-dependent protein kinase A (PKA), protein kinase C (PKC), and tyrosine kinase, and have been shown to be phosphorylated on multiple subunits by different protein kinases (Brandon et al., 2000; Macdonald, 1995; Angelotti et al., 1993; Macdonald et al., 1992). For example, expression of the $\alpha1\beta1\gamma2S$ receptor in a cell line with elevated levels of cAMP-dependent protein kinase (PKA) was shown to enhance GABAergic current in whole-cell recordings (Angelotti et al., 1993). However, diazepam sensitivity in this cell line was not significantly different from other normal- and low-level PKA expressing cell lines. It remains to be shown that post-translational modification via kinase activity can alter the functional properties of GABA$_A$-receptor modulators such as benzodiazepines and barbiturates.

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