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The endogenous cannabinoid system regulates seizure frequency and duration in a model of temporal lobe epilepsy.

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Running Title**: Endogenous cannabinoid system in epilepsy**

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Abbreviations:
2-AG: 2-arachidonylglycerol
AEA: arachidonylethanolamine
CB_1: cannabinoid CB_1 receptor
EEG: electroencephalographic
R(+)WIN55, 212: 4,5-dihydro-2-methyl-4(4-morpholinylmethyl)-1-(1-naphthalenyl-carbonyl)-6H-pyrrolo[3,2,1ij]quinolin-6-one
S(-)WIN55, 212: (S-[-]-[2,3-Dihydro-5-methyl-3-([4-morpholinyl]methyl(pyrrolo)1,2,3-de(-1,4--benzoxazinyl)-[1-napthalenyl]methanone)
SR141716A: N-(piperidin-1-yl-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride
THC: Δ^9-tetrahydrocannabinol
DSI: depolarization-induced suppression of inhibition
DSE: depolarization-induced suppression of excitation
SE: status epilepticus
HPLC-MS: High performance liquid chromatography-mass spectroscopy
i.p.: intraperitoneal

Section: Neuropharmacology

Key words: cannabinoids, epilepsy, pilocarpine, CB_1, anticonvulsant, hippocampus

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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 Δ^9-THC (10 mg/kg) as well as the cannabimimetic, R(+)WIN55,212 (5 mg/kg), completely abolished spontaneous epileptic seizures. Conversely, application of the CB_1 receptor antagonist, SR141716A, significantly increased both seizure duration and frequency. In some animals, CB_1 receptor antagonism resulted in seizure durations that were protracted to a level consistent with the clinical condition status epilepticus (SE). Furthermore, we determined that during an acute pilocarpine induced seizure, levels of the endogenous CB_1 ligand 2-AG 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_1 receptor. Furthermore, Western blot and immunohistochemical analyses revealed that CB_1 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_1 receptor occurs with epilepsy.

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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). Utilizing the maximal electroshock model of acute seizure, our laboratory determined that cannabinoid compounds block seizure spread via a cannabinoid CB_1 receptor dependent mechanism (Wallace et al. 2001, Wallace et al. 2002). Further study revealed that application of a CB_1 receptor antagonist lowered electroshock seizure threshold (Wallace et al. 2002), indicating that elimination of endogenous cannabinoid tone at the CB_1 receptor may increase seizure susceptibility.

The CB_1 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 (Ohno-Shosaku et al. 2002; Wilson and Nicoll, 2001). The endogenous cannabinoids, arachidonylethanolamine (AEA) 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 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_1 receptor activation. In light of cannabinoid effects on neurotransmission, increased CB_1 receptor activation could

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influence seizure activity. However, there are no studies evaluating the role of the endogenous cannabinoid system in an intact model of epilepsy.

This study was initiated to evaluate the role of the CB_1 receptor and the endogenous cannabinoid system in regulating seizure activity in a chronic model of epilepsy. We utilized 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_1 receptor agonists, 4,5-dihydro-2-methyl-4(4-morpholinylmethyl)-1-(1-naphthalenyl-carbonyl)-6H-pyrrolo[3,2,1ij]quinolin-6-one (R(+)WIN55,212) and Δ^9-tetrahydrocannabinol (THC) were evaluated for anticonvulsant efficacy. In addition to agonist effects on seizure activity, the effect of CB_1 receptor antagonism on seizure frequency and duration was evaluated using the specific antagonist, N-(piperidin-1-yl-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride (SR141716A). Hippocampal levels of 2-AG during acute, 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_1 receptor protein expression in the brains of chronically epileptic and sham control rats. The findings presented suggest an anticonvulsant role for

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the endogenous cannabinoid system and demonstrate that long-term plasticity of the CB_1 receptor occurs with epilepsy.

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METHODS

Pilocarpine Induced Status Epilepticus

Male Sprague-Dawley rats 200-250g were utilized in accordance with university animal care and use protocols. Animals were housed in single cages on a 12/12 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. (Mello et al., 1993) that is well established in our laboratory (Rice and DeLorenzo, 1998). Prior to 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 SE typically occurred within 20 to 40 min post 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 minutes or longer or intermittent seizures without regaining conciousness between seizures that lasted 30 minutes or longer. The severity of convulsions were evaluated and only those animals that displayed behaviors consistent with ongoing SE were utilized 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, 45% H_2O) at 1,3, and 5 hr post-onset of SE. Animals continuing to display seizure activity beyond 6 hr post-onset of SE were euthanized. Control groups were comprised 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**

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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 **two-three weeks after the initial episode of SE or after sham treatment** in a manner previously described (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, N.Y.) 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 one month prior to 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 characterized by video analysis of animals displaying moderate to**

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severe behavioral seizures characterized by forelimb clonus, rearing and falling in conjunction with electrographic seizure activity obtained from EEG analysis.

Two types of experiments were performed to evaluate the effects of cannabinoids and other agents on seizure frequency and duration: single injection and multiple injection experiments. The single injection experiments were designed to test the effects of a single experimental treatment in a given animal on seizure frequency and duration. The effect of each treatment in numerous animals was determined and expressed as the mean and standard error. Since variations in baseline seizure frequency were observed between epileptic animals, a multi-drug treatment regimen was carried out to increase statistical power of this study. This drug treatment paradigm allows for each epileptic animal to act as its own internal control, thus correcting for differences in epileptic manifestations within the experimental group. This regimen also allowed for the use of fewer experimental animals to obtain statistical analysis. Comparable results for each agent were obtained from the single and multiple injection experiments. These studies are extremely labor intensive and each animal was recorded for over a week. Video behavioral activity and EEG recordings were then analyzed over these extensive time periods for each animal and appropriate statistical analysis of the data was performed.

For single injection experiments, animals were given a 2-3 hr 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.),

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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 (Rhone-Poulenc, Inc., Princeton, NJ) and 0.9% saline at a ratio of 1:1:18. **Brief halothane anesthesia was induced in animals prior to drug injections to minimize unnecessary stress, pain or trauma. Animals fully recovered from anesthesia within two minutes 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 prior to initiation of the dosing regimen. Animals were then consecutively treated with vehicle solution for one 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 Chemical (St. Louis, MO). SR141716A and THC were supplied through the NIDA Chemical Synthesis and Drug Supply Program.

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Measurement of hippocampal 2-AG levels

Pilocarpine was utilized to acutely induce seizure activity in naive, male, Sprague-Dawley rats weighing 200-250g. In these studies, animals were injected with scopolamine and 375 mg/kg i.p. pilocarpine as described earlier (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 **HPLC-MS** according to published methods (Di Marzo et al., 2000).

Western Blot Protocol

Gel electrophoresis was carried out on rat hippocampal neuronal membrane preparations from 1-year post-SE, epileptic and age-matched, sham treated animals. Following 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 PMSF and neuronal membranes were isolated by centrifugation (Morris et al., 2001). Prior to electrophoresis, membrane samples were thawed on ice and protein concentration per sample was calculated using the MicroBradford reagent System (Biorad Inc. Hercules, CA) quantified using a UV-2101PC ultraviolet spectrophotometer (Shamadzu Corp. Japan). Samples were balanced to 5 μg protein / gel lane and were denatured in β-mercaptoethanol and loading dye buffer. Samples were then heated to 90°C for 5 min in a programmable thermal controller PTC100 (MT Research Inc. Watertown, MA) and were allowed to cool to room temperature prior to loading onto a 10% Tris-HCl Ready Gel (Biorad Inc. Hercules, CA). A colorimetric molecular weight marker including

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standards ranging from 10–182 kDA (ProSieve, BMA Rockland, MA) was loaded onto the last lane of the gel to aid in determining protein size. Gels were assembled into a MiniProtean II Electrophoresis System (Biorad Inc. Hercules, CA) and resolved for 90 min at 220 constant voltage in Tris Buffer (Biorad Inc. Hercules, CA). Following electrophoresis, gels were Western blot transferred to Immobilon nylon membrane (Millipore Inc. Bedford, MA) for 2 hr 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. Following transfer, the Western blot was stored in phosphate buffered saline at 4°C overnight. Gels were stained for protein and quantitated for MAP2 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 comprised of 3% Blotting Grade Blocker (Biorad Inc. Hercules, CA) and 0.05% Tween 20 in phosphate buffered saline for 45 min at room temperature. Rabbit (polyclonal) anti-cannabinoid CB_1 receptor unconjugated primary antibody (Biosource Inc. Camarillo, CA) was added to the blocking solution at a concentration of 1μg/ml and the membrane was incubated for 90 min at room temperature. Following primary antibody incubation, the membrane was washed for a total of 15 min (3 x 5 min) in phosphate buffered saline. The membrane was then re-blocked in fresh blocking buffer for 30 min. Anti-Rabbit IgG-HRP 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 (3 x 5 min) in PBS and incubated 5 min in Super Signal (Pierce Inc. Rockford, IL) for

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enhanced chemi-luminescent analysis. Chemi-luminescent images were visualized using Kodak X-Omat Blue XB-1 X-ray film (Kodak Rochester, NY) and developed using a Kodak M35A X-Omat Processor (Kodak Rochester, NY). Film images were digitized using a gel scanner and analyzed by computer-assisted densitometry (Molecular Dynamics Inc. Sunnyvale, CA). Using the molecular weight marker as reference, the protein band was determined to correspond to a weight of 64 kDa.

Immunohistochemistry

Four epileptic and 4 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 μM) were fixed in acetone and prepared for immunostaining using established techniques (Pettit et al., 1998). CB_1 receptor protein immunoreactivity for each animal was evaluated using greater than 15 tissue sections. Briefly, tissue sections were blocked in bovine serum for 1 hr and then incubated with CB_1 antiserum at 5.0 μg/ml for 1hr 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_1 receptor immunoreactivity was visualized by exposure to avidin-biotin complex and 3-3'diaminobenzidine (DAB)(Vector Laboratories Inc., CA). Adjacent tissue sections were evaluated morphologically using Nissl stain. Stained tissue sections were evaluated using a binocular microscope (Olympus Inc., NY) and were photographed using a digital camera (Olympus Inc., NY). Images were analyzed using Analysis® software (Soft Imaging System, CO).

Statistical Analyses

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Results are given as means ± standard error of the mean (SEM). 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 One-Way Analysis of Variance (RM ANOVA) in conjunction with the *post-hoc* Tukey test were utilized where appropriate. Graphs were generated using Origin 6.1 Software (Microcal Software, Inc. Northampton, MA).

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RESULTS

Modulation of the CB_1 Receptor Alters Seizure Frequency in the Rat Pilocarpine Model of Epilepsy

Epileptic rats manifested an average of 3.0 (±0.9) seizures per 10 hr period. Representative control and epileptic seizure EEG patterns are shown in Figure 1. Control animals never manifested EEG or behavioral seizures. Administration of the CB_1 receptor agonists R(+)WIN55,212 **(5 mg/kg i.p.)** (Fig. 1 & 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 mg/kg i.p. and 5 mg/kg i.p. The dose response evaluation of these compounds revealed approximate ED50 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 to inhibit seizures in this model is similar in effect and dose as its 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 (Colasanti BK et al., 1982; Karler R and Turkanis SA, 1980; Wada JA et al., 1975). 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 ED50 values are below the ED50 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 concentration range of some of the**

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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 & 2A). The enantioselectivity of R(+)WIN55,212's anticonvulsant effect strongly indicates that this compound is acting via CB_1 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 statndard 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 that completely abolished seizures. Phenobarbital 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 8 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**

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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 1-3 per 12 hr recording interval. Treatment with vehicle or the inactive isomer S(-)WIN55,212 had no statistically significant effect on seizure frequency (Fig. 3A & B). Conversely, treatment with R(+)WIN55,212 abolished seizures in all 8 animals utilized in this treatment paradigm (Fig. 3B $p \leq 0.05$). During the drug free period following treatment with R(+)WIN55,212, seizure frequency increased slightly above baseline (Fig. 3A & B). The observed increase in seizure frequency following 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 **when compared to baseline or the drug withdrawal seizure frequency** (Fig. 3A & 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 (Figure 2A). The multiple treatment experiments demonstrated a tolerance effect. Chronic administration of cannabinoids affected seizure frequency when the cannabinoids were discontinued. Further study of the tolerance effect of the cannabinoids on the CB1 receptor in epileptic animals is an**

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important area for further investigation, but is beyond the scope of the present study.

Antagonism of the CB_1 Receptor Significantly Increases Seizure Frequency and Duration

Administration of SR141716A, to epileptic rats resulted in a statistically significant increase in seizure frequency (Fig. 2A & 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 status epilepticus (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 and animals were unresponsive to external stimuli with loss of righting-reflex for 30 min or more. **SR141716A (10mg/kg i.p.) has also been shown to inhibit the anticonvulsant effects of cannabinoids and endocannabinoids (Wallace et al., 2001 Wallace et al., 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 (Figure 3). 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_1 receptor activation on seizure termination, we quantified the duration of individual seizure events within each drug treatment period (Fig. 5A & B). In all animals monitored, EEG seizures directly

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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_1 receptor antagonist, SR141716A, caused a significant increase in seizure duration ($p \leq 0.01$ Fig. 5A & 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^{++} dependent manner (Stella et al., 1997). Sustained neuronal depolarization and elevated intracellular Ca^{++} 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 to 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**

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

Increased Hippocampal CB_1 Receptor Expression in Epileptic Rats

Because CB_1 receptor activation was shown to alter seizure frequency and duration, we sought to evaluate possible changes in CB_1 receptor expression in the hippocampi of epileptic animals. Using Western blot analyses, we compared sham control to epileptic hippocampal neuronal membranes and found a significant increase in epileptic brains of the expression of the 64-kDa molecular weight CB_1 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 to sham treated animals (Fig. 6B, $p \leq 0.01$), indicating that a long term plasticity change in the expression of the CB_1 receptor occurs with epilepsy. Western blot values were further corrected using the internal membrane protein markers tubulin and MAP 2 proteins (data not shown). After correcting cannabinoid protein levels to internal protein standards, we still observed a significant increase in the CB_1 receptor expression in epileptic animals (data not shown).

To evaluate the anatomical distribution of this increase in CB_1 receptor expression in epileptic brains, we conducted immunohistochemical staining of CB_1 receptor protein on coronal hippocampal sections using established techniques (Pettit et al., 1998). Figure 7A & B show 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).

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Representative pseudocolor enhanced images of CB_1 receptor protein staining of epileptic and sham control hippocampal sections illustrate a dramatic increase in CB_1 receptor expression in epileptic hippocampi (Fig. 7C & D). CB_1 receptor protein staining was most dramatically increased in the CA1-CA3 regions of the hippocampus with the highest increase localized to the dendritic synaptic areas of CA2 and CA3 (Fig. 7E-G). The dentate gyrus did not show a corresponding increase in CB_1 receptor expression.

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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 R(+)-WIN55,212, were anticonvulsant in the rat pilocarpine model of acquired, refractory epilepsy. Antagonism of the CB_1 receptor by SR141716A caused a marked increase in seizure frequency and duration, indicating that endogenous activity of the CB_1 receptor strongly influences seizure activity. By antagonizing the CB_1 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 occured during acute, 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_1 receptor expression in epileptic animals was significantly increased over that of sham controls. These changes were primarily observed in CA1 - CA3 regions of 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_1 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**

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produce unwanted side effects is an important area for further research to develop novel therapuetic agents to treat intractable seizures.

Epilepsy Increases Hippocampal CB_1 Receptor Expression

The findings presented in this study demonstrate that a significant change in CB_1 receptor expression occurs with the epileptic phenotype. The animals utilized in this study had been epileptic for nearly one year, indicating that this change in CB_1 receptor expression is prolonged and, most likely, permanent. CB_1 receptor expression has also been shown to increase in an animal model of stroke (Jin et al., 2000), a condition that, like epilepsy, is associated with excessive glutamate release and the development of seizures. The observation that the cannabinoid receptor is upregulated 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 Δ^9-THC, as well as the proconvulsant action of the CB_1 receptor antagonist in this epilepsy model, we propose that the increase in CB_1 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_1 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_1 receptor expression was demonstrated up to one 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_1 receptor expression may be revealed by further study.

The Anticonvulsant Action of Cannabinoids

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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_1 receptor activation significantly reduced spasticity and tremor, and exogenous application of 2-AG has been shown to be neuroprotective following 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_1 receptor activation, we believe that this mechanism may underlie cannabinoid anticonvulsant properties. CB_1 receptor activation is known to decrease calcium influx through N and P/Q type Ca^{++} channels (Mackie and Hille, 1992), the result of which is decreased Ca^{++}-dependent glutamate release. Glutamate is the primary excitatory neurotransmitter of the central nervous system. Though 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_1 receptor activation also increases the conductance of presynaptic A-type (Hampson et al., 1995) and G-protein coupled inward rectifying K^+ channels (Mackie et al., 1995). Increased K^+ channel permeability attenuates neuronal bursting and stabilizes membrane potential, additional

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factors that would contribute to decreased epileptiform discharge. **Preliminary data from our group indicates that CB1 knock out animals have spontaneous seizures, further suggesting an endogenous role for the CB1 receptor in controlling neuronal excitability.**

CB_1 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 likely 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_1 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 (DSE) can be induced in hippocampal tissue (Ohno-Shosaku 2002). The induction of this phenomenon was dependent upon the sensitivity of the presynaptic neuron to cannabinoids as well as the duration of

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postsynaptic depolarization. With extended depolarization, the result of CB_1 receptor activation was a shift from DSI to DSE. 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^{++} 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_1 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_1 receptor to terminate seizure discharge, forming a negative feed-back loop. **This increase in 2-AG occurred in comparison to 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 following injection of the chemoconvulsant picrotoxin (Sugiura et al., 2000). 2-AG is known to bind the CB_1 receptor with high affinity in a manner that is blocked by co-administration 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 compounds

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catalysis. Increased receptor sensitivity and reducing cannabinoid catabolism during seizure activity could also account for the net increase in 2-AG observed following 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 (Consroe et al., 1975). This has prompted several countries to consider the legalization of marijuana for epilepsy treatment (National Institutes of Health, 1997; R vs. 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 (Morris et al., 2001; Leite and Cavalheiro, 1995). Our results demonstrate that activation of the CB_1 receptor by cannabinoid drugs and possibly endogenous ligands significantly alters seizure activity and was more effective than conventional anticonvulsants in treating the refractory seizures produced in the pilocarpine model. Although the dose dependency and chronic 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

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mechanisms of endogenous cannabinoid mediated anticonvulsant action may lead to the development of novel compounds that do not manifest behavioral toxicity. Further investigation of 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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Footnotes

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Figure 1. The effects of CB_1 receptor modulation on epileptiform activity in control and epileptic animals. Representative EEG recordings of control, epileptic, S(-)WIN55,212, (-)WIN **(5 mg/kg i.p.)** treated epileptic and R(+)WIN55,212, (+) WIN **(5 mg/kg i.p.)** treated epileptic. Treatment with R(+)-WIN55,212 **(5 mg/kg i.p.)** completely abolished seizure activity.

Figure 2. The effects of CB_1 receptor activation and blockade on the seizure frequency of epileptic rats **demonstrated in single injection eperiments**. **(A)** Seizure frequency per 10 hr 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 Δ^9-THC, THC **(30 mg/kg i.p.).** Data represent mean ± standard error. (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 Δ^9-THC, 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 to 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.**

Figure 3. The effects of CB_1 receptor activation and blockade on the seizure frequency in 8 epileptic rats sequentially treated with a multiple drug regimen that includes a CB_1 receptor agonist and antagonist. **These experiments evaluate the effects of each drug in comparison to the other drugs in the same animal.** **(A)** Seizure frequency per 12 hr

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in a representative epileptic animal following 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). The bars represent the number of seizures observed in a representative epileptic animal for each 12 hr monitoring period. **(B)** Mean seizure frequency (per 12 hr) of 8 epileptic animals treated with the same drug regimen shown in Figure 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 ± SE (seizures per 12 hr)(n=8, RM ANOVA, $*p \leq 0.05$, $**p \leq 0.01$).

Figure 4. Antagonism of the CB_1 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 hr recording period for epileptic and epileptic + SR conditions. These recordings representative continuous EEG recordings from an epileptic rat 60 min prior to and 60 min following treatment with SR141716A. Arrows represent individual seizures. **The representative EEG recording from an epileptic animal manifested one spontaneous recurrent seizure in the one hour of recording. SR treatment in epileptic animals caused a marked increase in seizure frequency. During the numerous seizures shown for SR141716A treatment in the one hour recording the animal was not responsive in between seizures for more than 30 minutes. Thus, SR141716A produced status epilepticus in this animal, employing the standard definition of SE that includes intermittent seizure activity lasting for more than 30 minutes without regaining conciousness between seizures.**

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The Control + SR representative EEG recording demonstrates that treatment of control (non-epileptic) animals with SR141716A did not produce seizure activity.

Figure 5. CB_1 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 Figure 3B. Data represent the mean $\pm$ SE (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 $\pm$ SE (n=7,*$p \leq 0.01$, Student's t-test)

Figure 6. CB_1 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_1 receptor protein (Cichewicz et al., 2001). **(B)** Mean optical densities (arbitrary units) of CB_1 receptor protein expression in hippocampal membranes of epileptic and sham control rats (n=6, *$p \leq 0.01$, Student's t-test). Epileptic CB_1 receptor protein was increased by 183%.

Figure 7. Immunohistochemical detection of CB_1 receptor expression in control and epileptic hippocampi. Representative Nissl-staining of control **(A)** and epileptic **(B)** sections. Representative pseudocolor enhanced immunohistochemical staining of the

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CB_1 receptor protein in control **(C)** and epileptic **(D)** sections. The increase in CB_1 receptor protein expression observed in epileptic hippocampi was representative of 4 epileptic versus 4 control animals, 15 tissue sections per animal. High magnification of CB_1 receptor immunoreactivity of sham control **(E)** and epileptic **(G)** hippocampal formation demonstrating increased staining in the dendritic fields of the CA2 and CA3 regions of epileptic animals. Arrows indicate the location of the CA2-CA3 pyramidal neurons. High magnification of pseudocolor enhanced images of sham control **(F)** and epileptic **(H)** CA2 and CA3 regions. Bars in **A-D** represent 2 mm. Bars in **E-H** represent 200 µm. Red: highest level on color scale. The results shown are representative of several experiments.

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Figure 1

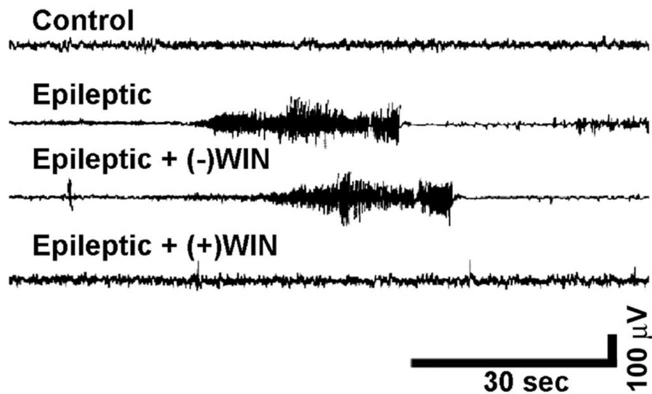
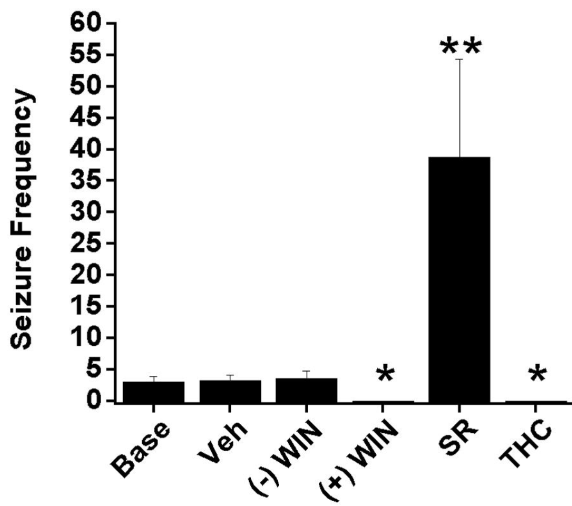
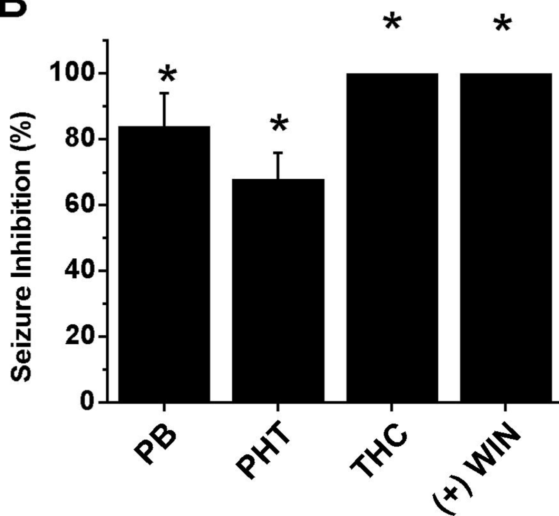


Figure 2





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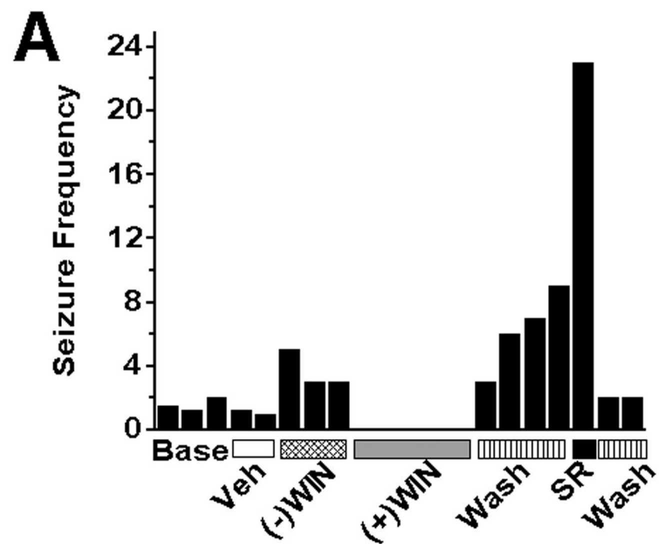


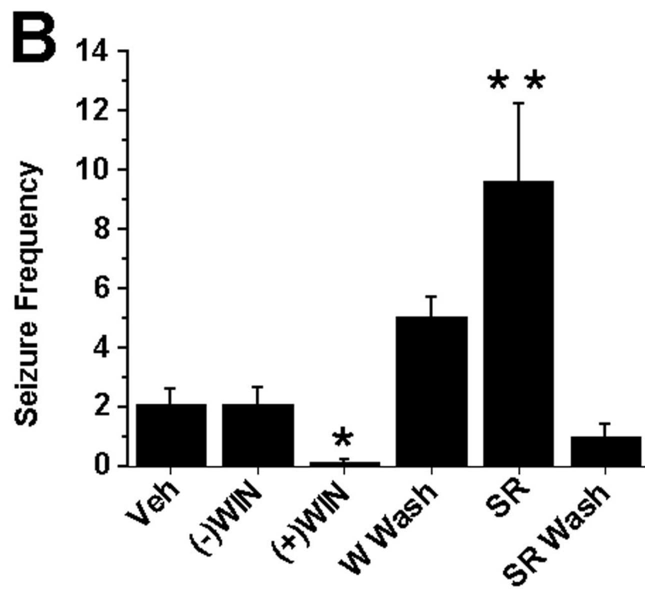
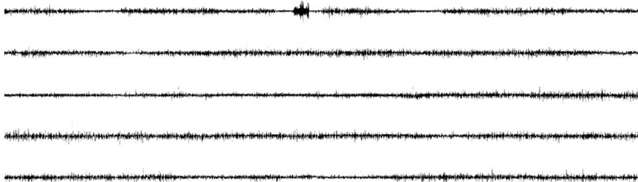
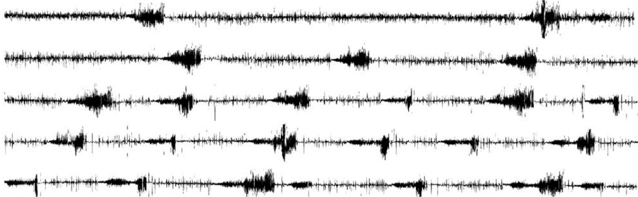


Figure 4

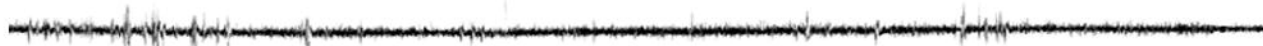
Epileptic



Epileptic + SR



Control + SR



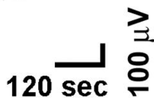
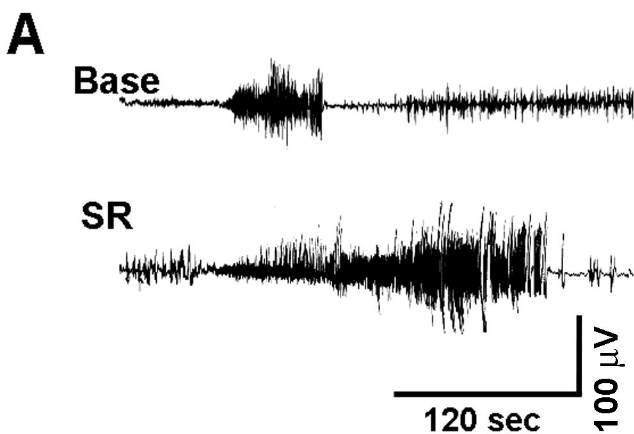
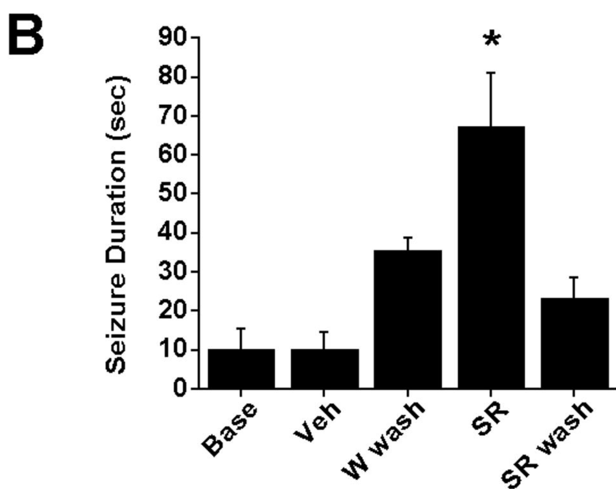


Figure 5





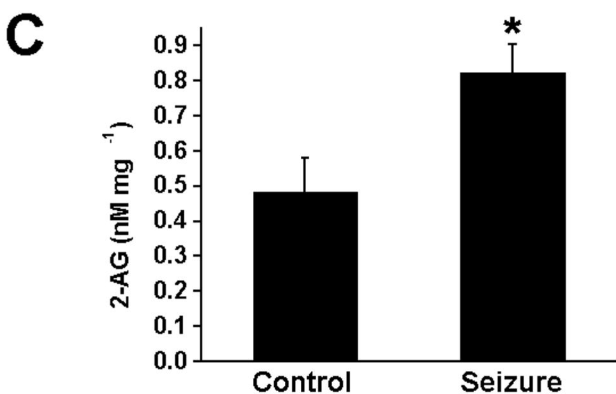


Figure 6

C E C E C E C E

A

64 kDa

B

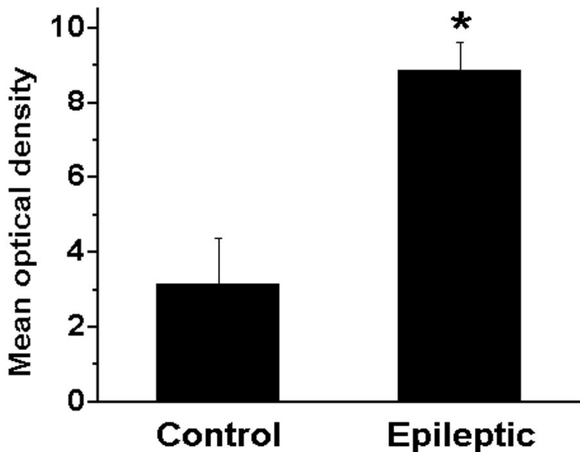


Figure 7