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Statistical Parametric Mapping reveals ligand and region specific activation of G-proteins by CB₁ receptors and non-CB₁ sites in the 3D reconstructed mouse brain

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Abstract

CB₁ receptors mediate the CNS effects of Δ⁹-tetrahydrocannabinol and synthetic cannabinoids. Previous studies have investigated cannabinoid-mediated G-protein activity in a subset of brain regions thought to mediate the behavioral effects of cannabinoids, but a detailed regional comparison of the effects of multiple ligands has not been conducted. This study used a novel approach, Statistical Parametric Mapping (SPM), to analyze 3D reconstructed brain images derived from agonist-stimulated [³⁵S]GTPγS autoradiography in a whole-brain unbiased manner. SPM analysis demonstrated regional differences in the relative efficacies of cannabinoid agonists methanandamide (M-AEA), CP55,940 (CP) and WIN55,212-2 (WIN) in CB₁^{+/+} mouse brains. To assess the potential contribution of novel cannabinoid binding sites, experiments were performed in CB₁^{-/-} mouse brains. SPM analysis revealed that the aminoalkylindole WIN, but not the bicyclic cannabinoid CP or the endocannabinoid analogue M-AEA, stimulated [³⁵S]GTPγS binding in cortex, hippocampus, hypothalamus, amygdala, cerebellum and certain brainstem areas (dorsal tegmental complex and locus coeruleus). No differences between WIN-stimulated G-protein activity and basal activity were found in basal ganglia. Pharmacological experiments using the CB₁ antagonist SR141716A in CB₁^{+/+} mice showed that SR141716A blocked WIN-stimulated G-protein activity in all brain regions, suggesting that it binds to both CB₁ and putative non-CB₁ sites. These studies show ligand and region specific cannabinoid-mediated G-protein activity at both CB₁ and non-CB₁ sites and demonstrate that SPM is a powerful approach for the analysis of reconstructed brain imaging data derived from agonist-stimulated [³⁵S]GTPγS autoradiography.

Introduction

Cannabinoids offer therapeutic potential for a spectrum of medical conditions, however therapeutic use is limited by their central side effects and development of tolerance following chronic administration (Hollister, 1986). Cannabinoid receptors belong to the superfamily of 7 transmembrane spanning G-protein coupled receptors, and are primarily linked to inhibitory Gα_{i/o} proteins (Howlett et al., 2002). Two cannabinoid receptors have been cloned to date. CB₁ receptors are densely distributed throughout the central nervous

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system (CNS) (Devane et al., 1988; Herkenham et al., 1991; Matsuda et al., 1990), whereas CB₂ receptors are found mainly in the immune system with limited CNS expression (Munro et al., 1993; Van Sickle et al., 2005). Moreover, evidence suggests that novel non-CB₁/non-CB₂ cannabinoid receptor(s) are also expressed in the CNS (Mackie and Stella, 2006). The CNS distribution of CB₁ receptors is widespread, with greatest abundance in basal ganglia, hippocampus, and cerebellum (Herkenham et al., 1991; Tsou et al., 1998). Previous studies have focused on these and other regions with moderate to high levels of CB₁ receptors that contribute to the in vivo effects of cannabinoids. However, the endocannabinoid system is implicated in diverse physiological functions (Howlett et al., 2002), highlighting the importance of conducting an anatomically inclusive analysis.

Previously our group developed agonist-stimulated [³⁵S]GTPγS autoradiography for the examination of receptor-activated G-proteins in brain tissue sections (Sim et al., 1995). While agonist-stimulated [³⁵S]GTPγS autoradiography provides anatomical resolution of functional receptor activity, anatomical analysis may be limited by study design. Conventionally, autoradiographic datasets are analyzed manually by sampling regions of interest (ROI) that demonstrate a large measurable signal for receptors or receptor-stimulated G-protein activity. Defining ROIs, however, not only poses a potential inter-rater bias, but does not maximize the sensitivity to detect differences in signal that do not overlap within predefined ROIs. Furthermore, ROI-based approaches are usually hypothesis-driven, thus significant effects outside the boundaries of predefined ROIs may be missed. For a heterogeneous signal, for example, defining ROIs may be particularly difficult. This may be the case when receptor-mediated G-protein activity is not homogeneously distributed within a brain region.

Our previous findings have demonstrated that CB₁ receptor signaling varies by brain region when examined following either acute or chronic activation of CB₁ receptors (Breivogel et al., 1999; Sim-Selley, 2003; Sim et al., 1996a). However, a comprehensive regional profile of receptor activity for both CB₁ and putative non-CB₁ sites has not been elucidated and systematically compared for different classes of cannabinoids. Such a study further requires an anatomical approach that addresses limitations of current strategies for design and analysis of autoradiographic imaging data. Statistical Parametric Mapping (SPM) is a well established statistical technique for the analysis of human neuroimaging data (Friston et al., 1995a; Friston et al., 1990). SPM facilitates an unbiased approach for anatomically localizing significant effects of interest on a whole-brain basis. SPM has also been successfully adapted previously for the analysis of 3D reconstructed autoradiographic datasets mapping cerebral blood flow (Dubois et al., 2008; Holschneider et al., 2006; Nguyen et al., 2004), but not for receptor or agonist-stimulated [³⁵S]GTPγS autoradiography.

A whole brain regional study of cannabinoid receptor function is essential for understanding the endogenous cannabinoid system and identifying potential therapeutic targets. Because signaling at CB₁ receptors (Breivogel and Childers, 2000; Breivogel et al., 1998) and non-CB₁ sites (Mackie and Stella, 2006; Ross, 2009) varies by agonist, it is important to assess different agonists, including methanandamide (M-AEA), a partial agonist and stable analog of the endocannabinoid anandamide, and synthetic compounds WIN55,212-2 (WIN) and CP55,940 (CP) which are aminoalkylindole full and bicyclic high efficacy partial agonists, respectively. This study extends previous findings by comparing differences in cannabinoid-mediated G-protein activity produced by different classes of cannabinoids in reconstructed brain images of wild-type and CB₁^{-/-} mice.

Materials and Methods

Subjects

Transgenic adult $CB_1^{-/-}$ male mice and their wild-type controls (backcrossed onto a C57/Bl6J background (13 generations)) were provided by the National Institute on Drug Abuse Center Transgenic Colony at Virginia Commonwealth University (Richmond, VA). Mice were housed four to six per cage and maintained on a 12-hr light/dark cycle in a temperature controlled environment (20-22°C), with free access to food and water. All experiments were performed with the approval of the Institutional Animal Care and Use Committee at Virginia Commonwealth University in accordance with the *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996).

Drugs and Chemicals

[35 S]GTP γ S (1250 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). CP55,940 and SR141716A were provided by the Drug Supply Program of the National Institute on Drug Abuse. Bovine serum albumin (BSA), GDP, and WIN55,212-2 were purchased from Sigma-Aldrich (St. Louis, MO) and methanandamide (R-1) was purchased from Cayman Chemicals. All other chemicals were obtained from Sigma-Aldrich or Fisher Scientific. *Agonist-Stimulated [35 S]GTP γ S Autoradiography*

Mice were sacrificed by rapid decapitation, and brains were removed and immediately frozen in isopentane at -30°C and stored at -80°C . Autoradiographic assays were conducted as previously published from our laboratory (Sim et al., 1995). Briefly, coronal sections (20 μm) were cut on a cryostat maintained at -20°C , thaw-mounted onto gelatin-subbed slides, and stored desiccated at 4°C overnight. Slides were then stored desiccated at -80°C until use. To minimize variation in assay conditions, slides from each experimental condition within a single animal were processed concurrently. This allowed for identical assay conditions for basal and agonist incubated sections from each animal. For [35 S]GTP γ S autoradiography assay, slides were brought to room temperature ($\sim 22^{\circ}\text{C}$) for 40 min, then equilibrated in 50 mM Tris-HCl buffer (pH 7.4) with 3 mM MgCl_2 , 0.2 mM EGTA, and 100 mM NaCl (Assay Buffer) for 10 min at 25°C . Next, slides were transferred to Assay Buffer + 0.5% BSA, with 2 mM GDP and 10 mU/ml adenosine deaminase for 15 min at 25°C . Slides were then incubated in Assay Buffer + 0.5% BSA containing 0.04 nM [35 S]GTP γ S in the presence or absence (basal) of appropriate drug(s) and/or vehicle for 2 hrs at 25°C . Maximally effective concentrations of agonists were used, as determined in concentration-effect curves, using 0.003-3 μM CP, 0.01-10 μM WIN, or 0.1-30 μM M-AEA in membrane preparations from mouse whole brain (Fig. S1) or cerebellum (data not shown). Membrane agonist-stimulated [35 S]GTP γ S binding experiments were conducted as previously published (Sim-Selley et al., 2006). Results from whole brain and cerebellum membranes were similar, and thus concentrations used for agonist-stimulated autoradiographic assays in all studies ($CB_1^{+/+}$ and $CB_1^{-/-}$ animal studies) were: CP (3 μM), WIN (10 μM), M-AEA (25 μM). In CB_1 antagonist studies 0.5 μM SR141716A was used, which is 1,000-fold greater than its K_D value and at this concentration does not exhibit inverse agonism in [35 S]GTP γ S autoradiographic assays, as previously determined in our laboratory (Sim-Selley et al., 2001). After final incubation, slides were rinsed twice in 50 mM Tris buffer (pH 7.4) at 4°C , then in deionized water. Slides from each condition were then dried and loaded together in the same cassette with a [^{14}C] standard, and exposed to Kodak BioMax MR film for 24-36 hrs. Films were digitized at 8-bits per pixel with a Sony XC-77 video camera.

Slice Registration and 3D Image Reconstruction

Brain image reconstruction of autoradiographic data was conducted as previously published (Holschneider et al., 2006; Nguyen et al., 2004), with modifications (outlined in Fig. 1). For each study, reconstructed brain images from each condition (agonist or basal (absence of agonist)) were created from coronal sections collected throughout the neuroaxis from bregma 2.34 mm to -6.84 mm (Franklin and Paxinos, 2008) with an inter-slice distance of 200 μ m. Serial adjacent sections from a single brain were collected for different assay conditions in duplicate sets. This scheme allowed for duplicate sets of at most 4 different conditions (reconstructions) from a single mouse brain. For sections with extensive artifact(s) the other duplicate section was utilized. In these special cases, the true inter-slice distance varied between 100 to 260 μ m. Individual sections were digitized at 8-bits per pixel and saved in TIFF format. Image reconstructions were generated in ImageJ (version 1.38, <http://rsb.info.nih.gov/ij/>) by creating an image stack of the coronal sections. To make brain reconstructions spatially consistent in 3D, a section-to-section registration technique was used. Briefly, a section in the middle of the brain was used as the initial target to align adjacent sections in a stepwise fashion. Each registered section was then used as a new target to register the next adjacent section in either the anterior or posterior direction. This registration algorithm utilizes the intensity of all image pixels for a section and searches for the transformation that maximizes a measure of intensity similarity between corresponding pixels of an adjacent section (Thévenaz et al., 1998). Volumetric images were saved in Analyze format with a voxel resolution of $40 \times 40 \times 200 \mu\text{m}^3$. Gray level intensities of the raw image stack were then quantitated to activity values (nCi/g of tissue) as previously described (Sim et al., 1996b). Briefly, [^{35}S] was incorporated into sections of frozen brain paste and sections were then weighed to obtain nCi/g of tissue. Radioactivity in each section was determined by liquid scintillation spectrophotometry. [^{14}C] microscale standards and [^{35}S] sections were then exposed to film and correction factors were calculated to convert [^{14}C] values to [^{35}S]. Raw optical density values (OD) are quantitated to radioactivity values (Y) using the following linear equation, where m is the slope and b is the intercept, as calculated from [^{14}C] standards:

$$Y = m \bullet OD + b$$

Quantitated images were then saved at a dynamic range of 16-bit in order to encompass the full data range of our images, which ranged from 100 to 1700 nCi/g. Quality of brain reconstructions was assessed by visual inspection of internal structures viewed in different orthogonal angles and correspondence of landmarks across reconstructions of different conditions and/or subjects.

Voxel-based Analysis using Statistical Parametric Mapping (SPM)

SPM analyses (version SPM5, <http://www.fil.ion.ucl.ac.uk/spm/>) for reconstructed autoradiographs were conducted as previously published (Nguyen et al., 2004), with modifications. For each animal within each study, reconstructed brain images from the basal, M-AEA, CP (studies 1 & 2); or basal, SR1, or WIN/SR1 (study 3) conditions were coregistered (using normalized mutual information) to the WIN condition using the coregistration tool within the SPM software. The best reconstructed WIN image that was free from artifact related to preparation of the tissue was then smoothed with a Gaussian kernel (FWHM = 3 times its voxel dimension) and used as a preliminary brain template for spatial normalization. WIN reconstructions were used to estimate spatial normalization (Ashburner J., 1999; Friston et al., 1995a) parameters as these images had the greatest amount of structural information because WIN is a full agonist at CB₁ receptors (Breivogel et al., 1998). The initial spatially normalized WIN images were then averaged and smoothed

(FWHM = 3 times the voxel dimension) to create the final study-specific brain template. This template was subsequently used to spatially normalize the original un-normalized WIN image reconstructions within each study. Transformation parameters estimated from the spatial normalization of each subject's WIN image reconstruction were then applied to the remaining coregistered images (basal, M-AEA, CP (studies 1 & 2); or basal, SR1, and/or WIN/SR1 (study 3)) within each subject. All normalized brain image reconstructions were smoothed with a Gaussian kernel of FWHM equal to three times its voxel size, which was compatible with our estimate of misregistration error and anatomical variability. An image threshold was specified to create a mask image using the SPM software. The mask was visually inspected and overlaid onto brain images, and the threshold value was calculated such that voxels less than 10% of the mean voxel value within the brain were not included for SPM analysis. This calculated threshold value of 10% ensured that only voxels in the background and within the ventricles from all brain images (basal and agonist) were excluded from SPM analysis. Because amounts of radiolabeled [³⁵S]GTPγS for in situ binding assays were kept constant, global normalization in signal was omitted from SPM analysis. At each voxel, a general linear model was then used to describe the data in terms of experimental and confounding effects, and residual variability (Friston et al., 1995b). A voxel-by-voxel statistical analysis was performed to localize significant differences in receptor-mediated G-protein activation. The multiple comparisons problem was addressed using gaussian random field theory (Worsley et al., 1992). This analysis results in inference based on corrected *p*-values (Friston et al., 1991; Friston et al., 1996; Friston et al., 1994). Each study used a one-way repeated measures ANOVA at each voxel to test the effect of drug in activating G-proteins, and specific contrasts were evaluated. For all studies, significance (*p* < 0.05) was established at the voxel and/or cluster level (minimum cluster extent of 100 contiguous voxels) after correction for multiple comparisons. Statistical *p*-values were corrected using the false-discovery rate and were adjusted for small search volume for each CB₁ receptor containing region, including cortex, striatum, globus pallidus, substantia nigra, hippocampus, amygdala, hypothalamus, periaqueductal gray, and cerebellum. Small volume corrections used a sphere volume of interest to surround each CB₁ receptor containing region. Significance for novel regions not considered a priori were corrected for multiple comparisons for the whole brain search volume or used an uncorrected threshold of *p* < 0.001 and minimum cluster extent size of 100 contiguous voxels. Nissl staining of brain tissue sections from [³⁵S]GTPγS autoradiographic studies was also conducted to identify nuclei of smaller brain areas. Significant clusters of voxels representing brain regions of interest were also verified to span at least 2-3 adjacent sections. To validate and compare regions found to be significant with SPM analysis, a separate ROI analysis was performed. ROI measurements were conducted on the original unprocessed images, averaged bilaterally across hemispheres, and analyzed with GraphPad Prism Version 5 using repeated measures ANOVA and Tukey's post-hoc analysis. ROI anatomical boundaries were defined by a mouse brain atlas (Franklin and Paxinos, 2008). The following brain nuclei were included within each ROI measurement: amygdala (basomedial, basolateral, & medial, lateral, central nuclei), auditory cortex (primary, secondary), caudate-putamen, cerebellum, cingulate cortex (primary, secondary), globus pallidus, hippocampus (CA1-3, dentate gyrus), hypothalamus (medial), motor cortex (primary, secondary), periaqueductal gray, somatosensory cortex (primary, secondary), substantia nigra, thalamus (central, ventral posteromedial & posterolateral, ventromedial, and ventrolateral thalamic nuclei), and visual cortex (primary, secondary).

Results

Cannabinoid-mediated G-protein activation is ligand and region specific

Differences in the efficacy of various cannabinoid agonists to activate G-proteins have previously been studied using agonist-stimulated [35 S]GTP γ S binding in membranes prepared from various brain regions (Breivogel and Childers, 2000). However it is unclear whether the relative efficacy of cannabinoid agonists to activate G-proteins varies by region as determined autoradiographically in brain tissue sections. SPM was used to determine regional differences in cannabinoid-mediated G-protein activity in reconstructed wild-type C57/Bl6J mouse brains (N=5) using maximally effective concentrations of cannabinoid agonists differing in efficacy and structure, including the aminoalkylindole WIN55,212-2 (WIN), bicyclic CP55,940 (CP), and the metabolically stable endocannabinoid analogue methanandamide (M-AEA). Volumetric reconstructions of autoradiographic sections provided 3D anatomical visualization and localization of cannabinoid-mediated G-protein activity (Fig. 2 & Supplementary Fig. S2). The spatial extent of cannabinoid-stimulated G-protein activity as illustrated in Fig. 2 is shown in red, and the surface of the brain is rendered in gray for the right hemisphere. All three cannabinoid agonists significantly stimulated greater [35 S]GTP γ S binding than basal (no agonist) in all CB $_1$ receptor containing regions (Fig. S3) as determined by SPM analysis. As shown for WIN (Fig. 2), cannabinoid-stimulated G-protein activity was widespread in the cortex, densely distributed in the output nuclei of the basal ganglia (globus pallidus, substantia nigra) and seen in bands in the cerebellum that presumably correspond to the molecular layer. To determine the regional relative efficacy, SPM was then used to compare G-protein activity produced by the three cannabinoid agonists. WIN-stimulated G-protein activity was significantly greater than M-AEA in almost all CB $_1$ receptor containing regions, with exception of the thalamus (Fig. 3A). SPM provided a detailed anatomical comparison, as seen in the cortex (cingulate, somatosensory, and motor cortex) where WIN produced significantly greater G-protein activation than M-AEA in both superficial and deep laminae (Fig. 3A). A similar regional activation profile (data not shown) was seen comparing CP and M-AEA, where CP stimulated significantly greater G-protein activity than M-AEA. When comparing WIN and CP however, region-specific differences in the relative stimulation produced by these agonists were identified by SPM. For example, WIN stimulated significantly greater G-protein activity than CP in some CB $_1$ containing regions including globus pallidus, hypothalamus and periaqueductal gray (Fig. 3B). In contrast, WIN- and CP-stimulated G-protein activity were equivalent in the caudate-putamen, hippocampus, amygdala, cerebellum and cortical regions including motor, somatosensory, and cingulate cortices (Fig. 3B). Overall, SPM revealed that in some regions, including globus pallidus, hypothalamus, and periaqueductal gray, the activity profile was WIN > CP > M-AEA. In contrast, for most regions including caudate-putamen, hippocampus, amygdala, cerebellum and cortex (motor, somatosensory, and cingulate cortices) the relationship was WIN = CP > M-AEA. Interestingly, a different relative efficacy profile was found in the thalamus compared to all other brain regions. SPM revealed that WIN-, CP-, and M-AEA-stimulated [35 S]GTP γ S binding did not significantly differ in thalamic nuclei, including ventral posteromedial and posterolateral, ventromedial, and ventrolateral thalamic nuclei. Subsequent ROI analyses substantiated almost all of the SPM findings, except in hypothalamus where WIN and CP did not differ in stimulating [35 S]GTP γ S binding. In this brain region, ROI analysis revealed similar qualitative differences between WIN and CP as determined using SPM analysis, but failed to reach significance at the $p < 0.05$ level. This could be explained by differences in the analytical technique, where significance by SPM is based at the individual voxel-level as opposed to a user-defined region of interest, which contains the average of many image pixels or subnuclei.

While the anatomical distribution of agonist-stimulated G-protein activity was similar for each of the three agonists compared to basal (no agonist), the relative magnitude of activity varied by brain region, as determined by an independent ROI analysis of cannabinoid-stimulated [35 S]GTP γ S binding for selected brain regions (Table 1). The relative efficacies of the three agonists were calculated by expressing net agonist-stimulated [35 S]GTP γ S binding produced by CP or M-AEA as percent of [35 S]GTP γ S binding stimulated by the full agonist WIN (Table 1). For example, the relative efficacy of M-AEA ranged from 32% of WIN in periaqueductal gray to 76% of WIN in thalamus. In addition, the relative efficacy of CP ranged from 60% of WIN in periaqueductal gray to 105% in thalamus. Due to the potential contribution of WIN acting at non-CB $_1$ sites (see next section), [35 S]GTP γ S binding data is also reported as %CP (Supplementary Table 1). A similar relative efficacy relationship was revealed when using CP as the standard agonist. The high relative efficacy of WIN in specific brain regions did not correlate with having non-CB $_1$ -mediated activity, indicating that CB $_1$ receptors contribute to the majority of the signal detected in WIN-stimulated [35 S]GTP γ S autoradiography. When normalized to the full CB $_1$ agonist WIN, there was no significant correlation between the relative efficacies of M-AEA- and CP-stimulated G-protein activation (supplementary material Fig. S4) across the sampled brain regions of interest. In addition, a significant interaction of drug $\times$ region ($p < 0.01$) was found when analyzed by a two way repeated measures ANOVA. A significant interaction between the factors drug and region and lack of correlation in the relative efficacy profile, suggests that there may be regional differences in the relative efficacies of M-AEA and CP, which cannot be explained by receptor levels or G-protein abundance. These results thus indicate that the relative G-protein activity produced by these cannabinoid agonists was region-dependent, as revealed by both SPM and ROI analysis of 3D reconstructed agonist-stimulated [35 S]GTP γ S autoradiographic data.

WIN55,212-2 stimulates G-protein activity in a subset of brain regions of mice lacking CB $_1$ receptors

Results in wild-type mice suggested that the relative efficacy of cannabinoid agonists varies by brain region. However, the interpretation of these findings is complicated by the possible contribution of non-CB $_1$ binding sites to receptor-mediated G-protein activity. This question was addressed using SPM analysis of M-AEA-, CP-, and WIN-stimulated [35 S]GTP γ S binding in reconstructed CB $_1^{-/-}$ mouse brain (N=8). SPM analysis showed that agonist-stimulated [35 S]GTP γ S binding produced by CP or M-AEA did not significantly differ from basal activity (in the absence of agonist) in any brain region from CB $_1^{-/-}$ mice (data not shown). However, WIN-stimulated [35 S]GTP γ S binding significantly differed from basal binding in a number of areas in CB $_1^{-/-}$ mice as determined by SPM and subsequently confirmed by ROI analyses (Table 2). As seen in Fig. 4, WIN-stimulated [35 S]GTP γ S binding was significantly greater than basal binding in regions that partially overlapped the distribution of CB $_1$ receptors. SPM analysis revealed WIN-stimulated [35 S]GTP γ S binding in visual (V) and auditory cortices (Au) that appeared to be localized to deeper laminae. In the hippocampus, WIN-stimulated [35 S]GTP γ S binding was noted in the caudal, ventral CA1 and CA3, and to a lesser extent rostral, dorsal CA3. Although ROI analysis of the whole hippocampus showed a similar trend as in the SPM analysis, it failed to reach significance at the $p < 0.05$ criterion, possibly due to the heterogeneity of signal in this brain region as demonstrated in the SPM map (Fig. 4). WIN-stimulated [35 S]GTP γ S binding was widely distributed in the amygdaloid complex, and appeared to be distributed in areas that corresponded to anterior portions of the basomedial and basolateral, and medial, lateral and central nuclei. WIN-stimulated [35 S]GTP γ S binding was also found in the medial hypothalamus. Interestingly, the greatest magnitude of WIN-stimulated [35 S]GTP γ S binding in CB $_1^{-/-}$ mouse brains was found within the brain stem including tegmental nuclei that appeared to correspond to lateral and dorsal tegmentum, and areas adjacent to the fourth

ventricle, which corresponds to the locus coeruleus (Table 2). Significant WIN-stimulated G-protein activity was also found in the cerebellum that appeared to correspond to the molecular layer. In contrast, no differences between WIN-stimulated G-protein activity and basal binding were detected in basal ganglia (globus pallidus, substantia nigra, caudate-putamen), brain areas that normally contain among the highest levels of CB₁ receptors. It is important to note that the average magnitude of net WIN-stimulated G-protein activation in significant brain areas was approximately 4-5 fold less in CB₁^{-/-} mice compared to wild-type mice (Table 1 versus 2). For example, net WIN-stimulated G-protein activity (WIN stimulated – basal) in cerebellum was 83 ± 20 nCi/g in CB₁^{-/-}, compared to 456 ± 37 nCi/g in wild-type mice. Thus, data from reconstructed CB₁^{-/-} mouse brains suggest that the aminoalkylindole WIN, but not the endocannabinoid analogue M-AEA or bicyclic CP, activates non-CB₁ sites in a subset of brain areas that only partially overlaps with CB₁ receptor containing regions.

WIN55,212-2-stimulated G-protein activity in wild-type mice is blocked by SR141716

The aminoalkylindole WIN activated G-proteins in CB₁^{-/-} mice, providing evidence for non-CB₁ sites in a genetic model. To validate this finding, a pharmacological approach was applied by incubating wild-type mouse brain sections with WIN in the presence or absence of the CB₁ antagonist SR141716A (SR1), and resulting G-protein activity was compared using SPM in the reconstructed CB₁^{+/+} mouse brain (N=8). SR1 (0.5 μM) alone did not significantly stimulate or inhibit [³⁵S]GTPγS binding compared to basal binding in any brain region (data not shown). WIN-stimulated G-protein activity was robust and widespread in CB₁ receptor containing regions in the absence of SR1, as discussed above (data not shown). WIN-stimulated [³⁵S]GTPγS binding in the presence of SR1 (WIN/SR1) was reduced in all brain regions and did not significantly differ from basal as determined by SPM. It is possible that WIN might exhibit lower potency and/or efficacy for putative non-CB₁ sites (Breivogel et al., 2001). Therefore, a higher concentration of WIN (50 μM) was used in the presence or absence of 0.5 μM SR1 in select brain regions that demonstrated non-CB₁ binding sites as determined in CB₁^{-/-} mice (Table 2, Fig. 4). ROI analyses of these regions, however, showed no significant stimulation of [³⁵S]GTPγS binding in the presence of 50 μM WIN + SR1 (data not shown). These findings suggest that SR1 might also bind to putative non-CB₁ sites.

Discussion

The primary goal of this study was to elucidate regional differences in the relative efficacy of receptor-mediated G-protein activity produced by cannabinoids differing in intrinsic efficacy and chemical structure. The potential contribution of non-CB₁ sites to agonist-stimulated G-protein activity was then evaluated in the CB₁^{-/-} mouse model. This was accomplished using a novel approach, in which Statistical Parametric Mapping (SPM) was adapted for whole-brain analysis of 3D reconstructed agonist-stimulated [³⁵S]GTPγS autoradiographic data. SPM revealed regional differences in the efficacy of cannabinoids to activate G-proteins in brains of wild-type mice and localized novel WIN-stimulated [³⁵S]GTPγS binding sites in CB₁^{-/-} mouse brains. This approach provided the dual advantages of creating a functional map of receptor-activated G-proteins in the reconstructed brain, with an unbiased statistical comparison of levels of G-protein activity, thereby providing new data on cannabinoid-mediated activity in the mouse brain.

SPM has previously been adapted for the analysis of reconstructed autoradiographic datasets mapping cerebral blood flow (Nguyen et al., 2004), and successfully applied to functionally map brain activity in small animals (Dubois et al., 2008; Holschneider et al., 2006; Soto-Montenegro et al., 2008; Yang et al., 2007). Applying SPM analysis to *in vitro* agonist-stimulated [³⁵S]GTPγS autoradiographic data offers unique advantages compared to

conventional region of interest (ROI) analysis. ROI analysis is generally assessed within predefined anatomical boundaries, but effects of interest might not always conform to anatomically defined regions. For heterogeneous distributions of receptor-mediated activity, the size and shape of ROIs and plane(s) of data might be difficult to define a priori in order to maximize signal detection. In addition, if regions of receptor activity are substantially smaller than the defined ROI, significance might be missed when signal is averaged with surrounding background that is included within the ROI. Moreover, the anatomical precision of this approach is illustrated in the motor and somatosensory cortices, where voxels with the most significant and greatest magnitude of cannabinoid-stimulated [^{35}S]GTP γ S binding in CB $_1^{+/+}$ mice were localized in specific cortical laminae (Fig. 3A) in a pattern consistent with previous reports showing the highest concentration of CB $_1$ receptor protein in the superficial (II, III) and deep (VI) layers of cortex (Herkenham et al., 1991; Tsou et al., 1998). It is important to note that most of the significant differences between cannabinoid-stimulated [^{35}S]GTP γ S binding showed bilateral symmetry. This neuroanatomical property, in addition to the known expression profile of CB $_1$ receptor protein, further strengthens the validity of SPM for analyzing agonist-stimulated [^{35}S]GTP γ S binding autoradiographic data.

Initial studies revealed regional differences in the relative efficacies of WIN-, CP-, and M-AEA-stimulated [^{35}S]GTP γ S binding. SPM comparison identified an efficacy profile of WIN > CP > M-AEA in areas including globus pallidus, hypothalamus, and periaqueductal gray. However in other brain areas including caudate-putamen, cerebellum, hippocampus, amygdala, substantia nigra and cortex, the relative efficacy for cannabinoid-stimulated G protein activity was WIN = CP > M-AEA. A different finding emerged in the thalamus, where the profile WIN = CP = M-AEA was found. A previous study using membrane preparations from cerebellum, hippocampus, and hypothalamus also reported regional differences in the relative efficacy and potency of WIN, CP, and M-AEA (Breivogel and Childers, 2000). However, the relative stimulation of receptor-activated G-proteins for these cannabinoid agonists differed somewhat from the current study. This discrepancy could be due to differences in the anatomical resolution of autoradiography versus [^{35}S]GTP γ S binding in membranes prepared from grossly dissected brain regions. While the mechanism(s) underlying regional differences in relative agonist efficacy are not known, CB $_1$ receptors have been shown to exhibit domain selectivity for coupling to different subtypes of G $\alpha_{i/o}$, and in various brain regions, activate different subtypes of G-proteins with varying efficacy and potency after agonist stimulation (Mukhopadhyay et al., 2000; Prather et al., 2000). Cannabinoid-selective G-protein signaling has also been demonstrated using recombinant expression of CB $_1$ receptors in situ with reconstitution of purified G-protein subunits (Glass and Northup, 1999). In that study, the relative efficacies of different cannabinoid agonists differed between G α_i versus G α_o activation. One can thus envision ligand- and region-specific CB $_1$ receptor signaling based on the stoichiometric complement of various G α subtypes.

An alternative explanation for the higher efficacy of certain cannabinoids is the contribution of multiple receptors (e.g. CB $_1$ + non-CB $_1$ receptors) to the overall agonist-stimulated activity. To explore this question further, SPM was used to determine the extent to which WIN, CP, and M-AEA, would activate G-proteins in CB $_1^{-/-}$ mouse brains. Of these cannabinoid agonists, only the aminoalkylindole WIN significantly stimulated [^{35}S]GTP γ S binding when compared to basal activity (absence of agonist). In this study, WIN stimulated [^{35}S]GTP γ S binding in a subset of brain regions in CB $_1^{-/-}$ mouse brains that only partially overlapped the distribution of activity in CB $_1^{+/+}$ brains. The greatest magnitudes of WIN-stimulated G-protein activity were noted within the dorsal tegmental complex and locus coeruleus, which contain acetylcholine and norepinephrine producing neurons, respectively. Relatively high levels of G-protein activation were also found in the cerebellum, which

appear to correspond to the molecular layer. Modest G-protein activation was found in the cortex, amygdala, hippocampus, and hypothalamus. Interestingly, the hypothalamus was a brain region in which SPM analysis of brains from CB₁^{+/+} mice found significantly greater WIN- versus CP-stimulated [³⁵S]GTPγS binding, suggesting a possible contribution of additional non-CB₁ sites to the greater efficacy for WIN in G-protein activation. However, for other brain areas in CB₁^{-/-} mice with significant WIN-stimulated [³⁵S]GTPγS binding, such as amygdala and cerebellum, there were no differences in efficacy for G-protein activation between WIN and CP in the CB₁^{+/+} mice. For these brain areas, it is possible that differences in regional G-protein coupling may enhance the efficacy of CP in CB₁^{+/+} mice. Interestingly, no significant WIN-stimulated [³⁵S]GTPγS binding in CB₁^{-/-} mice was found in the nuclei of the basal ganglia, which normally contain the highest levels of CB₁ receptors (Herkenham et al., 1991). In contrast, the brainstem contains relatively low to moderate levels of CB₁ receptors (Herkenham et al., 1991) but the greatest magnitude of WIN-stimulated activity was detected in certain pontine nuclei. These findings are also consistent with previous reports that WIN stimulated [³⁵S]GTPγS binding, but CP had no effect, in membrane homogenates prepared from CB₁^{-/-} mice (Breivogel et al., 2001). Although previous studies have also found that anandamide (AEA) stimulated G-protein activity in CB₁^{-/-} mice (Breivogel et al., 2001; Di Marzo et al., 2000), the stable analog M-AEA had no effect in this study. Brain areas with significant WIN-stimulated [³⁵S]GTPγS binding compared to basal in this study (Table 2) were similar to those previously reported by Breivogel and colleagues (Breivogel et al., 2001). Both studies found WIN-stimulated G-protein activity in the brainstem, cortex, hypothalamus, and hippocampus, and no significant WIN-stimulated [³⁵S]GTPγS binding in the basal ganglia. However, no significant WIN-stimulated [³⁵S]GTPγS binding was found in the cerebellum by Breivogel and colleagues, whereas in this study significance was localized in bands that appear to correspond to the molecular layer of the cerebellum (Fig 4). The slight discrepancy in results could be due to differences in the analytical techniques, where SPM assesses changes at the individual voxel-level in intact brain tissue sections versus the [³⁵S]GTPγS binding assay for homogenized gross brain areas.

Interestingly, non-CB₁ WIN-stimulated [³⁵S]GTPγS binding sites detected by SPM appeared to be distributed in certain functional systems. The localization of non-CB₁ sites in the locus coeruleus (LC) and its projection regions including frontal cortex, amygdala, hypothalamus, hippocampus, and cerebellum, might suggest a possible neuromodulatory role of these non-CB₁ sites within the noradrenergic system. Noradrenergic projections from the LC to forebrain regions are especially relevant to regulation of cognition, attention and anxiety (Aston-Jones et al., 1999; Bremner et al., 1996; Foote et al., 1983). Previous studies by Van Bockstaele and colleagues have shown that systemic WIN administration increased cfos expression in tyrosine hydroxylase positive LC neurons, indicating that WIN was affecting the coeruleo-cortical pathway. In addition, administration of WIN systemically (Oropeza et al., 2005) or directly into the frontal cortex (Page et al., 2008) increased norepinephrine release in an SR1-sensitive manner. Interestingly, CB₁ receptors are generally associated with presynaptic inhibition of neurotransmitter release (Schlicker and Kathmann, 2001) and inhibit NE release in the hippocampus (Schlicker et al., 1997). While it is possible that enhanced WIN-mediated NE release in the frontal cortex occurs via an indirect mechanism, it is also possible that NE release is regulated by both CB₁ and non-CB₁ receptors in this region. Approximately 30% of CB₁ receptor immunoreactive terminals in the frontal cortex also contain the catecholamine-synthesizing enzyme dopamine-β-hydroxylase (Oropeza et al., 2007), and if non-CB₁ WIN sites predominated in remaining terminals, the net result could be enhanced NE release. However, this question would need to be further examined in CB₁^{-/-} mice. Thus, the anatomical profile of non-CB₁ WIN sites found in the current study could subserve a distinct modulatory function from the endogenous cannabinoid system that has yet to be fully characterized.

Because WIN was active in CB₁^{-/-} mice, pharmacological studies were conducted in CB₁^{+/+} mice to assess WIN-stimulated [³⁵S]GTPγS binding in the presence of the CB₁ receptor antagonist SR141716A (SR1). However, no significant G-protein activation was detected with WIN in the presence of SR1, nor did SR1 alone produce any change in [³⁵S]GTPγS binding compared to basal activity. Thus, it is possible that SR1 might bind to both CB₁ and non-CB₁/CB₂ sites. In fact, previous studies have shown that certain non-CB₁ mediated effects of WIN are inhibited by pretreatment with SR1 (Hajos et al., 2001; Hoffman et al., 2005; Pistis et al., 2004). It is further possible that multiple non-CB₁ WIN-stimulated [³⁵S]GTPγS binding sites exist, based on pharmacological and species specificity as defined in the literature (Hoffman et al 2005). Although the molecular identity of non-CB₁ WIN-binding sites in the current study is unknown, several conclusions can be reached regarding their pharmacology. WIN is an agonist of unknown efficacy, SR1 appears to be an antagonist or very low efficacy partial agonist, and CP and M-AEA are not agonists. Further, these sites are presumably coupled to inhibitory G-proteins of the Gα_{i/o} class because agonist-stimulated [³⁵S]GTPγS autoradiography in brain does not appear to detect other classes of receptor-activated G-proteins due to the high abundance of Gα_o in brain and the kinetics of binding under the conditions of the assay (Sim-Selley and Childers, 2002). Insensitivity to CP indicates that these sites are unlikely to be CB₂ receptors (Govaerts et al., 2004), despite previous reports that CB₂ receptors are expressed in brainstem (Van Sickle et al., 2005). These sites are also unlikely to be GPR55, which has been shown to activate G_q and G₁₂ and does not respond to WIN in various in vitro assays (Ross, 2009). Thus, the present results provide anatomical and functional evidence supporting the existence of a novel non-CB₁/CB₂/GRP55 WIN-stimulated [³⁵S]GTPγS binding site in defined regions of mouse brain.

In summary, Statistical Parametric Mapping (SPM) was used to assess and spatially map regional differences in cannabinoid-mediated G-protein activity in reconstructed mouse brain images derived from agonist-stimulated [³⁵S]GTPγS binding autoradiography. SPM analysis, combined with conventional ROI analysis, demonstrated regional differences in the relative efficacies of various cannabinoid agonists, and showed that certain cannabinoid agonists activate functional non-CB₁ sites, in addition to CB₁ receptors. The unique pharmacology and functional neuroanatomical distribution of non-CB₁ sites indicates that this novel system might have distinct physiological roles from the endogenous cannabinoid system that have yet to be characterized. Furthermore, the neuroanatomical distribution of these putative non-CB₁ sites suggests that this system could be exploited therapeutically. Lastly, this study demonstrates SPM as a powerful tool for the neuroanatomical analysis and functional mapping of G-protein coupled receptors in 3D reconstructed mouse brain images derived from [³⁵S]GTPγS autoradiography.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Flow chart of tissue preparation for autoradiography, 3D image reconstruction and SPM analysis.

* 20 μm coronal sections are cut in a cryostat maintained at -20°C . C = condition; S = slice level; a & b = set 1 & 2 of duplicate sections

† The section free from artifact (i.e. S1a or S1b) at each slice level is digitized at 8-bits per pixel and saved in TIFF format

‡ Image reconstruction and slice registration described in *Nguyen et al., NeuroImage 23 (2004)*. Registered image stacks are quantitated and saved as 16-bit images in Analyze image format.

§ Individual image reconstructions are spatially normalized to a study-specific brain template as described in *Nguyen et al., NeuroImage 23 (2004)*. Spatially normalized images are then smoothed with a Gaussian kernel (Full Width Half-Max = 3X the voxel value)

◆ Statistical Parametric Mapping (SPM) software used to setup experimental design and analysis of imaging data.

Figure 2.
3D image reconstruction and volumetric rendering of average ($n = 3$) cannabinoid-stimulated [^{35}S]GTP γ S binding (red) in mouse brain using a maximally effective concentration of the full cannabinoid agonist WIN ($10\ \mu\text{M}$). A threshold was applied to show the spatial extent of highest WIN-stimulated G-protein activity in brain regions, including the molecular layer of the cerebellum, cortex, and basal ganglia. AO (Anterior olfactory nucleus), Ent (entorhinal cortex), GP (globus pallidus), MO (medial orbital cortex), Pir (piriform cortex), SN (substantia nigra).

Figure 3.

Regional comparison of agonist-stimulated [³⁵S]GTPγS binding produced by the cannabinoid agonists WIN, CP, and M-AEA. Representative coronal sections illustrate Statistical Parametric Maps ($p < 0.05$, ANOVA, $n=5$) of significant differences in receptor-mediated G-protein activation, between WIN and M-AEA (**A**), or WIN and CP (**B**). WIN stimulated significantly greater G-protein activity than M-AEA in most CB₁ containing regions, with the exception of the thalamus. In addition, WIN-stimulated [³⁵S]GTPγS binding was greater than CP in globus pallidus, periaqueductal gray, and hypothalamus. Colored overlays (red to orange) correspond to significance level represented by a p -value scale. Au (auditory cortex), Hipp (hippocampus), CPu (caudate-putamen), GP (globus pallidus), Hyp (hypothalamus), M (motor cortex), PAG (periaqueductal gray), S (somatosensory cortex), SN (substantia nigra reticular), V (visual cortex), *M-AEA*, methanandamide; *WIN*, WIN55,212-2

Figure 4. SPM analysis showing the regional distribution of significant differences between WIN-stimulated and basal [^{35}S]GTP γ S binding ($p < 0.05$, ANOVA, $n=8$) in $\text{CB}_1^{-/-}$ mouse brains. Representative coronal sections with colored overlays (red to yellow) show significant differences (WIN > basal) corresponding to a p -value scale. Au (auditory ctx), BMA/BLA (basomedial and basolateral amygdala), CA1 (CA1 field of hippocampus), CBLM (cerebellum), CPu (caudate-putamen), DTg (tegmental nucleus, dorsal), LC (locus coeruleus), MH (medial hypothalamus), SN (substantia nigra), V (visual cortex), WIN, WIN55,212-2.

Regional differences in the relative efficacies of different cannabinoid agonists were found by both SPM and ROI analysis of agonist-stimulated [³⁵S]GTPγS autoradiographic images.

Table 1

Brain Region	Net-stimulated binding (nCi/g)				Net stimulation as %WIN			
	M-AEA	CP	WIN	WIN	M-AEA	CP	WIN	WIN
Motor cortex	263 ± 11 ^{Aa}	470 ± 14 ^{Bb}	494 ± 13 ^{Bb}	53 ± 2	95 ± 3	100 ± 3	100 ± 3	100 ± 3
Somatosensory cortex	146 ± 29 ^{Aa}	272 ± 44 ^{Bb}	264 ± 37 ^{Bb}	55 ± 11	103 ± 17	100 ± 14	100 ± 14	100 ± 14
Cingulate cortex	273 ± 17 ^{Aa}	488 ± 16 ^{Bb}	521 ± 30 ^{Bb}	52 ± 3	94 ± 3	100 ± 6	100 ± 6	100 ± 6
Hippocampus	170 ± 34 ^{Aa}	313 ± 50 ^{Bb}	377 ± 26 ^{Bb}	45 ± 9	83 ± 13	100 ± 7	100 ± 7	100 ± 7
Amygdala	200 ± 34 ^{Aa}	384 ± 36 ^{Bb}	457 ± 28 ^{Bb}	44 ± 7	84 ± 8	100 ± 6	100 ± 6	100 ± 6
Thalamus	59 ± 20 ^{Aa}	81 ± 24 ^{Aa}	77 ± 2 ^{Aa}	76 ± 25	105 ± 30	100 ± 27	100 ± 27	100 ± 27
Hypothalamus	83 ± 23 ^{Aa}	203 ± 31 ^{Bb}	235 ± 16 ^{Cb}	35 ± 10	86 ± 13	100 ± 7	100 ± 7	100 ± 7
Periaqueductal gray	100 ± 30 ^{Aa}	188 ± 19 ^{Bb}	313 ± 23 ^{Cc}	32 ± 10	60 ± 6	100 ± 7	100 ± 7	100 ± 7
Cerebellum	210 ± 45 ^{Aa}	472 ± 39 ^{Bb}	456 ± 37 ^{Bb}	46 ± 10	103 ± 8	100 ± 8	100 ± 8	100 ± 8
Substantia Nigra	597 ± 45 ^{Aa}	801 ± 28 ^{Bb}	863 ± 42 ^{Bb}	69 ± 5	93 ± 3	100 ± 5	100 ± 5	100 ± 5
Globus Pallidus	480 ± 28 ^{Aa}	607 ± 16 ^{Bb}	788 ± 38 ^{Cc}	61 ± 4	77 ± 2	100 ± 5	100 ± 5	100 ± 5
Caudate-Putamen	189 ± 9 ^{Aa}	373 ± 15 ^{Bb}	399 ± 26 ^{Bb}	47 ± 2	94 ± 4	100 ± 7	100 ± 7	100 ± 7

^ANet-stimulated binding values labeled with different letters within a brain region are significantly different from each other based on SPM (upper case; $p < 0.05$ corrected, ANOVA, $n=5$) or ROI analysis (lower case; $p < 0.05$, ANOVA, Tukey's post-hoc analysis) as described under *Methods*. Measurements in each column are mean net [³⁵S]GTPγS binding values (nCi/g) ± SEM for M-AEA-, CP-, and WIN-stimulated conditions as determined by ROI analysis. Net values were calculated by subtracting basal from agonist-stimulated [³⁵S]GTPγS binding. ROI anatomical boundaries are described in *Methods*.

^BNet-stimulated binding values labeled with different letters within a brain region are significantly different from each other based on SPM (upper case; $p < 0.05$ corrected, ANOVA, $n=5$) or ROI analysis (lower case; $p < 0.05$, ANOVA, Tukey's post-hoc analysis) as described under *Methods*. Measurements in each column are mean net [³⁵S]GTPγS binding values (nCi/g) ± SEM for M-AEA-, CP-, and WIN-stimulated conditions as determined by ROI analysis. Net values were calculated by subtracting basal from agonist-stimulated [³⁵S]GTPγS binding. ROI anatomical boundaries are described in *Methods*.

^CNet-stimulated binding values labeled with different letters within a brain region are significantly different from each other based on SPM (upper case; $p < 0.05$ corrected, ANOVA, $n=5$) or ROI analysis (lower case; $p < 0.05$, ANOVA, Tukey's post-hoc analysis) as described under *Methods*. Measurements in each column are mean net [³⁵S]GTPγS binding values (nCi/g) ± SEM for M-AEA-, CP-, and WIN-stimulated conditions as determined by ROI analysis. Net values were calculated by subtracting basal from agonist-stimulated [³⁵S]GTPγS binding. ROI anatomical boundaries are described in *Methods*.

^aNet-stimulated binding values labeled with different letters within a brain region are significantly different from each other based on SPM (upper case; $p < 0.05$ corrected, ANOVA, $n=5$) or ROI analysis (lower case; $p < 0.05$, n=5, ANOVA, Tukey's post-hoc analysis) as described under *Methods*. Measurements in each column are mean net [^{35}S]GTP- γ S binding values (nCi/g) $\pm$ SEM for M-AEA-, CP-, and WIN-stimulated conditions as determined by ROI analysis. Net values were calculated by subtracting basal from agonist-stimulated [^{35}S]GTP- γ S binding. ROI anatomical boundaries are described in *Methods*.

^bNet-stimulated binding values labeled with different letters within a brain region are significantly different from each other based on SPM (upper case; $p < 0.05$ corrected, ANOVA, $n=5$) or ROI analysis (lower case; $p < 0.05$, n=5, ANOVA, Tukey's post-hoc analysis) as described under *Methods*. Measurements in each column are mean net [^{35}S]GTP- γ S binding values (nCi/g) $\pm$ SEM for M-AEA-, CP-, and WIN-stimulated conditions as determined by ROI analysis. Net values were calculated by subtracting basal from agonist-stimulated [^{35}S]GTP- γ S binding. ROI anatomical boundaries are described in *Methods*.

^cNet-stimulated binding values labeled with different letters within a brain region are significantly different from each other based on SPM (upper case; $p < 0.05$ corrected, ANOVA, $n=5$) or ROI analysis (lower case; $p < 0.05$, n=5, ANOVA, Tukey's post-hoc analysis) as described under *Methods*. Measurements in each column are mean net [^{35}S]GTP- γ S binding values (nCi/g) $\pm$ SEM for M-AEA-, CP-, and WIN-stimulated conditions as determined by ROI analysis. Net values were calculated by subtracting basal from agonist-stimulated [^{35}S]GTP- γ S binding. ROI anatomical boundaries are described in *Methods*.

WIN (10 μ M) stimulated [35 S]GTP γ S binding in CB $_1^{-/-}$ brains in regions partially overlapping CB $_1$ receptors.

Table 2

Brain Region	[35 S]GTP γ S binding (nCi/g)						Sig.	ROI
	Basal	WIN	WIN Net	%Stim	SPM			
Auditory cortex	455 $\pm$ 17	533 $\pm$ 18	77 $\pm$ 21	18 $\pm$ 5	v,c		*	
Visual cortex	434 $\pm$ 20	494 $\pm$ 20	60 $\pm$ 30	16 $\pm$ 7	v,c		n.s.	
Amygdala	763 $\pm$ 20	911 $\pm$ 34	148 $\pm$ 27	19 $\pm$ 3	v,c		**	
Hippocampus	429 $\pm$ 16	478 $\pm$ 18	49 $\pm$ 24	12 $\pm$ 6	$p < 0.01$		n.s.	
Thalamus	390 $\pm$ 17	397 $\pm$ 8	7 $\pm$ 14	3 $\pm$ 3	n.s.		n.s.	
Hypothalamus	735 $\pm$ 29	863 $\pm$ 35	128 $\pm$ 40	19 $\pm$ 6	v		*	
Dorsal Tegmental complex	483 $\pm$ 12	687 $\pm$ 21	205 $\pm$ 19	43 $\pm$ 4	$p < 0.001$		***	
Locus Coeruleus	523 $\pm$ 23	720 $\pm$ 30	197 $\pm$ 29	39 $\pm$ 6	$p < 0.001$		***	
Globus Pallidus	508 $\pm$ 19	488 $\pm$ 12	-19 $\pm$ 14	-3 $\pm$ 3	n.s.		n.s.	
Substantia Nigra	404 $\pm$ 17	419 $\pm$ 13	16 $\pm$ 29	6 $\pm$ 8	n.s.		n.s.	
Caudate-Putamen	509 $\pm$ 24	515 $\pm$ 24	6 $\pm$ 20	2 $\pm$ 4	n.s.		n.s.	
Periaqueductal gray	758 $\pm$ 27	764 $\pm$ 16	6 $\pm$ 30	2 $\pm$ 4	n.s.		n.s.	
Cerebellum	223 $\pm$ 11	306 $\pm$ 20	83 $\pm$ 20	39 $\pm$ 10	v,c		**	

Measurements in columns 1 and 2 are mean [35 S]GTP γ S binding values (nCi/g) $\pm$ SEM for basal- and WIN-stimulated conditions sampled within each brain region by ROI. Net binding equals [WIN stimulated- basal binding]. Percent stimulation (%Stim) for each brain region is calculated as ((WIN - basal)/basal)*100%). Column five shows significance ($p < 0.05$) at the voxel (v) or cluster (c) level (corrected for multiple comparisons, as described in *Methods*, ANOVA, $n=8$) or uncorrected p -value for each brain region as determined by SPM analysis. Column six shows results obtained by ROI measurement with subsequent analysis using repeated measures ANOVA, followed by Tukey's post-hoc comparison.

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$.

ROI anatomical boundaries are described in *Methods*. WIN, WIN55,212-2; n.s. = not significant.