Statistical parametric mapping reveals regional alterations in cannabinoid CB₁ receptor distribution and G-protein activation in the 3D reconstructed epileptic rat brain

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Summary

Purpose—The endocannabinoid system is known to modulate seizure activity in several in vivo and in vitro models, and CB₁-receptor activation is anticonvulsant in the rat pilocarpine model of acquired epilepsy (AE). In these epileptic rats, a unique redistribution of the CB₁ receptor occurs within the hippocampus; however, an anatomically inclusive analysis of the effect of status epilepticus (SE)-induced AE on CB₁ receptors has not been thoroughly evaluated. Therefore, statistical parametric mapping (SPM), a whole-brain unbiased approach, was used to study the long-term effect of pilocarpine-induced SE on CB₁-receptor binding and G-protein activation in rats with AE.

Methods—Serial coronal sections from control and epileptic rats were cut at equal intervals throughout the neuraxis and processed for [³H]WIN55,212-2 (WIN) autoradiography, WIN-stimulated [³⁵S]GTP[S] autoradiography, and CB₁-receptor immunohistochemistry (IHC). The autoradiographic techniques were evaluated with both region of interest (ROI) and SPM analyses.

Key Findings—In rats with AE, regionally specific increases in CB₁-receptor binding and activity were detected in cortex, discrete thalamic nuclei, and other regions including caudate-putamen and septum, and confirmed by IHC. However, CB₁ receptors were unaltered in several brain regions, including substantia nigra and cerebellum, and did not exhibit regional decreases in rats with AE.

Significance—This study provides the first comprehensive evaluation of the regional distribution of changes in CB₁-receptor expression, binding, and G-protein activation in the rat pilocarpine model of AE. These regions may ultimately serve as targets for cannabinomimetic compounds or manipulation of the endocannabinoid system in epileptic brain.

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A large percentage of patients with epilepsy are refractory to conventional anticonvulsant treatment; therefore, epilepsy is a neurologic condition that requires novel insights for treatment and seizure prevention (Hauser, 1990; Annegers et al., 1996; Herman, 2002). Cannabinoids are known to have CB$_1$-receptor–dependent anticonvulsant effects in both in vitro and in vivo seizure models (Wallace et al., 2001, 2002; Marsicano et al., 2003; Blair et al., 2006; Gholizadeh et al., 2007; Armstrong et al., 2009), and the endogenous cannabinoid system has been shown to play a role in modulating epileptic seizure frequency and duration (Wallace et al., 2003). Previously our laboratory demonstrated that following pilocarpine-induced status epilepticus (SE), animals with acquired epilepsy (AE) exhibit a long-term redistribution of CB$_1$ receptors in the hippocampus, including layer-specific alterations in CB$_1$-receptor immunoreactivity (IR) and binding sites accompanied by changes in CB$_1$-receptor–mediated G-protein activity (Falenski et al., 2007). However, the regional whole-brain extent of CB$_1$-receptor adaptations in this model has not been reported.

The CB$_1$ receptor is among the most abundant G-protein–coupled receptors in brain, with a distribution consistent with behavioral effects of cannabinoid administration (Herkenham et al., 1991; Jansen et al., 1992; Tsou et al., 1998). Autoradiographic techniques such as [3H]ligand binding and agonist-stimulated [35S]GTP $\gamma$S autoradiography have been used to characterize regional changes in CB$_1$-receptor binding and G-protein activation in a number of paradigms (Sim et al., 1996; Berrendero et al., 1998; Dean et al., 2001; Sim-Selley & Martin, 2002). Conventionally, these data are analyzed using a region of interest (ROI) approach. However, ROI analyses are normally hypothesis driven, and effects of interest outside boundaries of predefined ROIs could be missed. To address this limitation, established whole-brain based approaches, such as statistical parametric mapping (SPM), have allowed for localization of significant effects of interest in an unbiased manner (Friston et al., 1990, 1995b). Previously, SPM has been utilized in autoradiographic data sets mapping cerebral blood flow (Nguyen et al., 2004; Dubois et al., 2008) and was recently adapted to study CB$_1$-receptor–mediated G-protein activity in three-dimensional (3D) reconstructed mouse brain (Nguyen et al., 2010, 2012).

In this study, the pilocarpine model was used to prepare rats with AE that were evaluated 1 year post-SE. Control and epileptic brains were evaluated using [3H]WIN55, 212 (WIN) and WIN-stimulated [35S]GTP $\gamma$S autoradiography to determine changes in receptor binding and G-protein activation, respectively. SPM was used to identify significant changes in CB$_1$-receptor binding and G-protein activation in reconstructed brain images, and subsequently confirmed by ROI analysis. CB$_1$ receptor expression was evaluated using immunohistochemistry (IHC). Examination of the plasticity of the CB$_1$ receptor at the whole-brain level in this model is critical for understanding the overall role that CB$_1$ receptors and the endogenous cannabinoid system plays in AE.

Materials and Methods

Induction of epilepsy in rats

All animal procedures were conducted in accordance with the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals and approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee. Adult male Sprague-Dawley rats (200–250 g, roughly 8 weeks) (Harlan, Indianapolis, IN, U.S.A.) were
housed on a 12 h light/dark cycle in single cages and were provided with food and water ad libitum. The pilocarpine model of AE was then used to induce spontaneous recurrent seizures (SRS) following SE by well-established procedures (Mello et al., 1993; Falenski et al., 2007). Briefly, methyl-scopolamine (1 mg/kg, i.p.) (Sigma, St. Louis, MO, U.S.A.) was administered 30 min before pilocarpine treatment to minimize peripheral effects. Pilocarpine (375 mg/kg, i.p.) (Sigma) was then given to induce SE, where onset was determined by the presence of continuous class 4–5 level seizures as assessed using the Racine scale (Racine, 1972). Diazepam (5 mg/kg, i.p.) (VCU Health Systems Pharmacy, Richmond, VA, U.S.A.) was administered to animals at 1, 3, and 5 h following SE onset to terminate seizures. Animals without SE were not used. Approximately 4 months post-SE, animals were screened by behavioral video monitoring to evaluate the presence of SRS activity, where seizures were assessed using the Racine scale (Racine, 1972). Only rats with SRS of class 3–5 observed within 24 continuous hours were determined to be epileptic. At 1-year post-SE, behavioral seizure frequency was observed in all epileptic animals for the 24 h immediately before sacrifice. Ten epileptic and 10 age-matched controls (five naive and five vehicle injected) were used in all experiments.

**Tissue preparation**

Rats were sacrificed by rapid decapitation. Brains were quickly removed and frozen in isopentane maintained at −25°C on dry ice. Preparation of tissue for image reconstruction was conducted as published previously, with slight modification (Nguyen et al., 2010). Briefly, 20-μm coronal sections were cut on a Leitz cryostat (Leica Microsystems, Wetzlar, Germany), maintained at −20°C, and mounted onto gelatin-subbed slides in duplicate. Alternating adjacent sections from a single brain were collected for different assay conditions. Sections were then utilized for Nissl staining, IHC, [3H]WIN autoradiography, or WIN-stimulated [35S]GTPγS autoradiography. Slides were stored at −80°C until assayed.

**Immunohistochemical analysis**

IHC was performed according to previously published techniques (Pettit et al., 1998; Wallace et al., 2003). Briefly, slides were blocked in Superblock (1 h; Pierce, Rockford, IL, U.S.A.), and then incubated in addition of an antibody to the N-terminus of the CB1 receptor (5 μg/ml, 1 h; Life Technologies, Inc., Grand Island, NY, U.S.A.) (Pettit et al., 1998). Sections were washed, incubated in biotinylated anti-Rabbit IgG (1:200, 30 min; Vector Laboratories, Burlingame, CA, U.S.A.) and avidin–biotin complex (1:100, 30 min; Vector), and visualized using 3′,3′-diaminobenzidine (DAB) peroxidase (Vector). Controls included absence of primary antibody and coabsorption with an immunizing peptide (12 μg/ml). Adjacent sections were Nissl stained. Sections were evaluated using a binocular microscope and photographed using a digital camera (Olympus Inc., Melville, NY, U.S.A.).

**[3H]WIN autoradiography**

[3H]WIN autoradiography was conducted as published previously (Jansen et al., 1992; Falenski et al., 2007). Briefly, slides were preincubated in Assay buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM MgCl2, and 0.5% w/v bovine serum albumin [BSA], pH 7.0) for 20 min at 30°C. Sections were then incubated for 80 min at 30°C in assay buffer containing 1 nM [3H]WIN (specific activity 52.2 Ci/mmol; Perkin-Elmer, Boston, MA, U.S.A.) in the absence or presence of 1 μM unlabeled WIN to assess total and nonspecific binding, respectively. WIN was used primarily because it is well-characterized in this model both in vitro and in vivo (Wallace et al., 2003; Falenski et al., 2007). Sections were washed in assay buffer (4 × 10 min, 25°C) followed by brief washing in ddH2O (4°C). Slides were dried overnight and exposed to Hyperfilm for 8 weeks in the presence of 3H microscales (Amersham, Arlington Heights, IL, U.S.A.).
WIN-stimulated $^{35}$S autoradiography

WIN-stimulated $^{35}$S autoradiography was performed as published previously (Sim et al., 1995; Falenski et al., 2007). Briefly, slides were incubated at 25°C in assay buffer (50 mM Tris–HCl, 3 mM MgCl$_2$, 0.2 mM ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid (EGTA), 100 mM NaCl, and 0.5% BSA, pH 7.4) for 10 min. Slides were transferred to assay buffer containing 2 mM guanosine diphosphate (GDP) and 10-mU/ml adenosine deaminase for 20 min. Sections were then incubated in addition of 40 pM $^{35}$S autoradiography (specific activity 1,250 Ci/mmol; Perkin-Elmer) and in the absence (basal) or presence of a maximally effective concentration of the full CB$_1$-receptor agonist WIN (10 μM) for 2 h at 25°C. Slides were rinsed in 50 mM Tris at 4°C (2 × 2 min each) and ddH$_2$O at 4°C (30 s) and dried overnight, then placed in cassettes with $^{14}$C microscales (Amersham) and exposed to Kodak Biomax MR film for 24 h. Films were digitized at 8-bits per pixel with a Sony XC-77 video camera (Sony, Tokyo, Japan).

3D Image reconstruction

Preparation of autoradiograms from WIN-stimulated $^{35}$S autoradiography and $^{3}$HWIN binding for SPM analysis was performed as described previously (Nguyen et al., 2004, 2010). Reconstructed brain images from each assay condition (WIN or basal for $^{35}$S autoradiography, and total $^{3}$HWIN binding for $^{3}$HWIN autoradiography) were collected in duplicate throughout the neuraxis (Bregma 4.70 to −11.60 mm) with an interslice distance of 300 μm. A representative section was selected from duplicate sections, thus excluding sections with extensive artifacts related to tissue preparation from analysis. Image reconstruction and slice registration was performed using ImageJ (version 1.38; http://rsb.info.nih.gov/ij/) as described previously (Thevenaz et al., 1998; Nguyen et al., 2004, 2010) (n = 6 animals/group were used based on adequate WIN stimulation, and 55 coronal levels were collected per animal). Reconstructed brain images were saved in Analyze format at an initial voxel resolution of $40 \times 40 \times 300 \ \mu m^3$ in 16-bit for $^{35}$S autoradiographic images and 32-bit for $^{3}$HWIN binding images. This was necessary to encompass the full data range and precision of the autoradiograms, which had an average maximum binding value of 1768 nCi/g and 10.2 nCi/mg for $^{35}$S autoradiography and $^{3}$HWIN binding assays, respectively. Quality of brain reconstructions were assessed by visual inspection of internal structures viewed in different orthogonal planes and correspondence of landmarks across reconstructions of different conditions and/or subjects.

Data analysis of reconstructed brain images

For both SPM and ROI analyses, quantitation of autoradiographic images were calculated into activity values, using a linear fit according to a $[^{14}$C] standard curve (corrected for $^{35}$S) into nCi/g for $^{35}$S autoradiography (Sim et al., 1995), and an exponential fit according to a $[^{3}$H] standard curve into nCi/mg for $^{3}$HWIN binding (Jansen et al., 1992 #646). Net stimulated $^{35}$S autoradiographic binding is defined as (WIN-stimulated binding, basal binding). Specific $^{3}$HWIN binding is defined as (total binding – nonspecific binding). SPM analysis (version SPM5, http://www.fil.ion.ucl.ac.uk/spm/) of 3D reconstructed autoradiographs was conducted as published previously (Nguyen et al., 2004, 2010), with minor modification. For $^{35}$S autoradiographic studies, the basal image of each brain was coregistered (using normalized mutual information) to the corresponding WIN condition using the SPM coregistration tool. WIN image reconstructions were used to estimate spatial normalization parameters (Friston et al., 1995a; Ashburner & Friston, 1999) to provide the greatest contrast. These images were spatially normalized to a study-specific brain template derived from the image average of all WIN reconstructions. A 3D mask outlining the ventricles was created using MRIcro (version 1.4; http://cml.web.arizona.edu/mricro.htm) to account for potential confounding effects of ventricular hypertrophy in some epileptic animals on spatial normalization. Transformation parameters were then applied to the coregistered basal image.
within each subject. Reconstruction of net-stimulated $[^{35}\text{S}]$GTP $\gamma$S binding images was derived by image subtracting basal-stimulated from their respective WIN-stimulated images. Inadequate overlap of basal-stimulated and WIN-stimulated images at section edges resulted in artifactual negative voxel values that were replaced and set to zero, allowing SPM to ignore these data points. For $[^{3}\text{H}]$WIN autoradiography, reconstructions were derived from coronal sections of total $[^{3}\text{H}]$WIN binding only, as low nonspecific binding in our assay (2 nCi/mg across all regions) and low contrast in these images resulted in inadequate anatomic information for optimal reconstructions.

All normalized brain image reconstructions were smoothed with a Gaussian kernel of full width half maximum (FWHM) equal to three times its voxel size, which was compatible with our estimate of misregistration error and anatomic variability. Using SPM, differences in AE-induced changes for $[^{3}\text{H}]$WIN or WIN-stimulated $[^{35}\text{S}]$GTP $\gamma$S binding were individually compared between epileptic versus control animals using an unpaired Student’s $t$-test at each voxel. Significance threshold was initially set at $p < 0.01$ (uncorrected for multiple comparisons) for individual voxels within clusters of contiguous voxels, and a minimum cluster size of 100 contiguous voxels. We then evaluated the corrected significance of individual voxels, clusters of contiguous voxels exceeding the threshold, and number of clusters detected in the entire SPM. The multiple comparisons problem was addressed using Gaussian random field theory and $p$-values corrected using the false discovery rate (Friston et al., 1991, 1996).

SPM findings were subsequently confirmed by an independent ROI analysis. ROI measurements were conducted on original unprocessed images (i.e., no spatial normalization) for total and nonspecific $[^{3}\text{H}]$WIN, as well as basal-stimulated and WIN-stimulated $[^{35}\text{S}]$GTP $\gamma$S autoradiograms, and anatomic boundaries were defined using a brain atlas (Paxinos, 1986). Data are reported as means ± standard error of the mean (SEM) of sections at three consecutive coronal levels ($n = 6$/group).

**Results**

**Epileptic rats exhibit regionally increased $[^{3}\text{H}]$WIN and WIN-stimulated $[^{35}\text{S}]$GTP$\gamma$S binding in 3D reconstructed brain with SPM**

In the rat pilocarpine model of AE, epileptic animals ($n = 10$) exhibited class 3–5 seizures (Racine, 1972), with an average seizure frequency of 7.07 ± 0.89 seizures per 24 h. Latency between final seizure and sacrifice ranged from 9 min to >24 h. SPM analysis was used to determine changes in cannabinoid receptor binding and CB$_1$-receptor–mediated G-protein activation in these animals. Figure S1 illustrates 3D results from SPM analysis showing the spatial extent of increases in cannabinoid receptor binding (Fig. S1A) and receptor-mediated G-protein activity (Fig. S1B) in epileptic rat brain when compared to control. Increases in both CB$_1$-receptor binding and activation were observed almost exclusively in forebrain, were distributed bilaterally, and were strikingly similar between the two measures. Specifically, the distribution of increased CB$_1$-receptor binding and activation appeared to be localized primarily to areas of cortex, nuclei of the thalamus, and in striatum (Fig. S1). $[^{3}\text{H}]$WIN binding and WIN-stimulated $[^{35}\text{S}]$GTP $\gamma$S autoradiography did not decrease in any major brain region of epileptic animals with SPM analysis (data not shown). These results highlight brain areas where CB$_1$ receptors are most dramatically changing with AE induction.

**Regional distribution of changes in cannabinoid receptor binding in epileptic rat brain**

Visual comparison of total $[^{3}\text{H}]$WIN binding in brain sections from control and epileptic rats revealed increases in receptor binding in several forebrain regions of rats with AE, including...
caudate-putamen, hippocampal layers throughout CA1-3, and most areas of cortex (Fig. 1). In the thalamus, increased \[^{3}H\]WIN binding was striking in the ventroposterior (VP) and lateral geniculate (LG) nuclei (Fig. 1). However, no apparent changes were observed in regions including globus pallidus, substantia nigra, and cerebellum (Fig. 1).

SPM and subsequent ROI analyses revealed widespread increases in total \[^{3}H\]WIN binding throughout the forebrain of epileptic animals when compared to control (Fig. 2, Table 1). In the cortex, SPM revealed increases primarily in the frontal, parietal (forelimb and hind limb [HL]), cingulate, entorhinal, and temporal areas of rats with AE, particularly in deep layers that appeared to correspond to lamina VI (Fig. 2). ROI analysis confirmed these observations; densitometric analysis of a sampled region of lamina VI in the HL cortex revealed a 2.7-fold increase in \[^{3}H\]WIN binding in rats with AE (Table 1). SPM revealed the most robust increases in \[^{3}H\]WIN binding with AE in the thalamus, particularly in the ventral lateral (VL), ventral medial (VM), ventroposterolateral/medial (VPL/VPM), dorsal/ventral lateral geniculate (DLG/VLG), medial geniculate (MGD/ MGV), and laterodorsal (LDDM/LDVL) nuclei (Fig. 2). In a sample of thalamus that included the VPL/VPM, posterior, lateroposterior (mediorostral), DLG, and LDVL (≈−3.6 mm Bregma), a greater than twofold increase in \[^{3}H\]WIN binding was measured in rats with AE; in the MGD/ MGV, a greater than threefold increase was observed. However, no differences were observed between epileptic and control animals in several midline thalamic nuclei including the intralaminar. SPM revealed increased total \[^{3}H\]WIN binding in the septum; ROI analysis demonstrated a greater than doubling of receptor binding in both septum and nucleus accumbens (Fig. 2, Table 1). Of interest, increased \[^{3}H\]WIN binding was not a global response to AE induction, as changes in \[^{3}H\]WIN binding did not occur in a number of brain regions containing the highest expression of CB\(_1\) receptors, such as globus pallidus, cerebellum, and substantia nigra (Fig. 2, Table 1).

**Regional plasticity of CB\(_1\)-receptor–mediated G-protein activity in epileptic rat brain**

Visual inspection of representative sections from \[^{35}S\]GTP \(_{\gamma}\) autoradiography revealed widespread increases in WIN-stimulated \[^{35}S\]GTP \(_{\gamma}\) binding throughout the forebrain with AE, particularly in thalamic, hippocampal, and cortical regions (Fig. 3). As assay conditions for \[^{35}S\]GTP \(_{\gamma}\) autoradiography likely preclude appreciable binding from an endogenous agonist (Sim et al., 1995), and previous work has illustrated an increased maximal effect of WIN-stimulated \[^{35}S\]GTP \(_{\gamma}\) binding in the hippocampus of rats with AE without a change in WIN-binding affinity (Falenski et al., 2007), increases in this measure are hypothesized to result from enhanced receptor efficacy. SPM analysis illustrated regionally specific increases in net \[^{35}S\]GTP \(_{\gamma}\) binding in epileptic animals when compared to control, with a similar anatomic distribution as \[^{3}H\]WIN binding (Fig. 4). In the cortex of rats with AE, SPM maps illustrated increases in net \[^{35}S\]GTP \(_{\gamma}\) binding in the cingulate, frontal, parietal, and temporal cortices that included a laminar pattern likely corresponding to layer VI of cortex (Fig. 4), although ROI analysis only showed an insignificant trend of increased WIN-stimulated \[^{35}S\]GTP \(_{\gamma}\) binding in the cingulate and entorhinal cortices (Table 1). SPM also revealed increased net \[^{35}S\]GTP \(_{\gamma}\) binding in several thalamic nuclei, particularly in the VL, VM, VPL/VPM, DLG/ VLG, MGD/ MGV, and LDDM/LDVL (Fig. 5). ROI analysis demonstrated that net \[^{35}S\]GTP \(_{\gamma}\) binding increased in all of these nuclei despite concurrent increases in basal G-protein activity (Table 1). SPM and ROI analyses showed that net \[^{35}S\]GTP \(_{\gamma}\) binding also increased in caudate-putamen and hippocampus, regions where basal binding was unchanged. Net \[^{35}S\]GTP \(_{\gamma}\) binding appeared to decrease in globus pallidus and periaqueductual gray (PAG) in AE, but these regions exhibited robust increases in basal activity. No alterations in CB\(_1\)-receptor–mediated G-protein activity were observed in the substantia nigra with AE (Fig. 4).
Evaluation of CB₁-receptor expression in the epileptic rat brain

IHC using a CB₁-receptor antibody was conducted to verify that increases in [³H]WIN binding and WIN-stimulated G-protein activity in AE resulted from increases in CB₁ receptors. Recent studies have identified other WIN binding sites in brain (Van Sickle et al., 2005; Nguyen et al., 2010) so this experiment provided important verification of the receptor target. Moreover, previous work demonstrated a heterogeneous redistribution of hippocampal CB₁ receptor expression in rats with AE (Falenski et al., 2007), but it was unclear as to whether these changes occurred in other brain areas. Therefore, IHC was conducted on sections from brains of control and epileptic animals using an N-terminus antibody well-characterized in unperfused flash-frozen tissue (Pettit et al., 1998), which allowed for the direct comparison of changes in CB₁-receptor expression and binding characteristics between adjacent sections from the same animal.

Overall, qualitative analysis revealed an increase in CB₁-receptor immunoreactivity (IR) throughout the forebrain of epileptic animals (Fig. 5, left panels). In rats with AE, increased CB₁-receptor IR was observed in frontal and parietal cortices as well as in caudate-putamen. In the thalamus, immunostaining in the VL, VM, VPL/VPM, DLG/VLG, and MGD/MGV was increased in rats with AE when compared to control (Fig. 5, left panels). In the hippocampus, CB₁-receptor IR increased in the CA1-3 strata oriens and radiatum of epileptic animals (Fig. 5), as previously shown (Wallace et al., 2003). Furthermore, CB₁-receptor IR decreased concurrently on the inner one third of the dentate gyrus molecular layer (Fig. 5), consistent with previous work (Falenski et al., 2007). CB₁-receptor expression did not appear to change upon induction of AE in substantia nigra or in nearly all brainstem nuclei (Fig. 5). Nissl stains conducted on adjacent sections revealed a small degree of cell loss consistent with previous work in this model (Fig. 5, right panels) (Rice & DeLorenzo, 1998; Falenski et al., 2007). These results are similar to the autoradiographic results and verify that adaptations in receptor binding and activity reflect CB₁ receptors.

Discussion

Results from this study demonstrate that throughout the rat forebrain, regionally specific long-term adaptations in CB₁ receptors and cannabinoid receptor–mediated G-protein activity persist following the induction of AE. SPM, which provided an unbiased whole-brain analysis, revealed alterations in both cannabinoid receptor binding and G-protein activation in the striatum, cortex, and several thalamic nuclei, confirmed by ROI analysis. The present findings also illustrate the functional redistribution of CB₁ receptors within the hippocampus in this model, a consistent finding in our laboratory (Falenski et al., 2007, 2009). These results suggest that the long-term increases in CB₁-receptor expression and function that occurs throughout the brain following SE may play a role in CB₁ receptor activation regulating seizure frequency and duration in epileptic animals (Wallace et al., 2003).

SPM has only recently been applied to receptor binding and agonist-stimulated [³⁵S]GTP S autoradiography (Nguyen et al., 2010, 2012), and this is the first known application of SPM to autoradiography in a pathologic model. SPM provided the advantage of quantitatively comparing binding in all regions of the rat brain, as well as in anatomic subdivisions of these areas. For example, in cortex, SPM revealed the novel finding that changes in the CB₁ receptor in AE were layer-specific. In fact, previous ROI analysis of WIN-stimulated [³⁵S]GTP S binding in selections containing all cortical layers revealed no effect between control and epileptic animals (unpublished observations). The most robust differences between epileptic and control animals were observed in deep laminae corresponding primarily to layer VI, containing the highest CB₁-receptor expression (Tsou et al., 1998), receptor binding (Herkenham et al., 1991; Mairieux & Vanderhaeghen, 1992; Bodor et al.,
2005), and net $^{35}$S-GTP $\gamma$ binding (Nguyen et al., 2010) in control tissue. This highlights the potential importance of CB$_1$ receptors on layer VI in modulating seizure activity. Other recent studies have demonstrated a role for cortical CB$_1$ receptors associated with epilepsy (Zurolo et al., 2010; Goffin et al., 2011). Collectively, these results warrant further investigation of CB$_1$ receptors in this region.

Increases in both total $[^3H]$WIN- and net $[^{35}S]$GTP $\gamma$-binding were observed throughout the epileptic forebrain; however, epileptic animals exhibited greater increases in $[^3H]$WIN binding than $[^{35}S]$GTP $\gamma$ binding. In addition, brain areas such as globus pallidus and PAG illustrated unchanged $[^3H]$WIN binding concomitant with decreased G-protein activation. Regional differences exist in receptor/transducer amplification factors (Breivogel et al., 1997), and a large CB$_1$-receptor reserve has been noted in the hippocampus (Gifford et al., 1999), suggesting that changes in receptor binding might not translate proportionally to changes in more functional measures. However, differences in the degree of change seen in $[^3H]$WIN and WIN-stimulated $[^{35}S]$GTP $\gamma$ binding may also be due to increased basal $[^{35}S]$GTP $\gamma$ binding in specific brain regions of epileptic rats. Although basal $[^{35}S]$GTP $\gamma$ binding is consistently unchanged within the hippocampus (Falenski et al., 2007), current results demonstrate that increases in basal $[^{35}S]$GTP $\gamma$ binding occur in other regions such as the thalamus, cortex, and PAG with AE. Increased basal $[^{35}S]$GTP $\gamma$ binding occurs in a model of peripheral nerve injury (Bantel et al., 2002); however, those changes were not likely applicable to the current study, which addressed the presence of endogenous adenosine. Alterations in glial cell activity have been noted following SE (Hosoi et al., 2010), but reactive gliosis and metabolic changes are less clear chronically (Garzillo & Mello, 2002; Melo et al., 2005); therefore, it is not clear whether increased basal $[^{35}S]$GTP $\gamma$ binding in our model is a result of increased G-protein–coupled receptor activation on glial cells. Previous studies demonstrated that a 0.3- $\mu$M concentration of the CB$_1$-receptor antagonist/inverse agonist SR141716A did not decrease basal $[^{35}S]$GTP $\gamma$ binding in control or epileptic animals (Falenski et al., 2007), but it is possible that higher concentrations are required to detect this effect in autoradiography (Sim-Selley et al., 2001). Future studies are necessary to further examine the nature of this regional increase in basal $[^{35}S]$GTP $\gamma$ binding and the contribution of CB$_1$-receptor activation therein.

No increases in CB$_1$-receptor binding and G-protein activation were observed between control and epileptic animals in brainstem nuclei or cerebellum, highlighting the regional specificity of this effect. The cerebellum has been utilized as a control region (Falenski et al., 2007) and does not exhibit many hallmarks of temporal lobe epilepsy (Morris et al., 1999; Churn et al., 2000). However, CB$_1$ receptors were also unaltered in globus pallidus and substantia nigra, which exhibit hypermetabolism during pilocarpine-induced SE (Handforth & Treiman, 1995) and contain high CB$_1$-receptor binding levels (Herkenham et al., 1991). Of interest, these regions also exhibit relatively less desensitization and down-regulation following repeated cannabinoid administration (Sim-Selley, 2003). It is possible that the relative expression of regulatory proteins differs in these regions, so that CB$_1$ receptors are less likely to exhibit neuroadaptations. Alternatively, it is possible that a sufficient number of receptors are expressed under normal conditions due to receptor reserve. CB$_1$ receptors in the globus pallidus and substantia nigra are very highly expressed, with 1.5–2-fold higher expression than hippocampus (Herkenham et al., 1991).

Results from this study support previous findings that CB$_1$ receptors are redistributed within the hippocampus, with expression increasing in layers such as CA3 stratum radiatum while concomitantly decreasing in the dentate gyrus inner molecular layer (Falenski et al., 2007). More recently, studies in this region have examined CB$_1$-receptor localization in the mouse pilocarpine model, reporting both increases (Magloczky et al., 2010) and decreases (Wyeth et al., 2010, 2011) in CB$_1$-receptor IR. In the hippocampus of control animals, CB$_1$
receptors are localized at both γ-aminobutyric acid (GABA)ergic (Tsou et al., 1999) and glutamatergic synapses (Kawamura et al., 2006). CB₁-receptor activation is anticonvulsant in the rat pilocarpine model of AE (Wallace et al., 2003), and neuroprotective specifically on glutamatergic synapses in the kainic acid model of seizure (Monory et al., 2006; Guggenhuber et al., 2010), suggesting that increased CB₁ receptor IR and activation observed in this study may be compensatory. However, recent studies in the mouse pilocarpine model have found that CB₁ receptors increase on glutamatergic and GABAergic synapses (Karlocai et al., 2011); therefore, the nature of these changes has yet to be fully elucidated. Nonetheless, the cannabinoid system has been implicated in the hippocampus in several other seizure models (Chen et al., 2003, 2007; Araujo et al., 2010), and potential clinical relevance has been corroborated with findings that changes in the endocannabinoid system occur in the hippocampus of patients with epilepsy (Ludanyi et al., 2008; Romigi et al., 2010).

It is well documented that cannabinoids exhibit anticonvulsive and neuroprotective properties (Wallace et al., 2001, 2003; Alger, 2004). However, recent studies have also found that CB₁-receptor blockade following insults can prevent long-term plasticity changes (Chen et al., 2007; Eccegoyen et al., 2009; van Rijn et al., 2011; Vinogradova et al., 2011). Furthermore, temporally specific changes in CB₁-receptor IR occur following pilocarpine-induced SE (Falenski et al., 2009), indicating that the endocannabinoid system may play an important role in the initial stages of epileptogenesis, in addition to any long-term compensatory role after AE development. Results from this study provide a regional map of not only the regions affected by long-term epilepsy but of potential targets for earlier therapeutic intervention.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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Figure 1.
Increased $[^3]H$WIN binding is evident throughout the forebrain of epileptic animals. Representative autoradiograms of total $[^3]H$WIN binding in control and epileptic animals taken at several different levels illustrate greater $[^3]H$WIN binding in many forebrain regions of epileptic rats, including cortex, caudate-putamen, hippocampus, and thalamus. Pseudocolored images represent lowest to highest levels of $[^3]H$WIN binding as shown in blue and red, respectively (scale bar- nCi/mg).

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Statistical parametric mapping (SPM) reveals increased $[^3]H$WIN binding in discrete forebrain regions of epileptic animals. SPM illustrates regions with significantly greater $[^3]H$WIN binding in epileptic rats when compared to control (n = 6 per group). Representative coronal, sagittal, and transverse images illustrate the spatial extent of differences in magnitude of cannabinoid receptor binding shown as colored overlays (red to yellow) that correspond to significance level. Fr1-3, frontal cortex, areas 1–3; I, insular cortex; Cg1-2, cingulate gyrus, areas 1–2; CPu, caudate putamen; FL, forelimb cortex; S, septum; Par, parietal cortex; HL, hind limb cortex; LD, laterodorsal thalamic nucleus; VL/M, ventrolateral/medial thalamic nuclei; Te, temporal cortex, hippocampal area CA3; DLG, dorsal lateral geniculate nucleus; VLG, ventral lateral geniculate nucleus; MG, medial geniculate nucleus; VPM/L, ventral posterolateral/medial thalamic nuclei; SnR, substantia nigra; PAG, periaqueductal gray; Cblm, cerebellum.

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Figure 3.
Increased WIN-stimulated\[^{35}\text{S}\]GTP \(\gamma\)S binding is evident throughout the forebrain of epileptic animals. Representative autoradiograms of WIN-stimulated \[^{35}\text{S}\]GTP \(\gamma\)S binding in control and epileptic animals taken at several different levels illustrate greater WIN-stimulated \[^{35}\text{S}\]GTP \(\gamma\)S binding in many forebrain regions of epileptic rats, including cortex, caudate-putamen, hippocampus, and thalamus. Pseudocolored images represent lowest to highest levels of \[^{35}\text{S}\]GTP \(\gamma\)S binding as shown in purple/blue and red, respectively. Scale bar- nCi/g.
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Figure 4.
SPM reveals greater net WIN-stimulated $[^{35}\text{S}]$GTP $\gamma$S binding in discrete forebrain regions of epileptic animals. Statistical parametric maps illustrate regions with significantly greater net $[^{35}\text{S}]$GTP $\gamma$S binding in epileptic rats when compared to control (n = 6 per group). Representative coronal, sagittal, and transverse images illustrate the spatial extent of differences in magnitude of G-protein activation shown as colored overlays (red to yellow) that correspond to significance level. Fr1-3, frontal cortex, areas 1–3; I, insular cortex; Cg1-2, cingulate gyrus, areas 1–2; CPu, caudate putamen; FL, forelimb cortex; S, septum; Par, parietal cortex; HL, hind limb cortex; LD, laterodorsal thalamic nucleus; VL/M, ventrolateral/medial thalamic nuclei; Te, temporal cortex, hippocampal area CA3; DLG, dorsal lateral geniculate nucleus; VLG, ventral lateral geniculate nucleus; MG, medial geniculate nucleus; VPM/L, ventral posterolateral/medial thalamic nuclei; SnR, substantia nigra; PAG, periaqueductal gray; Cblm, cerebellum.

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Figure 5.
Increased CB$_1$-receptor expression throughout the forebrain of epileptic rats as revealed with an N-terminus CB$_1$-receptor antibody. Representative immunostains in control and epileptic rats at several different levels illustrate greater CB$_1$-receptor immunoreactivity in many forebrain regions of epileptic rats when compared to control, including cortex, caudate-putamen, and thalamus (left panels). Furthermore, the “redistribution” of the receptor is evident in the hippocampus, whereby increases in CB$_1$-receptor expression in several strata, particularly the stratum radiatum of CA3, are concurrent with losses in CB$_1$-receptor expression in the dentate gyrus inner molecular layer. These changes occurred with a small degree of cell loss consistently observed in our model in a Nissl stain (right panels).

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Table 1

Region of interest (ROI) analyses reveal increased [\(^3\)H]WIN binding and WIN-stimulated [\(^35\)S]GTP \(\gamma\)S binding in several brain regions of epileptic animals when compared to control

<table>
<thead>
<tr>
<th>Brain region</th>
<th>[^{[3}]H]WIN binding (nCi/mg)</th>
<th>[^{[35}]S]GTP (\gamma)S binding (nCi/g)</th>
<th>Basal</th>
<th>Net WIN stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Epileptic</td>
<td>Control</td>
<td>Epileptic</td>
</tr>
<tr>
<td>Nucleus accumbens</td>
<td>0.98 ± 0.16</td>
<td>1.91 ± 0.30*</td>
<td>545 ± 42</td>
<td>583 ± 15</td>
</tr>
<tr>
<td>Caudate putamen</td>
<td>1.37 ± 0.20</td>
<td>2.40 ± 0.32*</td>
<td>556 ± 29</td>
<td>628 ± 23</td>
</tr>
<tr>
<td>Cingulate cortex</td>
<td>1.52 ± 0.23</td>
<td>2.33 ± 0.27*</td>
<td>520 ± 22</td>
<td>644 ± 36*</td>
</tr>
<tr>
<td>Globus pallidus</td>
<td>4.39 ± 0.84</td>
<td>5.70 ± 0.87</td>
<td>542 ± 27</td>
<td>798 ± 46*</td>
</tr>
<tr>
<td>Septal nuclei</td>
<td>1.04 ± 0.16</td>
<td>2.10 ± 0.24*</td>
<td>512 ± 19</td>
<td>709 ± 66*</td>
</tr>
<tr>
<td>Amygdala</td>
<td>0.67 ± 0.16</td>
<td>1.08 ± 0.14*</td>
<td>636 ± 27</td>
<td>784 ± 51*</td>
</tr>
<tr>
<td>Thalamus</td>
<td>0.50 ± 0.15</td>
<td>1.04 ± 0.18*</td>
<td>500 ± 14</td>
<td>653 ± 21*</td>
</tr>
<tr>
<td>VPM/VPL</td>
<td>0.63 ± 0.10</td>
<td>1.53 ± 0.24*</td>
<td>548 ± 16</td>
<td>722 ± 18*</td>
</tr>
<tr>
<td>LDDM/LDVL</td>
<td>0.59 ± 0.13</td>
<td>1.29 ± 0.20*</td>
<td>552 ± 32</td>
<td>761 ± 28*</td>
</tr>
<tr>
<td>DLG/VELG</td>
<td>0.52 ± 0.10</td>
<td>1.18 ± 0.19*</td>
<td>578 ± 25</td>
<td>776 ± 42*</td>
</tr>
<tr>
<td>MGID/MGV</td>
<td>0.50 ± 0.08</td>
<td>1.53 ± 0.21*</td>
<td>541 ± 24</td>
<td>715 ± 27*</td>
</tr>
<tr>
<td>Deep (HL) cortex</td>
<td>1.30 ± 0.29</td>
<td>3.55 ± 0.28*</td>
<td>594 ± 29</td>
<td>715 ± 30*</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>2.10 ± 0.35</td>
<td>2.80 ± 0.23</td>
<td>591 ± 35</td>
<td>634 ± 30</td>
</tr>
<tr>
<td>CA3 Strat. Rad.</td>
<td>1.92 ± 0.32</td>
<td>3.33 ± 0.35*</td>
<td>694 ± 41</td>
<td>810 ± 47</td>
</tr>
<tr>
<td>Entorhinal cortex</td>
<td>0.86 ± 0.10</td>
<td>1.53 ± 0.21*</td>
<td>570 ± 14</td>
<td>735 ± 34*</td>
</tr>
<tr>
<td>Substantia nigra</td>
<td>5.20 ± 1.00</td>
<td>5.04 ± 0.67</td>
<td>656 ± 15</td>
<td>732 ± 65</td>
</tr>
<tr>
<td>Periaqueductal gray</td>
<td>0.62 ± 0.15</td>
<td>0.80 ± 0.11</td>
<td>582 ± 23</td>
<td>729 ± 17*</td>
</tr>
<tr>
<td>Cerebellum mol layer</td>
<td>4.30 ± 0.72</td>
<td>4.38 ± 0.42</td>
<td>708 ± 19</td>
<td>633 ± 16*</td>
</tr>
</tbody>
</table>

ROI analyses confirmed increases in \[^{[3]}\]HWIN binding and net \[^{[35]}\]S[GTP \(\gamma\)S binding revealed by SPM analysis in epileptic animals compared to controls. Overall, several regions were confirmed using ROI analysis, such as the stratum radiatum of the hippocampus, thalamus, and caudate putamen. In addition, ROI analysis revealed significant increases in basal \[^{[35]}\]S[GTP \(\gamma\)S binding in several forebrain areas. Data shown are binding values ± SEM (n = 6 per group, * indicates a change from control at p < 0.05 using an unpaired Student’s t-test).