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Chronic Constriction Injury Reduces Cannabinoid Receptor 1 Activity in the Rostral Anterior Cingulate Cortex of Mice

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Abstract

The present studies examined the effect of chronic neuropathic pain on cannabinoid receptor density and receptor-mediated G-protein activity within supraspinal brain areas involved in pain processing and modulation in mice. Chronic constriction injury (CCI) produced a significant decrease in WIN 55, 212-2 -stimulated [³⁵S]GTPγS binding in membranes prepared from the rostral anterior cingulate cortex (rACC) of CCI mice when compared to sham operated controls. Saturation binding with [³H]SR 141716A in membranes of the rACC showed no significant differences in binding between CCI and sham mice. Analysis of levels of the endocannabinoids anandamide (AEA) or 2-arachidonoylglycerol (2-AG) in the rACC following CCI showed no significant differences between CCI and sham mice. These data suggest that CCI produced desensitization of the cannabinoid 1 receptor in the rACC in the absence of an overall decrease in cannabinoid 1 receptor density or change in levels of AEA or 2-AG. These data are the first to show alterations in cannabinoid receptor function in the rostral anterior cingulate cortex in response to a model of neuropathic pain.

Keywords

neuropathic pain; cannabinoids; rostral anterior cingulate cortex; WIN 55; 212-2

1. Introduction

Neuropathic pain is often difficult to treat, and while a number of potential pharmacological targets have been identified, many exhibit limited effectiveness and/or undesirable side effects (O'Connor, 2009). The endogenous cannabinoid system has been implicated in both acute and chronic pain (Cravatt and Lichtman, 2004) and administration of cannabinoids is effective in animal models of neuropathic pain (Herzberg et al., 1997; Bridges et al., 2001). In human clinical trials, cannabinoids have been shown to be effective in treating neuropathic pain conditions, and in some instances rival the analgesic efficacy of morphine (Ashton and Milligan, 2008)

The endogenous cannabinoid system is comprised of two known G-protein coupled receptors, CB₁ and CB₂, and endogenous lipid based ligands of which anandamide (AEA)

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and arachidonoylglycerol (2-AG) are best defined (Pertwee and Ross, 2002). CB₁ receptors are expressed in brain areas involved in nociceptive transmission and processing including the periaqueductal gray (PAG), anterior cingulate cortex (ACC), and thalamus in addition to the dorsal horn of the spinal cord and dorsal root ganglion (Farquhar-Smith et al., 2000; Glass et al., 1997; Herkenham, 1991; Herkenham et al., 1991; Hohmann and Herkenham, 1999). The ACC is part of the medial pain pathway and has been shown to be involved in the affective component of pain processing (Treede et al., 1999). Changes in regional cerebral blood flow (rCBF) in neuropathic pain patients with spontaneous pain have been shown to occur within this area (Hsieh et al., 1995). Conversely, no change in rCBF was observed in the somatosensory cortices I or II, which are part of the lateral pain pathway, in neuropathic pain patients with spontaneous pain (Di Piero et al., 1991; Hsieh et al., 1995), suggesting that spontaneous pain is derived from brain areas known to be involved in the affective component of pain. Consistent with this finding, studies have shown that the ACC modulates the aversive aspect of nociception in a rodent nerve injury model, without affecting sensory processing (LaBuda and Fuchs, 2005). A study assessing the effects of smoked marijuana on evoked pain responses reported that while smoked marijuana was effective in reducing pain ratings of neuropathic pain patients, they were not effective in reducing levels of evoked pain (Wilsey et al., 2008), suggesting that cannabinoid mediated analgesia may be at least partially mediated within the medial pain pathway.

Previous studies have shown upregulation of CB₁ in response to neuropathic pain at the level of the spinal cord in both mice and rats (Jones et al., 2004; Walczak et al., 2006; Wang et al., 2007), but little is known about the role of brain cannabinoid receptors in neuropathic pain. Based on reports that the ACC is involved in affective components of neuropathic pain and the high level of CB₁ receptors throughout this region (Sim-Selley et al., 2002), CB₁ receptor activity was examined in the ACC in a rat model of neuropathic pain. The ACC is an area that has a role in many types of emotional processing and regulation in addition to pain (Vogt, 2005). We selected the rostral ACC (rACC) for the present studies because previous studies have shown pain induced increases in the NR2A and NR2B subunits of the NMDA receptor and increases in phosphorylation of extracellular regulated kinases in the rostral portion of this region were associated with pain related aversion and the induction and expression of affective pain, respectively (Cao et al., 2009; Li et al., 2009). The thalamus was also examined as many medial thalamic nuclei project directly to the ACC (Hsu et al., 2000; Kung et al., 2002) and a recent study demonstrated a CB₂-mediated modulation of neuronal responses in the ventral posterior nucleus (VPL) of the thalamus in a rodent model of neuropathic pain (Jhaveri et al., 2008). Previous studies have shown that the PAG is involved in certain aspects of cannabinoid-mediated antinociception (Lichtman et al., 1996), so this region was included in the analysis. We hypothesize that neuropathic pain will induce alterations in cannabinoid receptor signaling in defined brain regions that might be related to the underlying neuropathology of this condition.

2. Results

Thermal Hyperalgesia Induced by CCI

Sciatic nerve ligation produced a significant reduction in paw withdrawal latency to thermal stimulus in the ipsilateral paw of CCI mice when compared to sham-operated controls. This effect was first observed at day 3 post-CCI surgery ($p < 0.0001$, $t = 6.938$, $df = 9$) and was also seen at day 10 ($p < 0.0001$, $t = 19.35$, $df = 19$) (Figure 1). Sciatic nerve ligation also produced a significant decrease in paw withdrawal latency in CCI mice when compared to baseline withdrawal latency at day 3 ($p < 0.0001$, $t = 11.03$, $df = 15$) and day 10 ($p < 0.0001$, $t = 25.46$, $df = 22$) post-surgery (Figure 1). Since thermal hyperalgesia was greatest at day 10-post CCI, that was the time point for which all in vitro experiments were conducted unless otherwise noted.

Cannabinoid Stimulated [^{35}S] GTP γ S Binding

WIN 55, 212-2-stimulated [^{35}S] GTP γ S binding was conducted on membranes prepared from the rACC, thalamus and PAG of CCI and sham operated mice at day 10 post surgery. WIN 55, 212-2 (10^{-5} – 10^{-8} M) produced a concentration dependent increase in [^{35}S]GTP γ S binding in both CCI and sham groups in all three brain areas (Figure 2a–c). Comparison of the E_{max} values revealed that WIN 55, 212-2-stimulated [^{35}S]GTP γ S binding was significantly decreased by 20% in the rACC of CCI mice when compared to sham mice ($p < 0.01$, $t = 3.211$, $df = 14$) (Figure 2a). In contrast, there were no significant differences in E_{max} values between sham and CCI mice in the PAG ($p = 0.38$, $t = 0.9365$, $df = 9$) (Figure 2b) or thalamus ($p = 0.69$, $t = 0.4207$, $df = 8$) (Figure 2c) and no statistically significant differences in EC_{50} values in any brain areas examined (Table 1).

To further investigate the decrease in E_{max} values in the rACC, a time-course analysis was conducted by assessing WIN 55,212-2 stimulated [^{35}S]GTP γ S binding at days 1 and 3 post-surgery. Time-course analysis showed that there were no statistically significant differences in the E_{max} or EC_{50} values for WIN55,212-2 to stimulate [^{35}S]GTP γ S binding at day 1 ($p = 0.1$, $t = 1.349$, $df = 10$) or day 3 ($p = 0.23$, $t = 0.8364$, $df = 4$) post surgery between CCI and sham mice (Figure 3). There were no differences in basal levels of [^{35}S]GTP γ S binding at day 1 or day 3 post-surgery (data not shown).

Recent reports suggest that CB $_2$ receptors are present in brain (Brusco et al., 2008; Morgan et al., 2009; Van Sickle et al., 2005). However, JWH-133, a CB $_2$ -selective agonist, did not stimulate [^{35}S]GTP γ S binding above basal in the rACC, thalamus, or PAG (data not shown). It is also possible that decreases in WIN 55,212-2 stimulated [^{35}S]GTP γ S binding reflect changes in basal G-protein activity. There were no statistically significant differences in basal levels of [^{35}S]GTP γ S binding in any of the brain areas examined at day 10 post-surgery (Table 1) which indicates that CCI induced desensitization in WIN 55,212-2-stimulated [^{35}S] GTP γ S binding in the rACC are not due to an overall decrease in GPCR-mediated G-protein activation.

[^3H] SR141716A Receptor Binding

In order to determine whether the decrease in WIN 55, 212-2 stimulated [^{35}S] GTP γ S binding in the rACC was due to a change in the levels of the CB $_1$ receptors, receptor binding experiments were conducted using the CB $_1$ antagonist [^3H]SR141716A. [^3H] SR141716A binding was conducted on membranes prepared from rACC at day 10 post CCI surgery, the time point at which decreased WIN55,212-2-stimulated [^{35}S]GTP γ S binding was observed. There were no significant differences in [^3H]SR141716A B_{max} ($p = 0.33$, $t = 1.044$, $df = 8$) or K_D ($p = 0.13$, $t = 1.330$, $df = 4$) values between CCI and sham mice (Figure 4) suggesting that the decrease in [^{35}S]GTP γ S binding in the rACC of CCI mice was due to desensitization of the CB $_1$ receptor and not a decrease in the number of receptors.

LC/MS Analysis

LC/MS analysis was used to test the hypothesis that the apparent desensitization of CB $_1$ receptors was due to an increase in the endogenous cannabinoids AEA and 2-AG following CCI surgery. There were no significant differences between CCI and sham mice in endocannabinoids levels measured in the rACC at either day 1 ($p = 0.85$, $t = 0.1925$, $df = 11$) for AEA, $p = 0.60$, $t = 0.5401$, $df = 11$ for 2-AG) or day 10 ($p = 0.48$, $t = 0.7418$, $df = 10$ for AEA, $p = 0.77$, $t = 0.2964$, $df = 10$ for 2-AG) post surgery (Figure 5). This suggests that the CCI induced desensitization of the CB $_1$ receptor in the rACC was not directly due to an increase in the endocannabinoids AEA or 2-AG.

3. Discussion

The results of this study revealed that cannabinoid receptor-mediated G-protein activity in the rACC is decreased after 10 days of CCI. To our knowledge, this is the first study to identify altered cannabinoid receptor activity in the cortex in response to a model of neuropathic pain. Previous studies in CB₁ receptor knockout mice have shown that CB₁ receptors are not required to produce the neuropathic state (Castane et al., 2006), but the current results suggest that reduced CB₁ receptor activity in the rACC might contribute to the negative affect associated with this condition. This possibility is supported by the observation that administration of cannabinoid receptor agonists alleviates pain and discomfort in humans with neuropathic pain (Blake et al., 2006; Nurmikko et al., 2007; Rog et al., 2007). The finding that cannabinoid receptor activity is reduced only in the rACC of the regions examined is particularly interesting, because this region appears to be associated with affective, rather than sensory components of nociception (Kulkarni et al., 2005; Kuo et al., 2009; LaBuda and Fuchs, 2005; Treede et al., 1999).

Studies were also conducted to examine potential mechanisms that might underlie the reduction of CB receptor-mediated G-protein activity after CCI. It is well known that cannabinoid receptor-mediated G-protein activity is reduced by chronic administration of cannabinoid receptor agonists and that apparent desensitization often occurs concomitantly with receptor downregulation (Sim-Selley, 2003). [³H]SR141716A binding was used to measure CB₁ receptor levels in the rACC of CCI and sham rats, but no significant difference was found between the experimental groups. It is interesting that upregulation of CB₁ receptors has been described in the spinal cord and DRG following CCI (Lim et al., 2003; Wang et al., 2007), which differs from our results in brain. CB₁ receptor mRNA has also been reported to increase in the thalamus (Siegling et al., 2001) but changes in mRNA might not be translated into changes in protein or function. These findings suggest that there are regional differences in CB₁ receptor adaptation and are consistent with previous studies showing region-specific adaptation in response to repeated cannabinoid treatment (Sim-Selley, 2003). Considering that prolonged exposure to cannabinoids can decrease CB₁-stimulated [³⁵S] GTPγS binding (Sim-Selley, 2003) and endocannabinoids were shown to be upregulated in supraspinal areas following CCI in the rat (Petrosino et al., 2007), it is possible that increased levels of AEA or 2-AG in the rACC following CCI could explain the decrease in WIN 55, 212-2 stimulated [³⁵S]GTPγS binding. However, LC/MS studies did not show any significant differences in levels of 2-AG or AEA in the ACC between CCI and sham operated mice. This result is consistent with a previous report showing that the levels of AEA and 2-AG were unchanged in the thalamus after spinal nerve ligation (Jhaveri et al., 2008). It is possible that changes in endocannabinoid levels were not detected because their synthesis and degradation are concurrently regulated, so that turnover is increased without altering net endocannabinoid levels. AEA and 2-AG are not stored in intracellular vesicles like many classical neurotransmitters but rather are synthesized “on demand” in response to specific stimuli as demonstrated in several different tissues (Panikashvili et al., 2001); (Basavarajappa et al., 2000); (Stella and Piomelli, 2001). Additionally, both AEA and 2-AG are rapidly internalized and degraded by the cell following release (Sugiura et al., 2002; Sugiura and Waku, 2002), which could counterbalance increased synthesis. Also, there are other endocannabinoids such as N-AEs—N-homo-c-linolenylethanolamine (HEA) and N-docosatetraenylethanolamine (DEA) which are found in brain tissue and have been shown to have nanomolar affinity for the CB₁ receptor (Hanus et al., 1993). Therefore, it could be that the apparent desensitization of the CB₁ receptor in the rACC of CCI mice was mediated by an increase in other endocannabinoids that were not examined.

The attenuation of cannabinoid receptor-mediated G-protein activity identified in the present study did not appear to be due to direct changes in the cannabinoid system (e.g. receptor

downregulation or increased endocannabinoid levels). However, the apparent desensitization of cannabinoid receptors in the rACC could be due to adaptations in other receptor systems. For example, development of persistent pain induced by nerve injury, but not acute pain, requires the presence of PKC γ (Malmberg et al., 1997) and PKC has been shown to disrupt the activity of CB₁ receptors via receptor phosphorylation (Garcia et al., 1998). Perhaps relevant to the current finding is the report that phosphorylation of mGluR1 which, like CB₁, is a G-protein coupled receptor, is increased in the ACC following peripheral nerve injury (Xu et al., 2008).

Taken together, these findings suggest that neuropathic pain can alter receptor signaling, at the level of the rACC, and the apparent desensitization of cannabinoid receptor-mediated G-protein activity in the present study might result from such changes. It is possible that reduced cannabinoid receptor activity in the rACC is associated with negative affective component of neuropathic pain and that this effect is attenuated by administration of cannabinoid receptor agonists.

4. Experimental Procedure

Animals

Male Swiss Webster mice (Harlan Laboratories, Indianapolis, IN) weighing 25–30g were housed 6 per cage in animal care quarters on a 12h light-dark cycle. Food and water were available *ad libitum*. Protocols and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University Medical Center and were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals.

CCI of the Sciatic Nerve

Mice were anesthetized under 2.5% isoflurane before shaving the lower back and right thigh. The shaved area was then cleansed with 2% povidine iodine and rinsed with 70% ethyl alcohol. A linear skin incision was made along the lateral surface of the biceps femoris and forceps were inserted into the muscle belly to split the muscle fibers and expose the sciatic nerve. The tips of the forceps were passed gently under the sciatic nerve and lifted to pass two 5-0 chromic gut sutures under the nerve, 1mm apart. The suture was then tied loosely around the nerve and knotted twice to prevent slippage. The incision was cleansed and the skin was closed with 2–3 ligatures of 5-0 dermalon. The mice recovered on a warmed surface and were checked routinely for 72 hours. A separate control group of sham-operated mice underwent the exact same surgical procedure with the exception of the ligation of the sciatic nerve.

Thermal Hyperalgesia Testing

Thermal hyperalgesia was assessed using a radiant heat source under a plexi-glass surface applied to the plantar surface of each hindpaw (Hargreaves et al., 1988). For two days prior to CCI surgery, mice were placed on the plexi-glass floor of the apparatus (Plantar Test, Ugo Basile, Comerio, Italy) and covered with an inverted clear plastic tube in order to familiarize the mice with the test. On the day prior to surgery, baseline measures of paw withdrawal latency were gathered. Mice were allowed to acclimate on the Plantar Test apparatus for approximately 20 minutes before the start of the radiant heat test. Five measures of paw-withdrawal latency were conducted with at least five minutes between each test with the average latency used for statistical purposes. Mice were again tested for paw-withdrawal latency at either, 1, 3, or 10 days post CCI or sham surgery. Paw-withdrawal latencies were expressed as relative values (%) to baseline latencies for each animal, as well as group means $\pm$ S.E.M. Data were analyzed via Student's t-test with differences considered

statistically significant at $p < 0.05$. Any CCI mouse that failed to demonstrate statistically significant withdrawal latency difference from baseline at day 3 or 10 post-surgery was excluded from further analysis.

Brain Dissection

On day 1, 3, or 10 post-surgery and following thermal hyperalgesia testing, mice were euthanized via cervical dislocation. Brains were removed and immediately placed on a glass plate on ice for dissection. The rACC was dissected from a 1.5 mm coronal section taken by first removing tissue at the rostral extent of the brain anterior to Bregma +2.5 then making a second cut at Bregma +1 using the middle cerebral artery as a landmark. Cingulate cortex was removed from the dorsal medial region of the resulting coronal section using the corpus callosum on the posterior surface as a landmark and a mouse brain atlas (Paxinos, 2001) as a guide. The resulting sample contained primarily Cg1 and Cg2, and likely included immediately adjacent portions of prelimbic and secondary motor cortices. Thalamus was collected from a section that extended from approximately Bregma -0.3 to Bregma -2.7 using the rostral extent of the stria medullaris as an anterior landmark and the superior colliculi as a posterior landmark. Cortical and hippocampal tissue was removed dorsal and lateral to the thalamus. Tissue ventral to the thalamus was removed by cutting at the midpoint of the section, dorsal to the third ventricle. The sample included medial and lateral nuclei throughout the rostral-caudal extent of the thalamus as well as habenula. The PAG was dissected from Bregma -3 to Bregma -5. This section was collected using the superior and inferior colliculi as anterior and posterior landmarks, respectively. Cortex and hippocampus were discarded and then the colliculi were removed. Tissue ventral to the PAG was removed at the midpoint of the section. The sample included PAG throughout its rostral-caudal extent, as well as surrounding reticulum. All tissue was immediately frozen in liquid nitrogen and stored at -80°C until further processing.

Cannabinoid Stimulated [^{35}S] GTP γ S Binding

On the day of the assay, tissue from individual mice was thawed and sonicated in 5 ml of buffer (50 mM Tris, 3 mM MgCl_2 , 1 mM EGTA, 100 mM NaCl, pH 7.7). The homogenate was centrifuged at $50,000 \times g$ at 4°C for 10 min and the resulting pellet was resuspended in 3–5 ml of membrane buffer and resonicated. Protein levels were determined by the Bradford assay (1976) using BSA as a standard. Membranes were then incubated with 4 mU/ml adenosine deaminase for 35 min at 30°C . Membranes (8–10 μg) were incubated in assay buffer containing 30 μM GDP, 0.1 nM [^{35}S]GTP γ S (PerkinElmer Life and Analytical Sciences, Boston, MA), and varying concentrations of the cannabinoid receptor agonist WIN 55, 212-2 or the CB2 receptor selective agonist JWH-133. Non-specific binding was assessed via the addition of 20 μM unlabeled GTP γ S and basal levels of binding were assessed via the omission of agonist. Membranes were incubated for 2 hr at 30°C with gentle agitation. The incubation was terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters, followed by three washes with ice-cold 50 mM Tris, pH 7.2. Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency for ^{35}S after extraction of the filters in 4 ml Budget Solve scintillation fluid and a 45 min shake cycle. Assays were conducted in triplicate.

[^3H] SR141716A Binding

Brain membranes from individual mice (10 μg) were diluted with buffer and prepared under the same conditions as for the [^{35}S]GTP γ S binding assays. Saturation binding analyses were performed by incubating 10 μg of membrane protein with 0.1–2.5 nM [^3H]SR141716A in the presence and absence of 1 μM unlabeled SR141716A to determine nonspecific and total binding, respectively. [^3H]SR141716A (GE Healthcare, Piscataway, NJ) was diluted in buffer (50 mM Tris, 3 mM MgCl_2 , 1 mM EGTA, 0.125% (wt/vol) BSA, pH 7.7) before

addition to the assay. Assays were conducted in duplicate and incubated for 1.5 h at 30°C. Reactions were terminated by rapid filtration under vacuum through Whatman GF/B glass-fiber filters that had been soaked in 50 mM Tris buffer, pH 7.4, containing 0.5% (wt/vol) BSA, followed by three washes with the Tris buffer. Bound radioactivity was determined by liquid scintillation spectrophotometry after extraction of the filters in 4ml Budget Solve scintillation fluid and a 45 min shake cycle. Assays were conducted in triplicate.

Liquid Chromatography-Mass Spectrometry Analysis (LC/MS)

At day 1 or 10 post surgery, the PAG and ACC were extracted from the brain from each mouse, weighed and flash frozen in dry ice and stored at -80°C until the time of processing. On the day of processing, tissues were weighed and homogenized with 1.4 ml chloroform:methanol (2:1 v/v containing 0.0348g PMFS/ml) after the addition of internal standards to each sample (2 pmol AEA -d8 and 1 nmol 2-AG-d8). Homogenates were then mixed with 0.3 ml of 0.73% w/v NaCl, vortexed and then centrifuged for 10 min at 4,000 rpm at 4°C. The aqueous phase plus debris were collected and extracted again twice with 0.8 ml chloroform. The organic phases from the three extractions were pooled and the organic solvents were evaporated under nitrogen gas. Dried samples were reconstituted with 0.1 ml chloroform and mixed with 1 ml cold acetone. The mixtures were then centrifuged for 5 min at 3000 rpm and 4 °C to precipitate the proteins. The upper layer of each sample was collected and evaporated under nitrogen. Dried samples were reconstituted with 0.1 ml methanol and placed in autosample vials for analysis.

LC/MS/MS was used to quantify AEA and 2-AG. The mobile phase consisted of (10:90) water: methanol with 0.1% ammonium acetate and 0.1% formic acid. The column used was a Discovery ® HS C18, 4.6* 15 cm, 3 micron (Supelco, USA). The mass spectrometer was run in Electrospray Ionization in positive mode. Ions were analyzed in multiple reaction monitoring mode and the following transitions were monitored: (348>62) and (348>91) for AEA ; (356>62) for AEA-d8; (379>287) and (279>269) for 2-AG ; (387>96) for 2AG-d8. A calibration curve was constructed for each assay based on linear regression using the peak area ratios of the calibrators. The extracted standard curves ranged from 0.03 pmol to 40 pmol for AEA and from 0.05nmol to 64 nmol for 2-AG. The recovery of 1 mol 2-AG-d8 is 85% and 2 pmol AEA-d8 is 73%.

Drugs and Chemicals

The non-selective cannabinoid receptor agonists WIN 55, 212-2 (Sigma-Aldrich, USA) which is CB1 and CB2 selective and JWH-133 (provided by Dr. John W. Huffman, Clemson University) which has an approximate 200-fold affinity for the CB2 receptor were utilized for the [³⁵S]GTPγS binding experiments. The CB1 selective antagonist SR141716A (Drug Supply Program of NIDA) was used for the ³H receptor binding experiment. For LC/MS experiments arachidonoyl ethanolamide-d8 (AEA-8) and 2-Arachidonoyl glycerol-d8 (2-AG-d8) (Cayman Chemical, Ann Arbor, MI) were utilized. Each contains eight deuterium atoms at the 5, 6, 8, 9, 11, 12, 14, and 15 positions of the arachidonic acid moiety.

Data Analysis

Paw-withdrawal latencies were expressed as relative values (%) to baseline latencies for each animal, as well as group means ± S.E.M. Behavioral data was analyzed via Student's t-test with differences considered statistically significant at $p < 0.05$. For [³⁵S]GTPγS binding, basal binding is defined as specific [³⁵S]GTPγS binding in the absence of agonist. Net-stimulated [³⁵S]GTPγS binding is defined as specific [³⁵S]GTPγS binding in the presence of agonist minus basal binding. The percentage of stimulation is expressed as (net-stimulated [³⁵S]GTPγS binding/basal) × 100%. $E_{\max}$ and EC_{50} values were calculated from nonlinear regression analysis by iterative fitting of the concentration-effect curves to the

Langmuir equation [$E = E_{\max}/(EC_{50} + \text{agonist concentration}) \times \text{agonist concentration}$] using Prism 4.0 software (GraphPad Software Inc., San Diego, CA). For [^3H] SR141716A binding, specific binding was defined as non-specific binding subtracted from total binding. $B_{\max}$ and K_D values were calculated by iterative fitting of the saturation curves to the Langmuir equation [$B = B_{\max}/(K_D + \text{ligand concentration}) \times \text{ligand concentration}$] using Prism 4.0 software (GraphPad Software Inc.). $E_{\max}$ and EC_{50} and $B_{\max}$ and K_D values were then analyzed via Student's t-test for statistical significance between CCI and sham mice. For LC/MS experiments the amounts of AEA and 2-AG in each sample was calculated by linear regression using peak area ratios. The amount obtained for each sample, was per the weight of that sample which was then converted to per gram tissue. The values were then analyzed via Student's t-test for statistical significance between CCI and sham mice.

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Figure 1.

Sciatic nerve ligation produced a significant reduction in paw withdrawal latency to thermal stimulus in the ipsilateral paw of CCI mice when compared to sham control (*** $p < 0.0001$) and baseline (‡ $p < 0.0001$) measures. This effect was observed at Day 3 post-CCI surgery and Day 10.

Figure 2.

WIN 55, 212-2 stimulated stimulated [^{35}S] GTP γ S binding in the rACC shows a significant decrease in E_{max} values in CCI mice at day 10 post surgery when compared to sham operated controls ($n = 8$). There were no significant differences in WIN 55, 212-2 stimulated [^{35}S] GTP γ S binding in the PAG (panel b) ($n = 6$) or thalamus (panel c) ($n = 6$). Data are expressed as percent of net stimulated binding above basal binding.

Figure 3.

Time course evaluation showed that the $E_{\max}$ value of WIN 55, 212-2 was significantly decreased in the rACC of CCI relative to sham mice only on day 10 ($n = 8$, $**p < 0.01$) and was not significantly different at day 1 ($n = 6$) or day 3 ($n = 5$) post surgery. There were no significant differences in EC_{50} values at any time point examined. $E_{\max}$ and EC_{50} values are expressed as difference between the group means of CCI and sham mice.

Figure 4. $[^3\text{H}]$ SR 141716A receptor binding was conducted on the rACC at day 10 post CCI surgery. There were no significant differences in CB1 receptor binding between CCI ($B_{\text{max}} = 2.43$ pmol/mg $\pm$ 0.492, $K_D = 1.21$ nM $\pm$ 0.565) and sham mice ($B_{\text{max}} = 1.88$ pmol/mg $\pm$ 0.172, $K_D = 0.601$ nM $\pm$ 0.179) in the rACC. Data are expressed as group means $\pm$ S.E.M. (n = 5).

Figure 5.

CCI did not alter levels of the endocannabinoids AEA or 2-AG in the rACC at day 1 (n = 6) or day 10 (n = 6) post-surgery. Data are expressed as group means $\pm$ SEM.

Table 1

$E_{\max}$, EC_{50} and basal binding values from WIN 55, 212-2 stimulated [35 S] GTP γ S binding in brain areas of the mouse at day 10 post-surgery.

Region	Sham	CCI
rACC		
$E_{\max}$ (% stimulation)	132.4 $\pm$ 6.94	106.3 $\pm$ 4025 **
EC_{50} (μ M)	0.130 $\pm$ 0.022	0.193 $\pm$ 0.038
Basal (fmol/mg)	120.2 $\pm$ 6.712	129.0 $\pm$ 8.294
Thalamus		
$E_{\max}$ (% stimulation)	98.40 $\pm$ 5.19	102.8 $\pm$ 9.02
EC_{50} (μ M)	0.170 $\pm$ 0.014	0.215 $\pm$ 0.088
Basal (fmol/mg)	153.8 $\pm$ 11.34	121.5 $\pm$ 10.82
PAG		
$E_{\max}$ (% stimulation)	99.66 $\pm$ 0.024	106.6 $\pm$ 6.53
EC_{50} (μ M)	0.192 $\pm$ 0.024	0.227 $\pm$ 0.031
Basal (fmol/mg)	170.7 $\pm$ 3.925	156.6 $\pm$ 6.964

The $E_{\max}$ value of WIN 55, 212-2 was significantly decreased in the rACC of CCI relative to sham mice only on day 10 (**p < 0.01). Data are expressed as group means $\pm$ S.E.M of five to eight mice per group.