

# The Endogenous Cannabinoid System Regulates Seizure Frequency and Duration in a Model of Temporal Lobe Epilepsy

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## ABSTRACT

Several lines of evidence suggest that cannabinoid compounds are anticonvulsant. However, the anticonvulsant potential of cannabinoids and, moreover, the role of the endogenous cannabinoid system in regulating seizure activity has not been tested in an in vivo model of epilepsy that is characterized by spontaneous, recurrent seizures. Here, using the rat pilocarpine model of epilepsy, we show that the marijuana extract  $\Delta^9$ -tetrahydrocannabinol (10 mg/kg) as well as the cannabimimetic, 4,5-dihydro-2-methyl-4(4-morpholinylmethyl)-1-(1-naphthalenyl-carbonyl)-6H-pyrrolo[3,2,1-*i,j*]quinolin-6-one [*R*(+)-WIN55,212 (5 mg/kg)], completely abolished spontaneous epileptic seizures. Conversely, application of the cannabinoid CB<sub>1</sub> receptor (CB<sub>1</sub>) antagonist, *N*-(piperidin-1-yl-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride (SR141716A), significantly increased both seizure duration and frequency. In some animals, CB<sub>1</sub> receptor antagonism resulted in seizure dura-

tions that were protracted to a level consistent with the clinical condition status epilepticus. Furthermore, we determined that during an short-term pilocarpine-induced seizure, levels of the endogenous CB<sub>1</sub> ligand 2-arachidonylglycerol increased significantly within the hippocampal brain region. These data indicate not only anticonvulsant activity of exogenously applied cannabinoids but also suggest that endogenous cannabinoid tone modulates seizure termination and duration through activation of the CB<sub>1</sub> receptor. Furthermore, Western blot and immunohistochemical analyses revealed that CB<sub>1</sub> receptor protein expression was significantly increased throughout the CA regions of epileptic hippocampi. By demonstrating a role for the endogenous cannabinoid system in regulating seizure activity, these studies define a role for the endogenous cannabinoid system in modulating neuroexcitation and suggest that plasticity of the CB<sub>1</sub> receptor occurs with epilepsy.

Characterized by spontaneously recurrent seizures, epilepsy is one of the most common neurological conditions (Hauser and Hesdorffer, 1990). Understanding the factors that contribute to seizure initiation and termination has important implications for our ability to treat epilepsy and for the potential development of novel anticonvulsant agents. Previous evidence has suggested that the endogenous cannabinoid system may be a novel locus of anticonvulsant activity in the brain (Karler et al., 1974; Wallace et al., 2001). Using the maximal electroshock model of short-term seizure, our laboratory determined that cannabinoid compounds block seizure spread via a cannabinoid CB<sub>1</sub> receptor-dependent mechanism (Wallace et al., 2001, 2002). Further study re-

vealed that application of a CB<sub>1</sub> receptor antagonist lowered the electroshock seizure threshold (Wallace et al., 2002), indicating that elimination of endogenous cannabinoid tone at the CB<sub>1</sub> receptor may increase seizure susceptibility.

The CB<sub>1</sub> receptor is the most highly expressed G-protein-coupled receptor in brain (Herkenham et al., 1990) and has been implicated in regulation of neuronal excitability (Wilson and Nicoll, 2001; Ohno-Shosaku et al., 2002). The endogenous cannabinoids, arachidonylethanolamine and 2-arachidonylglycerol (2-AG) (Devane et al., 1992; Mechoulam et al., 1995), are synthesized "on demand" in response to sustained neuronal depolarization and elevated intracellular calcium levels (Stella et al., 1997); both of these events occur with seizure activity (Hauser and Hesdorffer, 1990; Raza et al., 2001). The neuronal hyperexcitability that accompanies seizure discharge may stimulate endogenous cannabinoid synthesis and subsequently result in CB<sub>1</sub> receptor activation. In light of cannabinoid effects on neurotransmission, increased

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**ABBREVIATIONS:** 2-AG, 2-arachidonylglycerol; CB<sub>1</sub>, cannabinoid CB<sub>1</sub> receptor; EEG, electroencephalographic; *R*(+)-WIN55,212, 4,5-dihydro-2-methyl-4(4-morpholinylmethyl)-1-(1-naphthalenyl-carbonyl)-6H-pyrrolo[3,2,1-*i,j*]quinolin-6-one; *S*(-)-WIN55,212, (*S*)-(-)-[2,3-dihydro-5-methyl-3-((4-morpholinyl)methyl)pyrrolo]-[1,2,3-*c,d,e*]-[1,4-benzoxazinyl]-[1-naphthalenyl]methanone); SR141716A, *N*-(piperidin-1-yl-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride; THC,  $\Delta^9$ -tetrahydrocannabinol; DSI, depolarization-induced suppression of inhibition; SE, status epilepticus; PBS, phosphate-buffered saline; ANOVA, analysis of variance; RM, repeated measures; CA, cornu ammonis.

CB<sub>1</sub> receptor activation could influence seizure activity. However, no studies have evaluated the role of the endogenous cannabinoid system in an intact model of epilepsy.

This study was initiated to evaluate the role of the CB<sub>1</sub> receptor and the endogenous cannabinoid system in regulating seizure activity in a long-term model of epilepsy. We used the pilocarpine model of temporal lobe, partial-complex epilepsy; a rat model of acquired, refractory epilepsy that produces spontaneous recurrent seizures for the lifetime of the animal (Mello et al., 1993; Rice and DeLorenzo, 1998). The pilocarpine model has been shown to closely resemble human refractory partial-complex epilepsy (Mello et al., 1993; Raza et al., 2001). In this study, seizure frequency and duration were determined by continuous electrographic and video recording of each epileptic animal (Rice and DeLorenzo, 1998). The CB<sub>1</sub> receptor agonists *R*(+)WIN55,212 and Δ<sup>9</sup>-tetrahydrocannabinol (THC) were evaluated for anticonvulsant efficacy. In addition to agonist effects on seizure activity, the effect of CB<sub>1</sub> receptor antagonism on seizure frequency and duration was evaluated using the specific antagonist SR141716A. Hippocampal levels of 2-AG during short-term, pilocarpine-induced seizures were measured to determine whether a correlation exists between endogenous cannabinoid synthesis and seizure activity. In addition, Western blot and immunohistochemical analyses were used to evaluate hippocampal CB<sub>1</sub> receptor protein expression in the brains of chronically epileptic and sham control rats. The findings presented suggest an anticonvulsant role for the endogenous cannabinoid system and demonstrate that long-term plasticity of the CB<sub>1</sub> receptor occurs with epilepsy.

## Materials and Methods

**Pilocarpine-Induced Status Epilepticus.** Male Sprague-Dawley rats weighing 200 to 250 g were used in accordance with university animal care and use protocols. Animals were housed in single cages on a 12-h/12-h light/dark cycle (lights on at 7:00 AM) and were provided food and water ad libitum. Animals were made epileptic using a modified protocol of Mello et al. (1993) that is well established in our laboratory (Rice and DeLorenzo, 1998). Before pilocarpine injections, animals were administered methylscopolamine nitrate (1 mg/kg i.p.) to minimize peripheral, parasympathetic effects of pilocarpine treatment. Pilocarpine nitrate (375 mg/kg i.p.) was then administered 30 min later. Onset of status epilepticus (SE) typically occurred within 20 to 40 min after pilocarpine injection and was determined when the animal displayed continuous moderate to severe behavioral seizures characterized by forelimb clonus, rearing, and falling.

SE was defined as continuous seizure activity that lasted 30 min or longer or intermittent seizures without regaining consciousness between seizures that lasted 30 min or longer. The severity of convulsions was evaluated, and only those animals that displayed behaviors consistent with ongoing SE were used in the study (Rice and DeLorenzo, 1998). Seizure activity was terminated by consecutive diazepam injections (5 mg/kg i.p., solubilized in 10% ethanol, 45% propylene glycol, and 45% H<sub>2</sub>O) at 1, 3, and 5 h post onset of SE. Animals continuing to display seizure activity beyond 6 h post onset of SE were euthanized. Control groups were composed of both naive and sham control animals that received methylscopolamine nitrate and diazepam injections only. Approximately 75% of the SE animals developed epilepsy under these conditions, and the mortality rate from SE was approximately 10%. SE animals that did not stop seizing with diazepam treatment were uncommon and represented less than 2% of the animals injected with pilocarpine.

**Epileptic Seizure Monitoring.** Seizures were monitored in freely moving animals via simultaneous electroencephalographic (EEG) and video monitoring at least 3 months after pilocarpine treatment (Rice and DeLorenzo, 1998). Electrographic seizures were detected via skull surface electrodes implanted 2 to 3 weeks after the initial episode of SE or after sham treatment in a manner described previously (Perlin et al., 1993). Briefly, animals were put under general ketamine/xylazine anesthesia (75 mg/kg ketamine i.p., 7.5 mg/kg xylazine i.p.), and a midline scalp incision was made to expose the skull. Four surface screw electrodes were implanted bilaterally 2.5 mm from midline, at 2.5 mm posterior to bregma and 2.5 mm anterior to lambda. Surface screw electrodes were connected via Teflon-coated stainless steel wire (Medwire, Mount Vernon, NY) to a male amphenol pin headset assembly, which was secured to the skull with dental acrylic (Hygenic, Akron, OH). Animals were allowed to recover for a minimum of 1 month before experimental analysis. Both electrographic and behavioral seizures were monitored with EEG and video recording, respectively, using a Biomedical Monitoring System Mobile EEG Unit (Campbell, CA).

Seizures were evaluated using established techniques (Rice and DeLorenzo, 1998) and confirmed by an observer blind to experimental treatment. Behavioral epileptic seizures were identified by video analysis of animals displaying moderate to severe behavioral seizures characterized by forelimb clonus, rearing, and falling in conjunction with electrographic seizure activity obtained from EEG analysis.

For single-injection experiments, animals were given a 2 to 3 h equilibration to the treatment setting and then were briefly anesthetized under halothane anesthesia (Halocarbon Laboratories, River Edge, NJ) and injected with either vehicle, *S*(-)WIN55,212 (5 mg/kg i.p.), *R*(+)WIN55,212 (5 mg/kg i.p.), SR141716A (10 mg/kg i.p.), phenobarbital (40 mg/kg i.p.), phenytoin (100 mg/kg i.p.), or THC (30 mg/kg i.p.). All drugs were suspended in a vehicle of absolute ethanol, Emulphor-620 (Rhône-Poulenc, Inc., Princeton, NJ), and 0.9% saline at a ratio of 1:1:18. Brief halothane anesthesia was induced in animals before drug injections to minimize unnecessary stress, pain, or trauma. Animals fully recovered from anesthesia within 2 min after induction. Sham control injected animals received identical treatment.

Multiple drug treatment experiments were conducted in a manner similar to single-injection experiments with the exception that each animal received, over a period of 10 days, the entire range of drugs analyzed in the single-injection experiments. All injections throughout the treatment period were administered twice daily at approximately 10:00 AM and 6:00 PM under brief halothane anesthesia. For the multidrug treatment experiments, animals were monitored for baseline seizure frequency and duration for 1.5 days before initiation of the dosing regimen. Animals were then consecutively treated with vehicle solution for 1 day, *S*(-)WIN55,212 (5 mg/kg i.p.) for 1.5 days, *R*(+)WIN55,212 (5 mg/kg i.p.) for 2.5 days, a 2-day drug-free period during which the animals received no injections, SR141716A (10 mg/kg i.p.) for 1 day, and finally a 1-day drug-free period. Only generalized tonic-clonic seizures were counted and later confirmed by an observer blind to experimental treatment. Methylscopolamine nitrate, pilocarpine nitrate, *S*(-)WIN55,212, *R*(+)WIN55,212, phenytoin, and diazepam were purchased from Sigma-Aldrich (St. Louis, MO). SR141716A and THC were supplied through the National Institute on Drug Abuse Chemical Synthesis and Drug Supply Program.

**Measurement of Hippocampal 2-AG Levels.** Pilocarpine was used to acutely induce seizure activity in naive, male, Sprague-Dawley rats weighing 200 to 250 g. In these studies, animals were injected with scopolamine and 375 mg/kg i.p. pilocarpine as described under *Pilocarpine-Induced Status Epilepticus* and were sacrificed at 15 min post onset of status epilepticus. Age-matched, sham control animals were also sacrificed. Hippocampi were immediately dissected and flash frozen in liquid nitrogen. 2-AG was isolated and detected using high-performance liquid chromatography-mass spec-

troscopy according to previously published methods (Di Marzo et al., 2000).

**Western Blot Protocol.** Gel electrophoresis was carried out on rat hippocampal neuronal membrane preparations from 1 year after SE, epileptic, and age-matched, sham-treated animals. After monitoring of epileptic animals to verify seizure activity, the rats were sacrificed, and hippocampal tissue was harvested on ice. Hippocampi were homogenized in 50 mM Tris, pH 7.5, 6 mM EGTA, 320 mM sucrose, 1 mM dithiothreitol, and 0.3 mM phenylmethylsulfonyl fluoride, and neuronal membranes were isolated by centrifugation (Morris et al., 2001). Before electrophoresis, membrane samples were thawed on ice, and protein concentration per sample was calculated using the MicroBradford reagent system (Bio-Rad, Hercules, CA) quantified using a UV-2101PC ultraviolet spectrophotometer (Shimadzu, Kyoto, Japan). Samples were balanced to 5  $\mu$ g protein/gel lane and denatured in  $\beta$ -mercaptoethanol and loading dye buffer. Samples were then heated to 90°C for 5 min in a programmable thermal controller PTC100 (MJ Research, Watertown, MA) and allowed to cool to room temperature before loading onto a 10% Tris-HCl Ready Gel (Bio-Rad). A colorimetric molecular mass marker including standards ranging from 10 to 182 kDa (ProSieve; Cambrex Bio Science Rockland, Inc., Rockland, ME) was loaded onto the last lane of the gel to aid in determining protein size. Gels were assembled into a MiniProtean II Electrophoresis System (Bio-Rad) and resolved for 90 min at 220 V constant in Tris buffer (Bio-Rad). After electrophoresis, gels were Western blot transferred to Immobilon nylon membrane (Millipore Corp., Bedford, MA) for 2 h at 4°C using a Genie transfer apparatus (IDEA Scientific, Minneapolis, MN) at a constant 200 V. Transfer buffer consisted of Tris-glycine buffer containing 10% methanol. After transfer, the Western blot was stored in phosphate-buffered saline at 4°C overnight. Gels were stained for protein and quantitated for microtubule-associated protein 2 and tubulin protein levels as described previously (Morris et al., 2001).

Immunostaining of the Western blot was performed by first blocking the membrane in buffer composed of 3% blotting grade blocker (Bio-Rad) and 0.05% Tween 20 in phosphate-buffered saline for 45 min at room temperature. Rabbit (polyclonal) anti-cannabinoid CB<sub>1</sub> receptor unconjugated primary antibody (Biosource International, Camarillo, CA) was added to the blocking solution at a concentration of 1  $\mu$ g/ml, and the membrane was incubated for 90 min at room temperature. After primary antibody incubation, the membrane was washed for a total of 15 min (three times for 5 min each) in phosphate-buffered saline. The membrane was then reblocked in fresh blocking buffer for 30 min. Anti-rabbit IgG-horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was then added to the blocking solution in a 1:2000 dilution, and the membrane was incubated for a final 45 min. Western blots were washed (three times for 5 min each) in PBS and incubated for 5 min in SuperSignal (Pierce Chemical, Rockford, IL) for enhanced chemiluminescent analysis. Chemiluminescent images were visualized using Kodak X-Omat Blue XB-1 X-ray film (Eastman Kodak, Rochester, NY) and developed using a Kodak M35A X-Omat Processor (Eastman Kodak). Film images were digitized using a gel scanner and analyzed by computer-assisted densitometry (Amersham Biosciences Inc., Piscataway, NJ). Using the molecular mass marker as reference, the protein band was determined to correspond to a mass of 64 kDa.

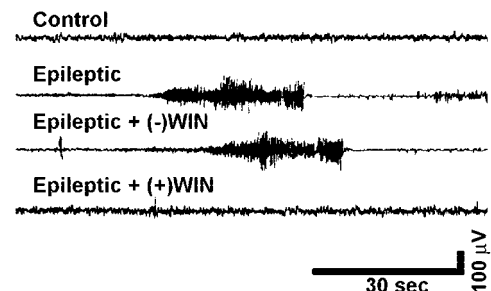
**Immunohistochemistry.** Four epileptic and four control rats were transcardially perfused with isotonic saline, and brains were quick frozen and stored at -80°C in embedding compound (Sakura Inc., Japan). Cryostat sections (10  $\mu$ M) were fixed in acetone and prepared for immunostaining using established techniques (Pettit et al., 1998). CB<sub>1</sub> receptor protein immunoreactivity for each animal was evaluated using more than 15 tissue sections. Briefly, tissue sections were blocked in bovine serum for 1 h and then incubated with CB<sub>1</sub> antiserum at 5.0  $\mu$ g/ml for 1 h at room temperature. Tissue slices were then washed in PBS (three washes, each for 5 min), followed by biotinylated anti-rabbit IgG at 1:200 dilution for 30 min

at room temperature. After again washing in PBS for 15 min, CB<sub>1</sub> receptor immunoreactivity was visualized by exposure to avidin-biotin complex and 3-3'-diaminobenzidine (Vector Laboratories, Burlingame, CA). Adjacent tissue sections were evaluated morphologically using Nissl stain. Stained tissue sections were evaluated using a binocular microscope (Olympus America Inc., Melville, NY) and were photographed using a digital camera (Olympus America Inc.). Images were analyzed using Analysis software (Soft Imaging System Corp., Lakewood, CO).

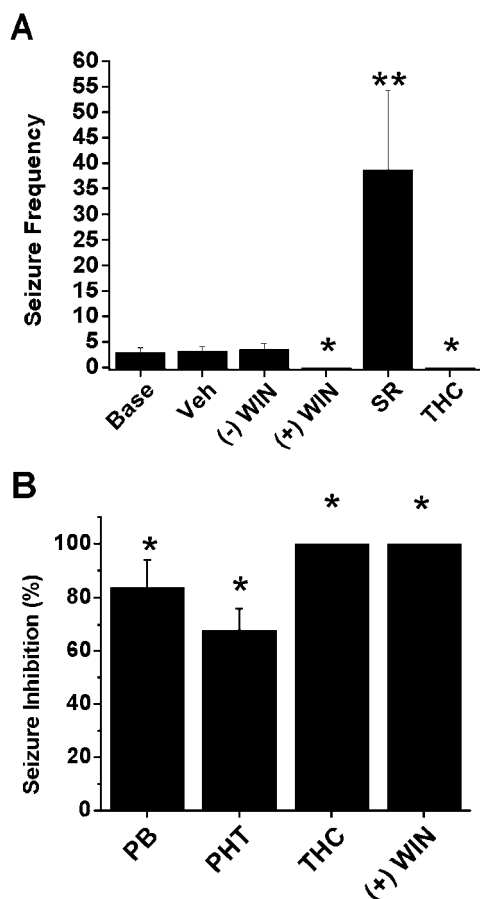
**Statistical Analyses.** Results are given as means  $\pm$  S.E.M. Statistical comparisons were made using SigmaStat software (SPSS Science, Chicago, IL). The Student's *t* test, one-way analysis of variance (ANOVA), and the repeated measures (RM) one-way ANOVA in conjunction with the post hoc Tukey test were used where appropriate. Graphs were generated using Origin 6.1 software (OriginLab Corp, Northampton, MA).

## Results

**Modulation of the CB<sub>1</sub> Receptor Alters Seizure Frequency in the Rat Pilocarpine Model of Epilepsy.** Epileptic rats manifested an average of 3.0 ( $\pm$  0.9) seizures per 10-h period. Representative control and epileptic seizure EEG patterns are shown in Fig. 1. Control animals never manifested EEG or behavioral seizures. Administration of the CB<sub>1</sub> receptor agonists *R*(+)WIN55,212 (5 mg/kg i.p.) (Fig. 1 and 2A) and THC (30 mg/kg i.p.) (Fig. 2A), the primary psychoactive active compound in marijuana, completely terminated both behavioral and electrographic seizures in this refractory seizure model ( $p \leq 0.05$ ). *R*(+)WIN55,212 and THC began having anticonvulsant effects at 0.5 and 5 mg/kg i.p., respectively. The dose response evaluation of these compounds revealed approximate ED<sub>50</sub> values of 1 mg/kg i.p. for *R*(+)WIN55,212 and 15 mg/kg i.p. for THC. The concentrations of THC and *R*(+)WIN55,212 required to inhibit seizures in this model were similar in effect and dose to their ability to inhibit maximal electric shock-induced seizures (Wallace et al., 2001), and this concentration of THC has been shown to have anticonvulsant effects in other seizure models (Wada et al., 1975; Karler and Turkkanis, 1980; Colasanti et al., 1982). At the maximal anticonvulsant doses of THC and *R*(+)WIN55,212, the animals were not significantly sedated and were alert enough to be able to move freely in the their cages. These ED<sub>50</sub> values are below the ED<sub>50</sub> values for *R*(+)WIN55,212 and THC in decreasing spontaneous activity and similar to the concentrations used to cause hypothermia and analgesia (Wallace et al., 2001). Thus, the anticonvulsant effects of THC and *R*(+)WIN55,212 are in the same



**Fig. 1.** The effects of CB<sub>1</sub> receptor modulation on epileptiform activity in control and epileptic animals. Representative EEG recordings of control, epileptic, *S*(-)WIN55,212-, [(-)WIN]-treated (5 mg/kg i.p.) epileptic animals and *R*(+)WIN55,212-, [(+)WIN]-treated (5 mg/kg i.p.) epileptic animals. Treatment with *R*(+)WIN55,212 (5 mg/kg i.p.) completely abolished seizure activity.



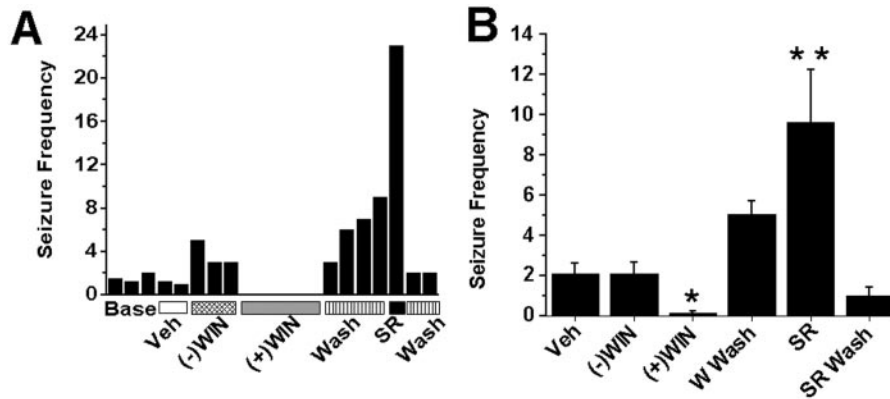
**Fig. 2.** The effects of CB<sub>1</sub> receptor activation and blockade on the seizure frequency of epileptic rats demonstrated in single-injection experiments. A, seizure frequency per 10 h for baseline (base) and treatment with vehicle (Veh), *S*(-)WIN55,212, [(-)WIN] (5 mg/kg i.p.), *R*(+)WIN55,212, [(+)WIN] (5 mg/kg i.p.), SR141716A (SR) (10 mg/kg i.p.), and THC (30 mg/kg i.p.). Data represent mean ± S.E. ( $n = 6$  per drug treatment; \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.001$ ). B, inhibition of seizure activity at high therapeutic concentrations (Morris et al., 2001) by the anticonvulsants phenobarbital (PB; 40 mg/kg i.p.) and phenytoin (PHT; 100 mg/kg i.p.) and the cannabinoids THC (30 mg/kg i.p.) and *R*(+)WIN55,212, (+)WIN (5 mg/kg i.p.) ( $n = 6$  per drug treatment; \*,  $p \leq 0.01$  in comparison with epileptic animals). Only THC and (+)WIN completely abolished seizure activity. These single-injection experiments directly evaluated the effects of each agent on seizure frequency in multiple animals.

concentration range of some of the other physiological effects of the cannabinoids but below sedative concentrations. The inactive isomer, *S*(-)WIN55,212 (5 mg/kg i.p.), and drug vehicle alone had no effect on seizure frequency (Fig. 1 and 2A). The enantioselectivity of the anticonvulsant effect of *R*(+)WIN55,212 strongly indicates that this compound is acting via a CB<sub>1</sub> receptor-specific mechanism. Maximally effective doses that produce high therapeutic blood levels of phenobarbital (40 mg/kg i.p.) and phenytoin (100 mg/kg i.p.), well established anticonvulsants, do not completely inhibit seizure activity in this model of refractory epilepsy (Leite and Cavalheiro, 1995; Morris et al., 2001). Because of the inability of these standard anticonvulsants to completely block seizures when used in high therapeutic levels in this model, the pilocarpine model of epilepsy is considered a model of refractory or difficult-to-control seizures with conventional anticonvulsant agents. The cannabinoids were very effective anticonvulsants in this model at a concentration that did not produce sedation but completely abolished seizures. Pheno-

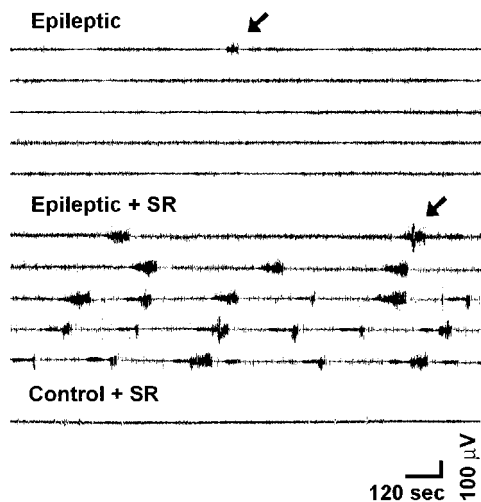
barbital and phenytoin at very high concentrations were not as effective. Thus, treatment of animals with phenobarbital and phenytoin was less efficacious than cannabinoids in preventing behavioral and electrographic seizures produced in this model (Fig. 2B), indicating that cannabinoids may offer unique advantages in treating seizures refractory to currently prescribed anticonvulsants.

Seizure characteristics can vary between animals, a limiting factor in the interpretation of data. Therefore, to increase the statistical power of the study, we tested the effects of cannabinoids on seizure frequency and duration by systematically treating a group of eight epileptic animals over a 10-day period with a multiple drug treatment regimen. This paradigm consisted of baseline (1.5 days), drug vehicle (1 day), *S*(-)WIN55,212 (5 mg/kg i.p.) (1.5 days), *R*(+)WIN55,212 (5 mg/kg i.p.) (2.5 days) followed by a drug-free period (2 days), SR141716A (10 mg/kg i.p.) (1 day), and ending with a drug-free period (1.5 days) (Fig. 3A). Seizure frequency for these epileptic animals during baseline recording ranged between one and three per 12-h recording interval. Treatment with vehicle or the inactive isomer *S*(-)WIN55,212 had no statistically significant effect on seizure frequency (Fig. 3, A and B). Conversely, treatment with *R*(+)WIN55,212 abolished seizures in all eight animals used in this treatment paradigm (Fig. 3B;  $p \leq 0.05$ ). During the drug-free period after treatment with *R*(+)WIN55,212, seizure frequency increased slightly above baseline (Fig. 3, A and B). The observed increase in seizure frequency after cannabinoid cessation is consistent with the withdrawal phenomenon and rebound hyperexcitability described in other behavioral studies (Karler et al., 1986). However, this rebound effect was transient, with seizure frequency in most animals returning to levels similar to baseline by the later half of the second day of drug withdrawal. On day 9, we administered a single injection of SR141716A that produced a significant but reversible increase in seizure frequency compared with baseline or the drug withdrawal seizure frequency (Fig. 3, A and B), supporting the hypothesis that endogenous cannabinoids act tonically to dampen neuronal hyperexcitability. The effect of SR141716A was significantly elevated above the baseline and the rebound periods. In addition, SR141716A treatment alone clearly produced increased seizure frequency in epileptic animals (Fig. 2A). The multiple treatment experiments demonstrated a tolerance effect. Long-term administration of cannabinoids affected seizure frequency when the cannabinoids were discontinued. Further study of the tolerance effect of the cannabinoids on the CB<sub>1</sub> receptor in epileptic animals is an important area for further investigation but is beyond the scope of the present study.

**Antagonism of the CB<sub>1</sub> Receptor Significantly Increases Seizure Frequency and Duration.** Administration of SR141716A to epileptic rats resulted in a statistically significant increase in seizure frequency (Fig. 2A and 3B;  $p \leq 0.01$ ). Figure 4 compares 60 min of continuous EEG recording before and after SR141716A treatment in an epileptic animal. This EEG recording is representative of the increased seizure frequency observed in all animals treated with SR141716A. Several SR141716A-treated animals developed SE, a severe prolonged seizure condition associated with a high morbidity and mortality (DeLorenzo et al., 1996). In these animals, EEG seizure activity was nearly continuous,



**Fig. 3.** The effects of CB<sub>1</sub> receptor activation and blockade on the seizure frequency in eight epileptic rats sequentially treated with a multiple drug regimen that includes a CB<sub>1</sub> receptor agonist and antagonist. These experiments evaluate the effects of each drug in comparison with the other drugs in the same animal. A, seizure frequency per 12 h in a representative epileptic animal after consecutive administration of vehicle, *S*(-)-WIN55,212, (-)WIN (5 mg/kg i.p.), *R*(+)-WIN55,212, (+)WIN (5 mg/kg i.p.), *R*(+)-WIN55,212 (5 mg/kg i.p.) washout (W wash), SR141716A, SR (10 mg/kg i.p.), and SR141716A washout (SR wash). Bars represent the number of seizures observed in a representative epileptic animal for each 12-h monitoring period. B, mean seizure frequency (per 12 h) of eight epileptic animals treated with the same drug regimen shown in Fig. 3A. This figure presents the mean data for the multiple drug experiments for each experimental condition and analyzes the data statistically. Data represent the mean ± S.E. (seizures per 12 h) ( $n = 8$ ; RM ANOVA; \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ).



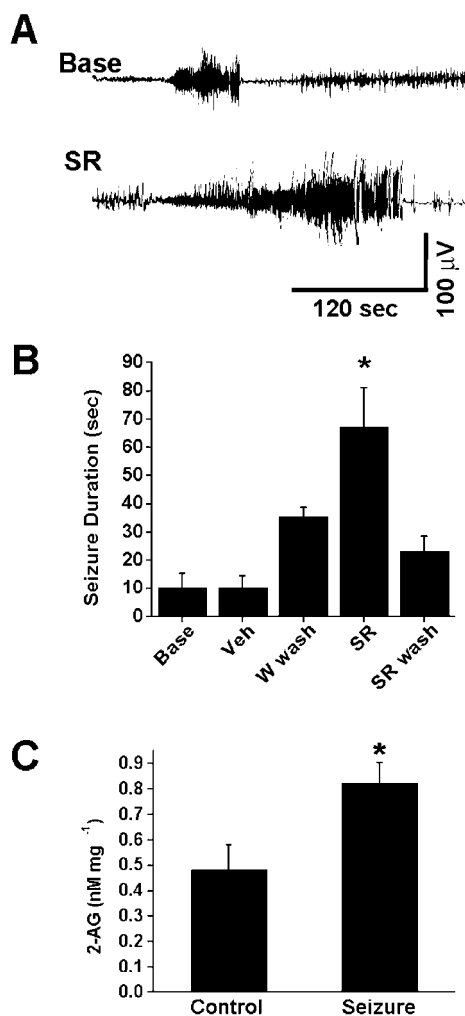
**Fig. 4.** Antagonism of the CB<sub>1</sub> receptor by SR141716A (10 mg/kg i.p.) caused increased seizure frequency and produced status epilepticus in some animals. The data represent EEG and behavioral seizures observed over the 1-h recording period for epileptic and epileptic + SR conditions. These recordings represent continuous EEG recordings from an epileptic rat 60 min before and 60 min after treatment with SR141716A. Arrows represent individual seizures. The representative EEG recording from an epileptic animal manifested one spontaneous recurrent seizure in the 1 h of recording. SR treatment in epileptic animals caused a marked increase in seizure frequency. During the numerous seizures shown for SR141716A treatment in the 1-h recording, the animal was not responsive in between seizures for more than 30 min. Thus, SR141716A produced status epilepticus in this animal, employing the standard definition of SE that includes intermittent seizure activity lasting for more than 30 min without regaining consciousness between seizures. The Control + SR representative EEG recording demonstrates that treatment of control (nonepileptic) animals with SR141716A did not produce seizure activity.

and animals were unresponsive to external stimuli with loss of righting reflex for 30 min or more. SR141716A (10 mg/kg i.p.) has been shown to inhibit the anticonvulsant effects of cannabinoids and endocannabinoids (Wallace et al., 2001, 2002). SR141716A was also effective in blocking the anticonvulsant effects of THC and *R*(+)-WIN55,212 at 5 mg/kg i.p. We choose to use the higher concentration of SR141716A to obtain a clear antagonist effect. In addition, SR141716A (10

mg/kg i.p.) did not induce seizures in control animals (Fig. 4). The effect of SR141716A was only observed in the epileptic animals, and this compound did not cause hyperexcitability in control or naive animals.

To further evaluate the role of endogenous CB<sub>1</sub> receptor activation on seizure termination, we quantified the duration of individual seizure events within each drug treatment period (Fig. 5, A and B). In all animals monitored, EEG seizures directly coincided with behavioral seizures observed on video recording. During vehicle and *S*(-)-WIN55,212 treatments, seizure duration was not significantly altered from baseline. Treatment with the CB<sub>1</sub> receptor antagonist, SR141716A, caused a significant increase in seizure duration ( $p \leq 0.01$ ; Fig. 5, A and B). Prolongation of seizure discharge by SR141716A is apparent in the EEG patterns of representative seizure events (Fig. 5A).

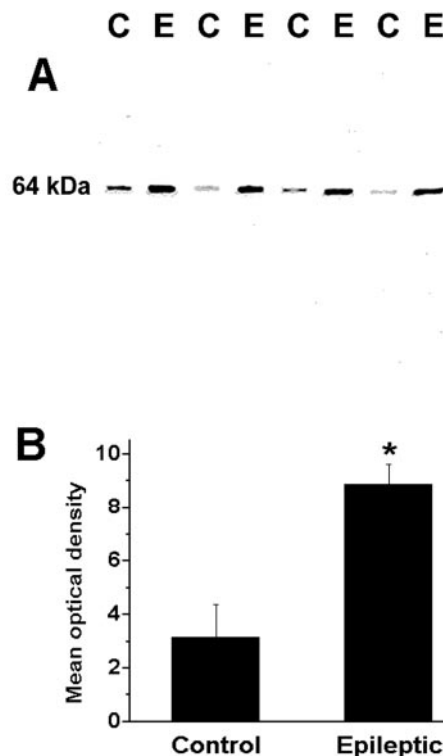
**Hippocampal Levels of 2-AG Increase during Seizure Activity.** 2-AG synthesis occurs during neuronal depolarization in a Ca<sup>2+</sup>-dependent manner (Stella et al., 1997). Sustained neuronal depolarization and elevated intracellular Ca<sup>2+</sup> are known to accompany seizure activity (Raza et al., 2001). We have previously shown that endogenous cannabinoids are anticonvulsant and this anticonvulsant activity of the endocannabinoids could be blocked by SR141716A (Wallace et al., 2002). If the endogenous cannabinoid system contributes to epileptic seizure termination, seizure activity in an intact animal would be expected to increase synthesis of endogenous cannabinoids. The hippocampal brain region is a locus of epileptic seizure activity (Lothman et al., 1991); therefore, we sought to determine the effect of status epilepticus on hippocampal levels of 2-AG. Pilocarpine-injected animals were sacrificed after 15 min of pilocarpine-induced seizure activity along with sham controls. Levels of 2-AG in hippocampal extracts were determined according to previously published methods (Di Marzo et al., 2000). In acutely seizing animals, endogenous 2-AG levels were significantly increased compared with controls (Fig. 5C;  $p \leq 0.05$ ). The data demonstrate that a single pilocarpine-induced seizure can increase the level of the endogenous cannabinoid 2-AG in hippocampal tissue. We have also demonstrated that



**Fig. 5.** CB<sub>1</sub> receptor-dependent regulation of seizure duration in epileptic rats. A, a representative EEG recording of a seizure in an epileptic (Base) and an epileptic animal treated with SR141716A (SR) (10 mg/kg i.p.) demonstrating increased seizure duration produced by SR. B, mean seizure duration for the treatments shown in Fig. 3B. Data represent the mean  $\pm$  S.E. ( $n = 8$  animals; RM ANOVA; \*,  $p \leq 0.01$ ). C, hippocampal endogenous 2-AG levels in control and seizure animals (15 min after seizure onset). The data represent the mean  $\pm$  S.E. ( $n = 7$ ; \*,  $p \leq 0.01$ ; Student's *t* test)

SR141716A (10 mg/kg i.p.) inhibited the anticonvulsant effects of endocannabinoids (Wallace et al., 2002).

**Increased Hippocampal CB<sub>1</sub> Receptor Expression in Epileptic Rats.** Because CB<sub>1</sub> receptor activation was shown to alter seizure frequency and duration, we sought to evaluate possible changes in CB<sub>1</sub> receptor expression in the hippocampi of epileptic animals. Using Western blot analyses, we compared sham control with epileptic hippocampal neuronal membranes and found a significant increase in epileptic brains of the expression of the 64-kDa molecular mass CB<sub>1</sub> receptor protein (Cichewicz et al., 2001) (Fig. 6A). Quantification of the bands shown in Fig. 6A revealed that expression of this protein was increased 183% in the hippocampi of epileptic rats compared with sham-treated animals (Fig. 6B;  $p \leq 0.01$ ), indicating that a long-term plasticity change in the expression of the CB<sub>1</sub> receptor occurs with epilepsy. Western blot values were further corrected using the internal membrane protein markers tubulin and microtubule-associated protein 2 proteins (data not shown). After correcting canna-



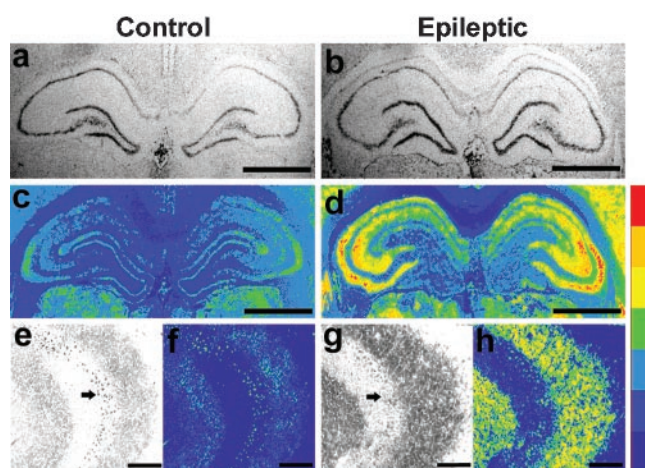
**Fig. 6.** CB<sub>1</sub> receptor expression is increased in hippocampal neuronal membranes of epileptic rats. A, representative Western blot of sham control (C) and epileptic (E) hippocampal neuronal membranes. The band visualized corresponded to the 64-kDa CB<sub>1</sub> receptor protein (Cichewicz et al., 2001). B, mean optical densities (arbitrary units) of CB<sub>1</sub> receptor protein expression in hippocampal membranes of epileptic and sham control rats ( $n = 6$ ; \*,  $p \leq 0.01$ ; Student's *t* test). Epileptic CB<sub>1</sub> receptor protein was increased by 183%.

binoid protein levels to internal protein standards, we still observed a significant increase in the CB<sub>1</sub> receptor expression in epileptic animals (data not shown).

To evaluate the anatomical distribution of this increase in CB<sub>1</sub> receptor expression in epileptic brains, we conducted immunohistochemical staining of CB<sub>1</sub> receptor protein on coronal hippocampal sections using established techniques (Pettit et al., 1998). Figure 7, A and B, shows representative patterns of cellular Nissl staining in sham control and epileptic hippocampi. No apparent changes in hippocampal morphology were observed in epileptic versus sham control animals, with the exception of minimal cell loss in the CA1 region (less than 10%), as described previously (Rice and DeLorenzo, 1998). Representative pseudocolor-enhanced images of CB<sub>1</sub> receptor protein staining of epileptic and sham control hippocampal sections illustrate a dramatic increase in CB<sub>1</sub> receptor expression in epileptic hippocampi (Fig. 7, C and D). CB<sub>1</sub> receptor protein staining was most dramatically increased in the CA1 through CA3 regions of the hippocampus, with the highest increase localized to the dendritic synaptic areas of CA2 and CA3 (Fig. 7, E-G). The dentate gyrus did not show a corresponding increase in CB<sub>1</sub> receptor expression.

## Discussion

In the present study, we report that the endogenous cannabinoid system plays a critical role in modulating seizure activity in epilepsy. Both cannabinoids, THC and



**Fig. 7.** Immunohistochemical detection of CB<sub>1</sub> receptor expression in control and epileptic hippocampi. Representative Nissl staining of control (A) and epileptic (B) sections. Representative pseudocolor-enhanced immunohistochemical staining of the CB<sub>1</sub> receptor protein in control (C) and epileptic (D) sections. The increase in CB<sub>1</sub> receptor protein expression observed in epileptic hippocampi was representative of four epileptic versus four control animals, 15 tissue sections per animal. High magnification of CB<sub>1</sub> receptor immunoreactivity of sham control (E) and epileptic (G) hippocampal formation demonstrated increased staining in the dendritic fields of the CA2 and CA3 regions of epileptic animals. Arrows indicate the location of the CA2 through CA3 pyramidal neurons. High magnification of pseudocolor-enhanced images of sham control (F) and epileptic (H) CA2 and CA3 regions. Bars in A through D represent 2 mm. Bars in E through H represent 200  $\mu$ m. Red, highest level on color scale. The results shown are representative of several experiments.

*R(+)*WIN55,212, were anticonvulsant in the rat pilocarpine model of acquired, refractory epilepsy. Antagonism of the CB<sub>1</sub> receptor by SR141716A caused a marked increase in seizure frequency and duration, indicating that endogenous activity of the CB<sub>1</sub> receptor strongly influences seizure activity. By antagonizing the CB<sub>1</sub> receptor, we blocked its activation by endogenous cannabinoids and thereby elicited a sustained seizure response that often resembled the clinical phenomenon status epilepticus. The rise in endogenous 2-AG levels that occurred during short-term, pilocarpine-induced seizure demonstrates that seizure activity can increase the levels of 2-AG and further suggests a modulatory role for endogenous cannabinoids in epilepsy. Additionally, using Western blot and immunohistochemical techniques, we determined that hippocampal CB<sub>1</sub> receptor expression in epileptic animals was significantly increased over sham controls. These changes were primarily observed in the CA1 through CA3 regions of the hippocampus, indicating that plasticity of the endogenous cannabinoid system occurs in this brain region in response to epilepsy. These data provide evidence that the CB<sub>1</sub> receptor and the endogenous cannabinoid system play a critical role in dampening epileptic neuroexcitation. Piomelli's group (Rodriguez de Fonseca et al., 2001) has demonstrated that it is possible to trigger physiological effects of cannabinoids without producing the unwanted behavioral or psychoactive effects of these compounds. Thus, the development of anticonvulsant cannabinoids that do not produce unwanted side effects is an important area for further research to develop novel therapeutic agents to treat intractable seizures.

**Epilepsy Increases Hippocampal CB<sub>1</sub> Receptor Expression.** The findings presented in this study demonstrate that a significant change in CB<sub>1</sub> receptor expression occurs

with the epileptic phenotype. The animals used in this study had been epileptic for nearly 1 year, indicating that this change in CB<sub>1</sub> receptor expression is prolonged and probably permanent. CB<sub>1</sub> receptor expression has also been shown to increase in an animal model of stroke (Jin et al., 2000), a condition, like epilepsy, that is associated with excessive glutamate release and the development of seizures. The observation that the cannabinoid receptor is up-regulated in ischemia and epilepsy implies a compensatory role for the receptor in mitigating excitotoxicity. In light of the anticonvulsant effect of both *R(+)*WIN55,212 and THC, as well as the proconvulsant action of the CB<sub>1</sub> receptor antagonist in this epilepsy model, we propose that the increase in CB<sub>1</sub> receptor expression displayed in epileptic brains was a compensatory rather than a causal factor of seizure manifestation and served to dampen seizure activity. The increased CB<sub>1</sub> receptor expression displayed in the hippocampi of epileptic animals was regionally specific, occurring in the CA dendritic field and not in the dentate gyrus. This increase in CB<sub>1</sub> receptor expression was demonstrated up to 1 year after the induction of epilepsy and thus demonstrates a long-lasting or permanent plasticity change in the brain that may play a role in the pathophysiology of epilepsy. The functional relevance of this differential increase in CB<sub>1</sub> receptor expression may be revealed by further study.

**Anticonvulsant Action of Cannabinoids.** Recent discoveries in the cannabinoid field have demonstrated that cannabinoids ameliorate symptoms associated with neuronal hyperexcitability. In models of multiple sclerosis (Baker et al., 2000) and Huntington's disease (Lastres-Becker et al., 2002), CB<sub>1</sub> receptor activation significantly reduced spasticity and tremor, and exogenous application of 2-AG has been shown to be neuroprotective after traumatic brain injury (Panikashvili et al., 2001). Furthermore, in *in vitro* and *in vivo* studies of ischemia, cannabinoids significantly decreased excitotoxic neuronal cell death that resulted from excessive glutamatergic transmission (Abood et al., 2001). These cannabinoid actions are believed to involve attenuation of glutamate release. At the molecular level, the anticonvulsant mechanism of cannabinoids is unknown. However, because modulation of presynaptic neurotransmitter release is believed to be a primary result of CB<sub>1</sub> receptor activation, we believe that this mechanism may underlie cannabinoid anticonvulsant properties. CB<sub>1</sub> receptor activation is known to decrease calcium influx through N- and P/Q-type Ca<sup>2+</sup> channels (Mackie and Hille, 1992), the result of which is decreased Ca<sup>2+</sup>-dependent glutamate release. Glutamate is the primary excitatory neurotransmitter of the central nervous system. Although critical for normal neurotransmission, elevated levels of glutamate are associated with excitotoxicity and excessive glutamatergic transmission is a hallmark of epilepsy (Lothman et al., 1991). With elevated levels of glutamate detected in epileptic tissue (Lothman et al., 1991), decreased release of this neurotransmitter would be a logical cannabinoid anticonvulsant mechanism. CB<sub>1</sub> receptor activation also increases the conductance of presynaptic A-type (Hampson et al., 1995) and G-protein-coupled inward rectifying K<sup>+</sup> channels (Mackie et al., 1995). Increased K<sup>+</sup> channel permeability attenuates neuronal bursting and stabilizes membrane potential, additional factors that would contribute to decreased epileptiform discharge. Preliminary data from our group indicates that CB<sub>1</sub>

knockout animals have spontaneous seizures, further suggesting an endogenous role for the CB<sub>1</sub> receptor in controlling neuronal excitability.

CB<sub>1</sub> receptor activation has also been shown to decrease GABAergic function in the hippocampus. In particular, endogenous cannabinoids are believed to be retrograde mediators of depolarization-induced suppression of inhibition (DSI) (Wilson and Nicoll, 2001). The overall effect of DSI at the synapse is disinhibition of the postsynaptic neuron and, therefore, facilitation of excitatory transmission. In light of the increased neuronal excitability that may result from this action, decreased GABAergic tone most probably does not mediate the anticonvulsant mechanism of cannabinoids. However, Cohen et al. (2002) recently demonstrated that the GABAergic system, normally an inhibitory neurotransmitter, can become a depolarizing force capable of synchronizing abnormal bursting in human epileptic, temporal lobe, brain slice preparations. If this phenomenon were to occur within the brains of animals with pilocarpine-induced epilepsy, then a cannabinoid-mediated decrease in GABAergic tone may indeed be anticonvulsant.

A more probable explanation for the anticonvulsant action of cannabinoids lies in the possibility that the pathology of epilepsy causes a compensatory shift to occur in the balance between CB<sub>1</sub> receptor-mediated inhibition of presynaptic glutamate and GABA release. In support of this, recent studies have shown that, in a manner similar to DSI, depolarization-induced suppression of excitation can be induced in hippocampal tissue (Ohno-Shosaku et al., 2002). The induction of this phenomenon was dependent on the sensitivity of the presynaptic neuron to cannabinoids as well as the duration of postsynaptic depolarization. With extended depolarization, the result of CB<sub>1</sub> receptor activation was a shift from DSI to depolarization-induced suppression of excitation. Therefore, the extended neuronal depolarization of an epileptiform discharge may cause a switch from suppression of GABA release to suppression of glutamate release.

Synthesis of the endogenous cannabinoid 2-AG is believed to occur in a calcium-dependent, "on-demand" fashion from arachidonic acid-enriched membrane phospholipids. During a seizure, elevated intracellular Ca<sup>2+</sup> results from prolonged neuronal depolarization (Raza et al., 2001). Because increased hippocampal levels of 2-AG were detected 15 min into a seizure and CB<sub>1</sub> receptor antagonism resulted in prolonged seizure duration, we believe that seizure-induced increases in intracellular calcium result in the de novo synthesis of endogenous cannabinoids that then bind the CB<sub>1</sub> receptor to terminate seizure discharge, forming a negative feedback loop. This increase in 2-AG occurred in comparison with sham animals and was shown to be dependent on seizure activity and not manipulation of the animals or drug-specific effects. Additional evidence of compensatory endogenous cannabinoid release during seizure activity is provided by studies that show elevated 2-AG after injection of the chemoconvulsant picrotoxin (Sugiura et al., 2000). 2-AG is known to bind the CB<sub>1</sub> receptor with high affinity in a manner that is blocked by coadministration of SR141716A.

Several factors in addition to increased production of 2-AG could explain seizure-induced increase in the levels of this compound. Alternatively, increased 2-AG levels during seizures may be the result of decreased function of the fatty acid amidohydrolase enzyme that is known to be responsible for

the catalysis of the compound. Increased receptor sensitivity and reducing cannabinoid catabolism during seizure activity could also account for the net increase in 2-AG observed after seizure activity. Further study may reveal which mechanism generates this increase in hippocampal 2-AG.

**Therapeutic Implications for Cannabinoids in the Treatment of Epilepsy.** Seizures in patients with refractory, partial-complex epilepsy can be difficult to control despite the use of currently available anticonvulsant medications and surgical interventions. Therefore, there is a clear need for the development of more effective anticonvulsant agents. Some epilepsy patients, seeking alternative treatments, have perceived improvement with marijuana (Consrøe et al., 1975). This has prompted several countries to consider the legalization of marijuana for epilepsy treatment (National Institutes of Health, 1997; R. v. Parker, 1997; House of Lords Select Committee on Science and Technology, 1998). The pilocarpine model represents a refractory epileptic condition that is not readily treated by conventional anticonvulsants (Leite and Cavalheiro, 1995; Morris et al., 2001). Our results demonstrate that activation of the CB<sub>1</sub> receptor by cannabinoid drugs and possibly endogenous ligands significantly alters seizure activity and is more effective than conventional anticonvulsants in treating the refractory seizures produced in the pilocarpine model. Although the dose dependence and long-term effects of cannabinoid administration on epilepsy must be further investigated, the results presented here provide evidence that warrants a comprehensive assessment of cannabinoid use in the control of refractory epilepsy via the use of animal models and placebo-controlled clinical trials. Although the psychoactive side effects of cannabinoids make their use in the treatment of epilepsy impractical, understanding the mechanisms of endogenous cannabinoid-mediated anticonvulsant action may lead to the development of novel compounds that do not manifest behavioral toxicity. Further investigation of the cannabinoid anticonvulsant phenomenon may illuminate novel therapeutic targets for the treatment of temporal lobe epilepsy as well as more clearly define the physiological function of the endogenous cannabinoid system in brain.

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