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SPINAL CANNABINOID CB2 RECEPTORS AS A TARGET FOR NEUROPATHIC PAIN: AN INVESTIGATION USING CHRONIC CONSTRICTION INJURY

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Abstract—Agonists for the cannabinoid CB2 receptor are antinociceptive in several rodent models and several reports have suggested that the target for these drugs is CB2 expressed in the spinal cord pain pathway. After confirming the efficacy of a systemically delivered CB2-selective agonist, GW405833, we tested this hypothesis by administering the CB2 agonists GW405833 and JWH-133, via intrathecal cannulation, to the lumbar spinal cord of rats that had undergone chronic constriction injury to induce mechanical allodynia. We found that although the non-selective CB1/CB2 cannabinoid receptor agonist WIN55,212-2 reversed mechanical allodynia in both ipsilateral and contralateral hind paws, neither GW405833 nor JWH-133 reversed mechanical allodynia. In addition, we investigated the expression of CB2 receptors in the neuropathic spinal cord using immunohistochemistry, Western blot and CB2 agonist stimulated [^{35}S]GTPγS binding. Although protein-based analysis of CB2 partially matched the results of earlier studies using the same antibody, we found evidence that this antibody may be insufficiently specific for the detection of CB2 in native tissue. Using [^{35}S]GTPγS binding assays, we found no evidence of functional CB2 in the spinal cord, in sham or surgery-treated tissue. However, WIN55,212-2 stimulated [^{35}S]GTPγS binding showed clear evidence of functional CB1 receptors consistent with the known distribution of elements of the pain pathway, and we concluded that spinal CB2 receptors are not a likely target for cannabinoid-mediated antinociception in this model. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: cannabinoid, CB2, chronic constriction injury, GW405833, neuropathic pain, spinal cord.

Drugs that activate the cannabinoid CB2 receptor have been reported to be antinociceptive in a variety of rodent models and may have potential in the treatment of neuropathic pain (Ashton and Milligan, 2008; Brownjohn and Ashton, 2009). Local or systemic delivery of CB2 receptor agonists reverses hyperalgesia in models of both postoperative pain (Romero-Sandoval and Eisenach, 2007) and neuropathic pain (Elmes et al., 2004; Leichsenring et al., 2009). Delivery of CB2 agonists has been reported to reduce chronic constriction injury (CCI)-induced sensitivity to light mechanical touch (allodynia) (Yao et al., 2008b; Hu et al., 2009; Yao et al., 2009) and reverse allodynia caused by tight ligation of spinal nerves (SNL) (Ibrahim et al., 2003; Elmes et al., 2004; Beltramo et al., 2006; Leichsenring et al., 2009), spinal nerve transaction (Romero-Sandoval et al., 2008), brachial plexus avulsion (Paszcuk et al., 2011), partial sciatic nerve ligation (PSL) (Valenzano et al., 2005; Whiteside et al., 2005; Yamamoto et al., 2008), and cancer (Curto-Reyes et al., 2010; Gu et al., 2011).

The studies that have employed intrathecal delivery of CB2 agonists in rodent models of neuropathic pain have suggested a spinal site of action for CB2-mediated antinociception (Romero-Sandoval et al., 2008; Yamamoto et al., 2008; Curto-Reyes et al., 2010; Gu et al., 2011; Hsieh et al., 2011; Paszcuk et al., 2011). In this study, we sought to further test this hypothesis using the CCI model in rats, which reliably induces allodynia but with considerably less neuronal degeneration than that caused by the SNL and PSL models. We addressed three questions: (i) does intrathecal delivery of CB2 agonists reverse allodynia in CCI? (ii) are previous reports of CB2 protein expression in the spinal cord in neuropathic pain reliable given the recent controversy over the inconsistency of antibodies raised against CB2 (Atwood and Mackie, 2010; Ashton, 2011, in press)? (iii) is functional CB2 protein expressed in the neuropathic spinal cord, as assessed by a ligand stimulated G protein-coupled receptor (GPCR) assay?

We initially aimed to confirm whether GW405833 is able to reduce mechanical allodynia in the CCI model when delivered systemically, as has previously been reported in both this model of neuropathic pain (Hu et al., 2009) and others (LaBuda et al., 2005; Whiteside et al., 2005; Beltramo et al., 2006). Then, with respect to each of these questions, first we aimed to test whether spinal delivery of GW405833 and the more potent and CB2 selective JWH-133 reverse allodynia in rats that have undergone CCI. The intrathecal delivery of GW405833 in the CCI model is not known, and similarly, although intrathecal delivery of the CB2 agonist JWH-133 has been found to reverse allodynia in the SNL model in mice (Yamamoto et al., 2008), it is not known whether JWH-133 also reduces allodynia in the CCI model. Second, the expression and cellular localization of CB2 with respect to the CNS is uncertain (Atwood and Mackie, 2010). CB2 receptors in the periphery (Anand et al., 2008), spinal cord (Beltramo et al., 2006; Romero-Sandoval et al., 2008), and the thala-

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Abbreviations: BSA, bovine serum albumin; CCI, chronic constriction injury; CHO, Irradiated Chinese hamster ovary; EDTA, Ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; GFAP, glial fibrillary acidic protein; PBS, phosphate buffered saline; PFA, paraformaldehyde; PSL, partial sciatic nerve ligation; SNL, ligation of spinal nerves.

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mus (Jhaveri et al., 2008) have all been implicated as targets. Current understanding of CB2 protein expression in the neuropathic spinal cord has been derived from immunolabeling, in particular, in studies by Wotherspoon et al. (2005) using immunohistochemistry and by Walczak et al. (2005) using Western blot, who employed an antibody (Cayman Chemical Company, MI, USA) that had previously been used to describe CB2 immunolabeling in naïve brain tissue (Gong et al., 2006). Therefore, we tested for CB2 immunolabeling in the lumbar spinal cords of both sham surgery and CCI rats using immunohistochemistry and Western blot and reassessed the antibody employed in the earlier studies (Walczak et al., 2005; Wotherspoon et al., 2005; Gong et al., 2006) for sensitivity and specificity of CB2 detection in spinal tissue. Third, we tested for functional CB2 expression in the neuropathic spinal cord using a ligand-stimulated [^{35}S]GTPγS binding assay, using both spinal cord homogenates and autoradiography for whole spinal cord sections.

EXPERIMENTAL PROCEDURES

Experimental design

We induced neuropathic pain in rats using the CCI model and tested the effects of intrathecal delivery of the CB1/CB2 nonselective agonist WIN55,212-2, and the CB2 selective agonists GW405833 and JWH-133, on mechanical allodynia following surgery. In addition, lumbar spinal tissue was harvested from CCI and sham-treated animals to examine CB2 receptor protein expression and functionality. Furthermore, using CB2 overexpressing and CB2-negative cells and tissue from wild-type CB2 receptor knockout mice, we tested the specificity of the antibody used in this study for CB2 receptor protein.

Animals

All procedures were approved by the University of Otago Animal Ethics Committee. Rats used in this study were male Wistars (200–350 g), which were kept in a 12- h light/dark cycle with *ad libitum* access to food and water. All protocols and experimental designs were chosen to minimize animal numbers and optimise animal welfare.

For validation of the N-terminus CB2 antibody used in this study, we obtained lumbar spinal tissue from male N-terminus CB2 knockout mice (the Jackson Laboratory, ME, USA) and wild-type mice of the same C57BL/6 strain. This tissue was generously gifted to us by Professor Ken Mackie, of the Gill Centre and Department of Psychological and Brain Sciences at Indiana University.

Chronic constriction injury surgery

CCI was performed using a previously described method (Bennett and Xie, 1988). Briefly, rats were anesthetized and maintained under 2.5% halothane, before a small incision was made along the mid thigh level of one hind leg. The underlying sciatic nerve was exposed by blunt dissection of biceps femoris. Four sutures of chromic gut (4/0, 2 metric) were loosely tied around the exposed nerve at 2-mm intervals, before the wound was closed in layers. In sham-operated animals, the sciatic nerve was exposed but not ligated. For acute postsurgical pain relief, buprenorphine (0.1 mg/kg, s.c.) was administered perioperatively, and 12 and 24 hours postoperatively.

Drugs

WIN55,212-2 ((*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate) was obtained from Sigma-Aldrich (MO, USA). GW405833 (1-(2,3-dichlorobenzoyl)-5-methoxy-2methyl-3-[2-(4-morpholinyl)ethyl]-1H-indole) and JWH-133 ((6aR,10aR)-3-(1,1-Dimethylbutyl)-6,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran) were obtained from Tocris Bioscience (UK). For systemic delivery, GW405833 was dissolved in 25% hydroxypropyl-β-cyclodextrin in 0.9% saline following acidification and rebalancing of the formulation back to a physiological pH range (pH 5.5–7.5), and administered i.p. in dosing volumes of 1 ml/kg for 1 mg/kg, and 4 ml/kg for 10 and 30 mg/kg. For intrathecal injection, all drugs were dissolved in a 1:1:1:17 solution of dimethyl sulphoxide:Cremophor:Ethanol:0.9% saline, and injected in volumes of 15–20 μl.

Drug administration

Acute lumbar punctures were performed as previously described (Milligan et al., 2005). Briefly, guide cannulae were made by removing the plastic hub from sterile 18-gauge spinal needles and catheters constructed of sterile polyethylene (PE-10) tubing, marked 7.7–7.8 cm from one end and attached at the other to a sterile Hamilton syringe via a 30-gauge needle. Ten days following CCI surgery, animals were anesthetized under 2.5% halothane, before shaving and cleaning of the dorsal lumbar region. A guide cannula was inserted through the L5/L6 inter-vertebral space to the intrathecal space. A catheter was then fed through the guide cannula to the L4–L6 lumbar spinal cord, using the 7.7–7.8-cm mark on the catheter as a guide. Drug or vehicle were administered at 1 μl/s. Catheter and cannula were then removed, and animals allowed to recover from anesthesia.

Behavioral testing

Mechanical allodynia was assessed using a Von Frey hair paradigm as previously described (Milligan et al., 2000). Behavioral testing was undertaken immediately before surgery (baseline), 3 and 10 days post surgery, and 30 min, 1, 3, and 5-hours post drug or vehicle administration. Animals were placed in a wire cylinder on an elevated wire mesh flooring (1.5-mm bars, 14-mm spacing) in a darkened room and allowed to habituate for approximately 30 min before testing. A logarithmically graded series of calibrated monofilaments (North Coast Medical, CA, USA) were used to determine paw withdrawal thresholds. The hairs presented had $\log_{10}(10{,}000\times g)$ intensities ranging from 3.61 (0.407 g) to 5.18 (15.136 g).

Using a modified form of the up/down testing procedure (Dixon, 1980), filaments were randomly applied alternately to the left and right ventral mid plantar aspects of the hind paws at a perpendicular angle for 8 s. Withdrawal rates to each tested stimulus were recorded. A withdrawal was considered as a paw flinch upon presentation, or sustained application, of a given monofilament, distinct from normal ambulatory movement. Behavioral testing was performed by an observer blind to drug treatment.

Data, in the form of the response rate observed for each tested stimulus, was fitted with a Gaussian integral psychometric function, using a maximum likelihood fitting method. This method of transformation produces an estimation of 50% paw withdrawal threshold, and data that is suitable for parametric analysis (Milligan et al., 2000). PsychoFit, a computer program created by Professor Lewis O. Harvey Jr at the University of Colorado, was used for this data transformation.

Cell culture

Irradiated Chinese hamster ovary (CHO) cells, overexpressing the rat CB2 receptor (EZCells™, Applied Cell Sciences Inc., MD,

USA) were grown in Ham's F12, supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids and 400 μg/ml G418, in 95% O_2, 5% CO_2 at 37 °C. Cells were harvested for immunocytochemistry and Western blot.

For a negative control, human embryonic kidney 293 (HEK 293) cells (American Type Tissue Collection, VA, USA) were grown on coverslips in Dulbecco's modified Eagle medium, supplemented with 10% FBS, 2 mM L-glutamine, 0.25 μg/ml amphotericin B, 100 U/ml penicillin, and 100 μg/ml streptomycin in 95% O_2, 5% CO_2 at 37 °C.

Immunohistochemistry

Immunocytochemistry. Cells were fixed for 10 min in 4% paraformaldehyde (PFA), blocked with 1% normal donkey serum and 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS), and incubated overnight (18–24 h) with a rabbit-derived polyclonal antibody raised against amino acids 20–33 of the N-terminus of the human CB2 receptor (#101550, Cayman Chemical Company), at a concentration of 1:200 in antibody diluent (0.1% BSA in PBS). Cells were washed in PBS containing 0.1% Triton X-100 (PBS-T) and incubated for 1 h with donkey anti-rabbit Alexa-Fluor 488 IgG (Invitrogen, CA, USA) at a concentration of 1:1000 in antibody diluent. Nuclei were labeled with Hoechst 33342 (Sigma, MO, USA). Adjacent sections were incubated with the CB2 antibody preadsorbed for 1 h with five times excess corresponding immunizing peptide (#301550, Cayman Chemical Company).

Rodent tissue preparation. Ten days following surgery, rats were anesthetized briefly with CO_2, before rapid decapitation and removal of the L4–L6 lumbar spinal cord by laminectomy. For knockout studies, mice were deeply anesthetized with sodium pentobarbital and transcardially perfused with 0.9% saline and 4% PFA. The L4–L6 spinal cord was excised and cryoprotected in 30% sucrose. Rat and mouse spinal cords were embedded in optimum cutting compound (OCT) and stored at −80 °C. Twenty-μm sections were cut at −20 °C on a Leica CM1850 cryostat (Meyer Instruments Inc., TX, USA) and thaw mounted on to chrome alum gelatin coated slides.

Immunohistochemistry. Rat spinal cord sections were fixed in 4% PFA for 20 min and endogenous peroxidases quenched by incubation for 12 min in 0.3% H_2O_2 in 1:1 methanol:PBS. Non-specific binding was blocked with a 30-min incubation in 1.5% normal goat serum. Sections were incubated overnight at 4 °C with the Cayman Chemical CB2 antibody, at a concentration of 1:250 in antibody diluent, before washing in PBS-T. Using the Vectastain Elite ABC kit (Vector Laboratories Inc., CA, USA), sections were incubated first for 30 min with biotinylated goat anti-rabbit IgG, and then for 1 h with avidin biotin complex at half the concentration recommended by the manufacturer. Immunolabeling was visualized with diaminobenzidine nickel (Vector Laboratories Inc.). Adjacent sections were incubated with the CB2 antibody preadsorbed with immunizing peptide. For the detection of Iba1, sections were treated identically, except for blocking with 1.5% normal horse serum, overnight incubation with goat anti-human Iba1 (#ab5076, Abcam, MA, USA) at 1:250 in 1% normal horse serum in PBS and secondary antibody incubation with biotinylated horse anti-goat IgG (Vector Laboratories Inc.). For the detection of glial fibrillary acidic protein (GFAP), sections were blocked with 1% donkey serum and 1% BSA, incubated for 4 h at room temperature with rabbit anti-cow GFAP (Dako, Denmark) at 1:500 in antibody diluent, then incubated for 1 h with donkey anti-rabbit Alexa-Fluor 488 IgG (Invitrogen, CA, USA) at 1:1000 in antibody diluent.

For experiments that employed wild-type and CB2 knockout mouse spinal cords, sections were permiabilized with 0.2% TritonX-100 in PBS for 15 min and endogenous peroxidases quenched by incubation for 30 min in 0.3% H_2O_2 in 1:1 methanol: PBS at 37 °C. Following quenching, all procedures and reagents were identical to those used for CB2 immunolabeling in fresh frozen rat tissue, except for an increase in primary antibody concentration from 1:250 to 1:100.

Image capture and densitometry. Images were captured using a Zeiss Axioplan microscope (Carl Zeiss, Germany). CB2 labeling was assessed with densitometry. Briefly, images were converted to 8-bit gray scale, then overlaid with a stereotaxic template of the rat spinal cord (Paxinos and Watson, 1986) to outline lamina boundaries and optical density measured with NIH ImageJ®, Version 1.40g. Optical density in the superficial dorsal horn (laminae I and II) was normalized to density in adjacent white matter (fasciculus cuneatus and fasciculus gracilis) on the same spinal section, which was unchanged with treatment (data not shown). Template fitting and densitometry were performed by an observer blinded to treatment.

Western blot

Rats were decapitated 10 days post surgery following brief CO_2 anesthesia and the L4–L6 lumbar spinal cord harvested by laminectomy. The spinal cord was divided into ipsilateral and contralateral halves, before homogenization and sonication in 50 mM Tris–HCl (pH 7.4) with complete protease inhibitors (Roche Applied Science, IN, USA). Samples were centrifuged at 13,000×*g* for 10 min at 4 °C, before standardization of supernatant using the Bradford method (Bradford, 1976). Standardized samples were combined 1:1 with gel loading buffer, containing 4% sodium dodecyl sulfate, 1.43 M β-mercaptoethanol, and 2 mM ethylenediaminetetraacetic acid. Spleen from naïve animals and CHO cells over expressing the rat CB2 receptor were prepared in the same way and used as positive controls.

Samples were electrophoresed on a 7.5% Tris-Bis gel alongside a molecular weight marker (Bio-Rad, CA, USA), and transferred to a polyvinylidene fluoride membrane. Membranes were blocked for 4 h with 10% skim milk powder and 0.1% BSA, in a solution of 0.1% TritonX-100 in Tris buffered saline (TTBS), and probed for CB2 using the Cayman Chemical antibody at 1:500 in Western antibody diluent (0.1% BSA in TTBS) overnight at 4 °C. Membranes were washed in TTBS, then incubated with horse radish peroxidase (HRP) conjugated sheep anti-rabbit IgG (Chemicon, USA) at 1:1000 in TTBS for 1 h at room temperature, and bands visualized on enhanced chemiluminescence (ECL) film with Western blot ECL reagents (GE Healthcare, UK). Membranes were again washed in TTBS, before overnight incubation with mouse anti-chicken β-actin (#sc-47778, Santa Cruz Biotechnology Inc., CA, USA) at 1:5000 in Western antibody diluent at 4 °C, then incubation with HRP-conjugated chicken anti-mouse IgG (Santa Cruz Biotechnology Inc.) at 1:2000 for 1 h in TTBS at room temperature, before band visualization. Adjacent membranes were incubated with primary antibody preadsorbed with 2.5 times excess of corresponding immunizing peptide. Bands of CB2 protein were normalized using β-actin as a loading control. Western blots were run in duplicate for analysis of band density, which was performed by a blinded observer, using Quantity One® software, version 4.5 (Bio-Rad, CA, USA).

Cannabinoid stimulated [35S]GTPγS binding

Ten days post surgery, rats were decapitated under light CO_2 anesthesia and the L4–L6 lumbar spinal cord rapidly removed. Lumbar spinal cords were snap frozen on powdered dry ice for membrane binding studies, or embedded in OCT for autoradiography, and stored at −80 °C until use.

Spinal cord homogenates. Spinal cords were homogenized and sonicated in ice cold homogenization buffer (3 mM $MgCl_2$, 1 mM EGTA, 100 mM NaCl in 50 mM Tris–HCl, pH 7.4). Homoge-

nized tissue was centrifuged at 48,000×*g* for 10 min at 4 °C, and the tissue pellet re-suspended in assay buffer (3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl in 50 mM Tris–HCl, pH 7.4) with an EDTA-free protease inhibitor cocktail (Roche Applied Science). After incubation on ice for 10 min, samples were re-centrifuged at 48,000×*g*, and the pellet re-suspended in assay buffer. Protein concentration was standardized using the Bradford protocol (Bradford, 1976).

Membrane homogenates (10 μg protein/well) were incubated with 50 pM [^{35}S]GTPγS (PerkinElmer, MA, USA), 30 μM guanosine-5′-diphospate disodium salt (GDP) (Sigma-Aldrich), and 1 μM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (Sigma-Aldrich) in assay buffer, in the presence of either 30 μM (R)-(+)-methanandamide, 10 μM [D-Ala^2, D-Leu5]-enkephalin (DADLE) (Sigma-Aldrich), or 100 pM to 10 μM JWH-133 for 2 h at 30 °C. Basal binding was determined in the absence of agonist, and non-specific binding determined in the presence of 10 μM unlabeled GTPγS (Roche Diagnostics, IN, USA). Incubation was terminated by rapid filtration through Whatman® GF/B glass fiber filters, and three consecutive washes with ice cold 50 mM Tris–HCl. Bound radioactivity was measured in duplicate by liquid scintillation spectrophotometry using a TopCount™ microplate scintillation and luminescence counter (Packard, CT, USA), following overnight extraction with Ecolite(+)™ scintillation fluid (MP Biomedicals, OH, USA). Non-specific binding was subtracted from all values, and stimulated values were normalized to basal binding values. JWH-133 concentration curve experiments were performed in triplicate.

Autoradiography. Transverse 20-μm sections of spinal cord were cut at −20 °C and thaw mounted on to chrome alum gelatin-coated slides. Sections were reanimated in assay buffer (3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl in 50 mM Tris–HCl, pH 7.4) containing 0.1 mM GDP for 15 min at 25 °C. Sections were then incubated in assay buffer containing 0.1 mM GDP, 50 pM [^{35}S]GTPγS (PerkinElmer) and 1 μM DPCPX, with either 10 μM WIN55,212-2 or 1 μM JWH-133 for 90 min. Basal binding was determined in the absence of agonist. Incubation was terminated by washing sections twice in ice cold 50 mM Tris–HCl for 5 min and once in ice cold dH_2O for 5 min. Sections were dried under moving air then desiccated overnight at −20 °C before exposure to ECL hyperfilm (GE Healthcare) for 120 h at room temperature. Films were developed and scanned for densitometric analysis, performed with NIH ImageJ®, Version 1.40g. Briefly, a stereotaxic template of the lumbar spinal cord (Paxinos and Watson, 1986) was overlaid on to the image, which was then converted to 8-bit gray scale. Using ImageJ® software, density was measured in the dorsal laminae (I–VI) and normalized to adjacent background film density. Template overlay and densitometry were performed by an observer blinded to treatment.

Statistical analysis

Behavioral and [^{35}S]GTPγS membrane-binding data were analyzed with two-way repeated-measures ANOVAs, and immunohistochemical and Western blot data were analyzed with two-way ANOVAs, all with Bonferroni post hoc tests, using Prism® version 5.01 (GraphPad, CA, USA). A three-factor general linear model with Bonferroni post hoc tests was performed to analyze [^{35}S]GTPγS autoradiography data, using Minitab® version 15 (Minitab Inc., PA, USA). All data are expressed as mean±SEM.

To assess the level of power achieved in the intrathecal intervention section, we conducted a power analysis. Using the variance of ipsilateral values obtained from the first three animals in each treatment group, we ran a two-factored analysis in nQuery Advisor® (Statistical Solutions, MA, USA), with time as a repeated measure and drug as the second factor. We estimated that with $n=5$ rats/group our analysis would have a power (β) of 0.89.

RESULTS

Effect of systemic GW405833 on allodynia

Systemic delivery of the CB2 partial agonist GW405833 significantly reversed mechanical allodynia in the ipsilateral hind paw of CCI-treated rats (Fig. 1A, B). A two-factor repeated-measures ANOVA of paw withdrawal threshold measured from 10 days post surgery indicated a significant interaction between drug and time ($P<0.01$). Conversion of paw withdrawal thresholds in to percentage reversal of mechanical allodynia (Fig. 1B) showed that 30 mg/kg GW405833 reversed allodynia maximally, by 66%, at 3 h post administration and by 59% at 5 h, which was significantly different to vehicle administration at both time points ($P<0.05$ and $P<0.01$, respectively).

Effect of intrathecal cannabinoids on allodynia

After establishing the efficacy of GW405833 in the CCI model, we further investigated the site of action using intrathecal administration. Intrathecal delivery of 30 μg of

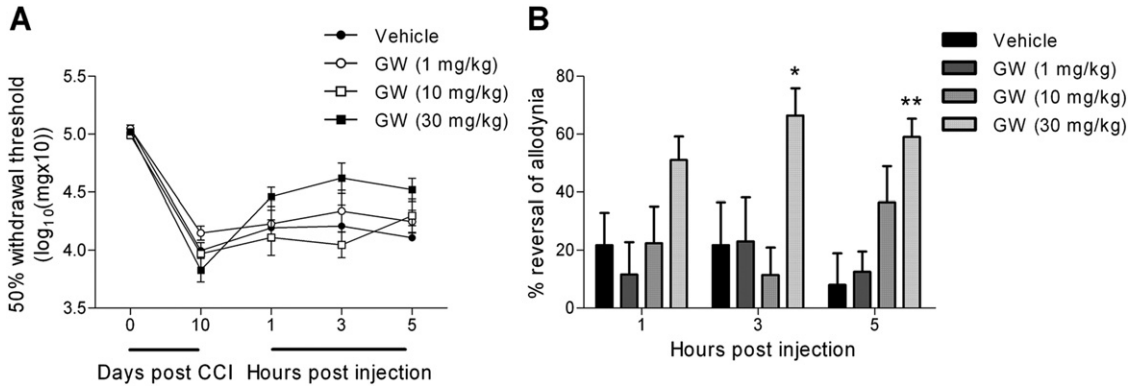

Fig. 1. Effect of the CB2 partial agonist GW405833 on CCI-induced mechanical allodynia in the ipsilateral hind paw, when delivered systemically via i.p. injection. (A) GW405833 significantly reduced mechanical allodynia ($P<0.01$ for the interaction between drug and time), peaking at 3 h post administration. (B) Converted in to percentage reversal of allodynia, 30 mg/kg GW405833 significantly reversed allodynia at 3 and 5 h post administration compared with vehicle (* $P<0.05$; ** $P<0.01$). $n=8$ for vehicle; $n=6$ for GW (1 mg/kg); $n=7$ for GW (10 mg/kg); $n=5$ for GW (30 mg/kg).

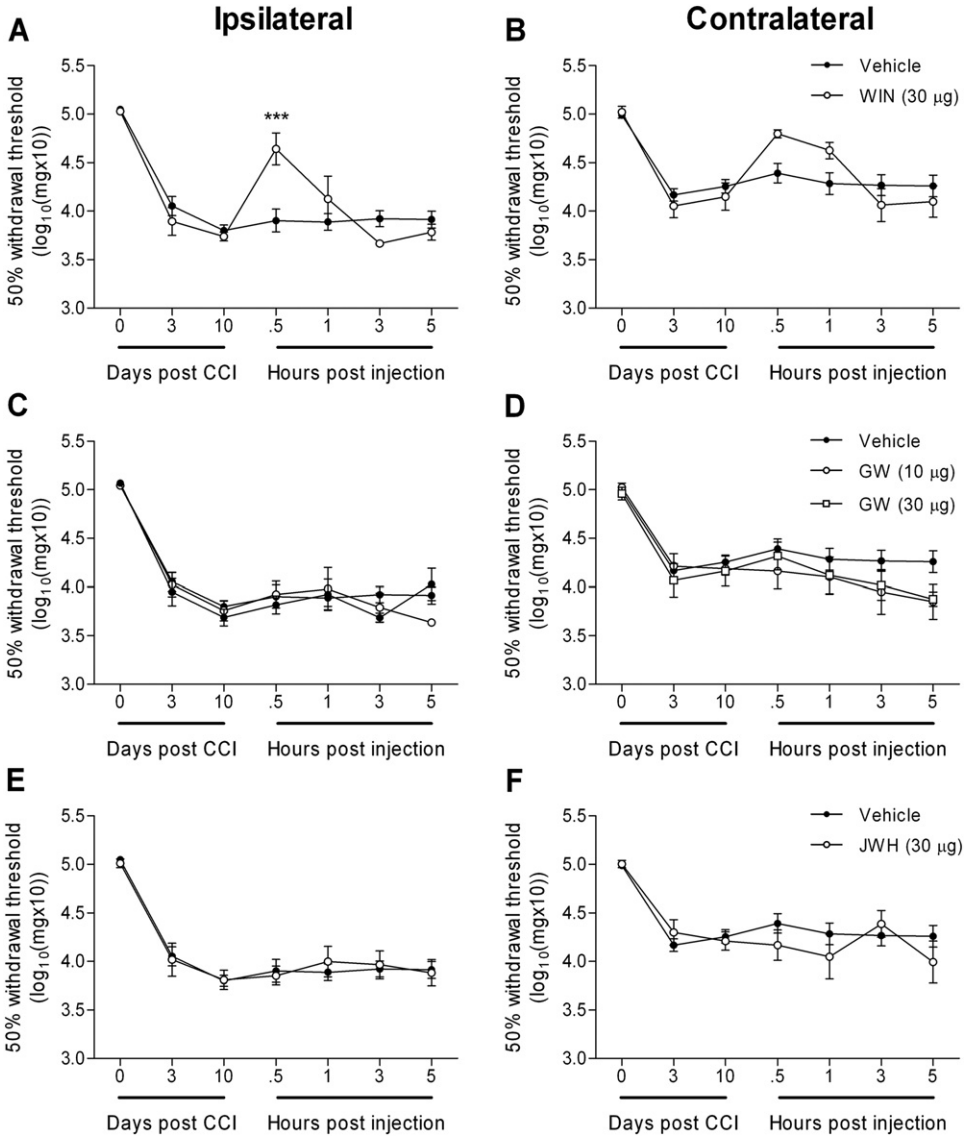

Fig. 2. Effect of the CB1/CB2 agonist WIN55,212-2, and the CB2-selective agonists GW405833 and JWH-133, on CCI-induced mechanical allodynia, when delivered intrathecally. WIN55,212-2, at a dose of 30 μg, ameliorated mechanical allodynia ipsilateral (A) and contralateral (B) to nerve injury that peaked at 30 min and returned to baseline levels within 5 h. Post hoc analysis indicated a significant effect of WIN55,212-2 ipsilateral at 30 min (*** $P<0.001$). GW405833, at doses of 10 and 30 μg (C, D), and JWH-133, at a dose of 30 μg (E, F), had no effect on mechanical allodynia in the same paradigm ($P>0.05$ for all relevant parameters). Error bars denote standard error. $n=14$ for vehicle; $n=5$ for all other groups.

the potent non-selective CB1/CB2 receptor agonist WIN55,212-2 significantly reversed allodynia in both the ipsilateral and contralateral hind paws of CCI rats (Fig. 2A, B). Specifically, in both ipsilateral and contralateral hind paws, two-factor ANOVA repeated measure analysis of paw withdrawal thresholds measured from day 10 after surgery through to 5 h after drug treatment showed that the interaction between drug treatment and time was significant ($P<0.001$). In addition, post hoc analysis showed that the difference between WIN55,212-2–treated and vehicle control rats was significant at 30 min following treatment for the ipsilateral hind paw ($P<0.001$). Sensory thresholds returned to original levels by 5 h. By contrast neither intrathecal delivery of 10 μg or 30 μg of the CB2 receptor partial agonist GW405833 nor 30 μg of the CB2 full agonist JWH-133 reduced allodynia in either hind paw ($P>0.05$ for all relevant parameters) (Fig. 2C–F).

Because of the lack of efficacy of intrathecal CB2 agonists in this model, we then used immunolabeling and a cannabinoid-stimulated GTPγS assay to assess whether CB2 protein is upregulated in the ipsilateral spinal cord following the induction of neuropathic pain as previously described (Wotherspoon et al., 2005).

CB2 immunolabeling in the neuropathic spinal cord

We initially determined whether we could replicate the pattern of immunolabeling described using immunohistochemistry by Wotherspoon et al. (2005) using the same antibody (Cayman Chemical Company and by Walczak et

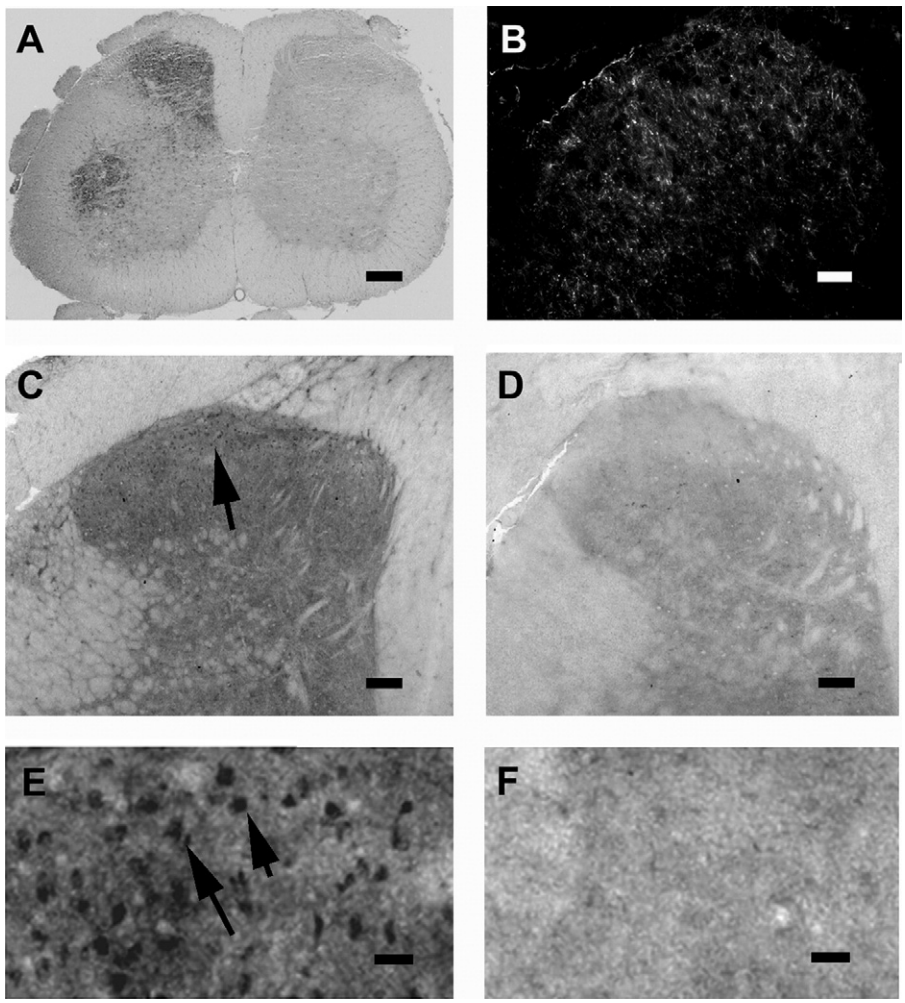

Fig. 3. Immunolabeling of the CB2 receptor and inflammatory markers in the lumbar spinal cord of CCI-treated rats. Labeling of the microglial/macrophage marker Iba1 (A) and the astroglial marker GFAP (B) occurs ipsilateral to nerve injury and is, particularly, dense in the superficial dorsal horn. CB2 receptor immunolabeling in the ipsilateral dorsal horn of CCI-treated rats (C, E) is abolished by preadsorption of the primary antibody with corresponding immunizing peptide (D, F). Immunolabeling is cellular, and membrane bound (E). Scale bars: (A) 200 μM; (B) 100 μM; (C, D) 75 μM; (E, F) 20 μM.

al. (2005) using Western blot. We then quantified immunolabeling to determine whether expression changed after induction of CCI. This antibody has been used to support the hypothesis of widespread neuronal CB2 expression in the CNS (Gong et al., 2006), and so we also tested the antibody on a range of positive and negative controls to assess sensitivity and specificity for CB2.

Using fresh-frozen sections (20 μm) postfixed with 4% PFA, we first determined that CCI reliably induced gliosis in the ipsilateral lumbar gray matter, both with respect to Iba1 labeling (microglia/macrophages) and GFAP labeling (astrocytes) (Fig. 3A, B). We then partially replicated the CB2 immunolabeling that Wotherspoon et al. (2005) described in the neuropathic spinal cord, with CB2 immunolabeling appearing as a band in the superficial dorsal horn (Fig. 3C). This was abolished by preadsorption with the immunizing peptide (Fig. 3D). Morphological integrity of the gray matter in these sections was sufficient to distinguish that staining consisted of both neuropil and cell soma (Fig. 3E, F). Earlier experiments had determined that fluorescent immunolabeling was insufficient to visualize the stained cells (data not shown), which foreclosed the use of double fluorescent immunolabeling to phenotype the cells. However, the distribution, morphology, and size of the cells were consistent with the soma of neurons and not with microglia or astrocytes.

When we carried out Western blot with the Cayman Chemical CB2 antibody using lumbar spinal cord from both sham and CCI rats, this revealed a single band at ~44 kDa, consistent with the predicted molecular weight of the 410aa rat isoform of CB2 (Fig. 4A). The antibody also detected this band in spleen homogenates, along with two additional bands at 59 and 37 kDa. The latter is consistent with the molecular weight of the 360aa rat CB2 isoform (Liu et al., 2009). Only the 37-kDa band was present in CB2 transfected CHO cells. When we compared the optical density of the 44-kDa bands between CCI and sham con-

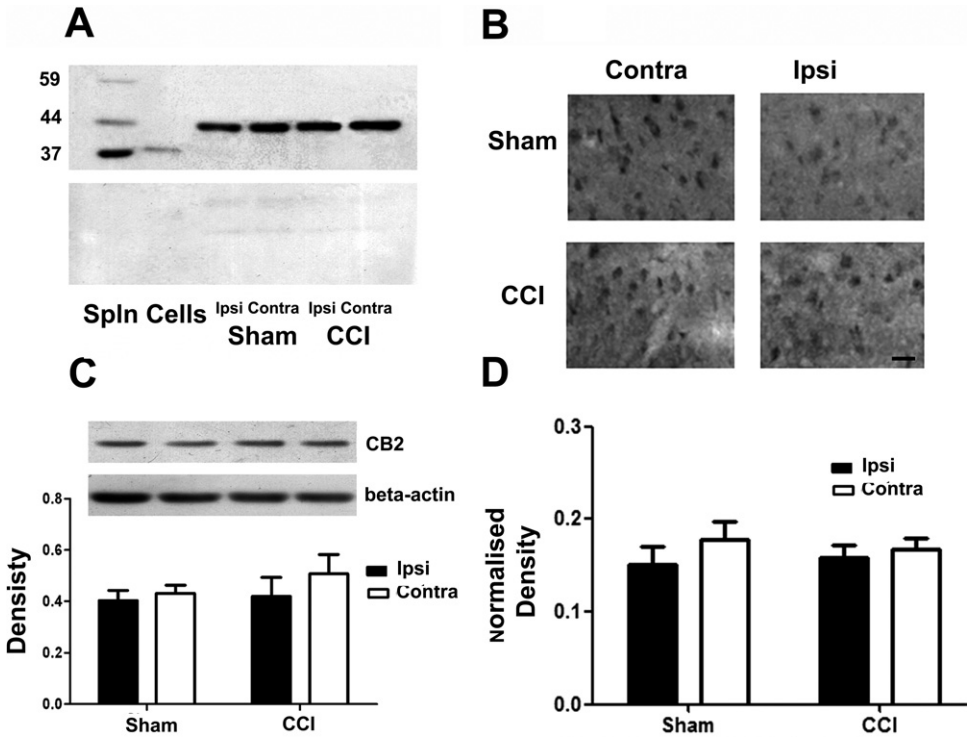

Fig. 4. Western blot analysis and corresponding immunolabeling of the CB2 receptor in lumbar spinal cords of CCI and sham-treated rats. Western blot of the CB2 receptor in the spleen, rat CB2 overexpressing CHO cells, and lumbar spinal cords of sham and CCI-treated rats, reveals bands of various weights (A, top), which are all abolished by preadsorption of the primary antibody with corresponding immunizing peptide (A, bottom). Densitometric analysis of CB2 receptor bands normalized to β-actin indicates no differences between surgery groups or sides (B) ($P>0.05$ for all relevant parameters) $n=5$ for sham; $n=6$ for CCI. Immunolabeling of the CB2 receptor in lumbar spinal cords of sham- and CCI-treated rats is concentrated in the superficial dorsal horn in the ipsilateral and contralateral sides of both sham and CCI rats (C), and does not significantly differ with respect to optical density (D) ($P>0.05$). All error bars denote standard error. Scale bar 20 μM. $n=4$ for sham; $n=6$ for CCI.

trol rats, statistical analysis (two-factor ANOVA; using laterality and surgery treatment as factors) revealed no significant differences in band density for CB2 between treatment groups ($P>0.05$) (Fig. 4B).

Quantitative analysis of immunohistochemistry for the CB2 antibody had similar results, with similar labeling appearing in both CCI and sham surgery rats, and in both contralateral as well as ipsilateral sides (Fig. 4C). Consistent with the results for Western blot, no changes in CB2 immunolabeling were detected using quantitative densitometry for CB2 immunohistochemistry. Statistical analysis (two-factor ANOVA; using laterality and surgery treatment as factors) revealed no significant differences in the optical density of immunolabeling for CB2 for any relevant parameter ($P>0.05$) (Fig. 4D). Therefore, quantification of CB2 immunolabeling for comparison between neuropathic and sham rats did not match those hypothesized by Wotherspoon et al. (2005).

Because of the discrepancy of our results with Wotherspoon et al. (2005), we reassessed the reliability of the antibody through tests on several positive and negative controls. We found that the antibody clearly labeled rat CB2 overexpressing CHO cells and that this was significantly reduced by incubation of the antibody with the immunizing peptide (Fig. 5A, B). Moreover, there was no labeling above weak autofluorescence in CB2-negative HEK293 cells (Fig. 5C). High magnification microscopy of CB2 immunolabeled CB2 overexpressing CHO cells revealed strong labeling in most parts of the cell apart from the nucleus, consistent with a membrane bound receptor (Fig. 5D–F). However, CB2 labeling in the dorsal horn of wild-type mice was identical to that seen in CB2 $-/-$ mice (Fig. 6), with membrane staining of cells in the superficially laminae appearing in both wild-type and knockout mice (insets Fig. 6F, G). We concluded that although the antibody is sufficiently sensitive to detect CB2 in overexpressing cells, it is likely insufficiently specific for CB2 to yield reliable results in native tissue in our experiments. Therefore, the antibody used in our study and by Wotherspoon et al. (2005), Walczak et al. (2005), and Gong et al. (2006) may not be specific for the CB2 receptor, labeling at least one other unidentified protein. To further investigate the expression of functional CB2 protein in the spinal cord, we then used a cannabinoid-stimulated GTPγS assay to measure CB2 agonist-induced G protein activity in the spinal cords of CCI and sham control rats.

Cannabinoid-stimulated G protein activity in the neuropathic spinal cord

Using a ligand-stimulated [^{35}S]GTPγS assay, we measured significant levels of cannabinoid receptor-induced G protein activity induced by the selective CB1 receptor agonist methanandamide (Fig. 7A). Activity above basal lev-

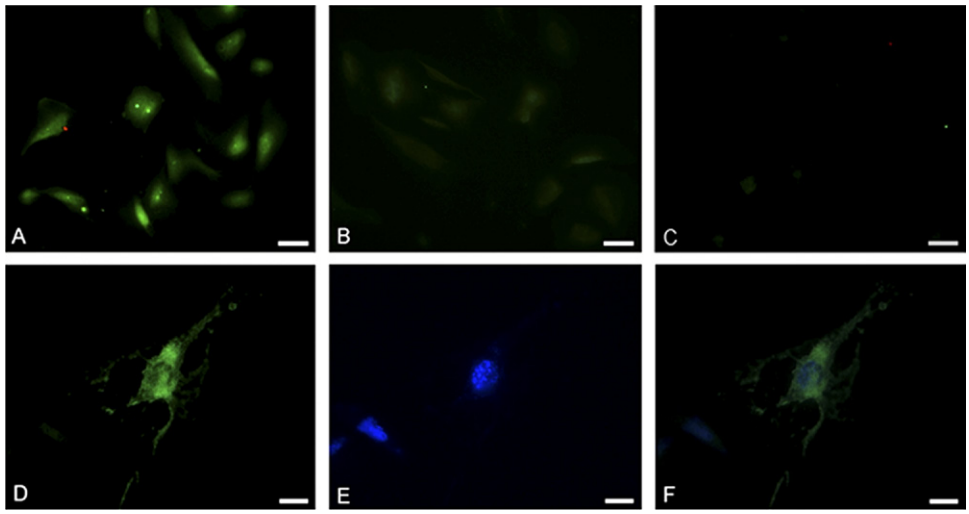

Fig. 5. CB2 receptor immunocytochemistry. CB2 receptor immunolabeling in rat CB2 overexpressing CHO cells (A) is abolished following preadsorption of primary antibody with corresponding immunizing peptide (B) and is absent in CB2 negative HEK293 cells (C). Receptor labeling in CHO cells is membrane bound and cytosolic (D) and merge with a nuclear stain (E) indicates that labeling is not nuclear (F). Scale bars: (A–C) 20 μM; (D–F) 50 μM. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

els was similar to that obtained using DADLE, a δ-opioid receptor agonist, which we used as a positive control (Fig. 7A). However, although two-factor ANOVA with repeated-measures revealed a significant effect from the concentration of the CB2-selective agonist JWH-133 ($P<0.001$) (Fig. 7B), the effect was entirely due to the G protein activity induced by 10 μM of the drug. This is a concentration far exceeding saturation binding for both CB1 as well as CB2 receptors. By contrast, no significant activity was detected at lower CB2-selective concentrations. In addition, there was no significant difference between tissue from sham and CCI rats ($P>0.05$).

Using fresh-frozen spinal cord sections, we then performed an *in situ* version of the assay and visualized the results using autoradiography (Fig. 8A–D). We again used DADLE as a positive control and found that it stimulated opioid activity in the superficial dorsal horn as similar to that previously described (Lemberg et al., 2006) (data not shown). As with the results using spinal cord homogenates, quantitative densitometry showed that a non-selective CB1/CB2 agonist (WIN55,212-2) stimulated significant G protein activity, whereas the CB2-selective agonist JWH-133 did not. Moreover, JWH-133 did not stimulate significant activity in spinal cords from either sham or CCI rats, on either the ipsilateral or contralateral side (Fig. 8A, B). We hypothesized that as WIN55,212-2 is a potent high affinity agonist for both CB1 and CB2 receptors, then if CB2 receptor expression were to be induced in CCI spinal cords and specifically in the ipsilateral dorsal horn, then this would show up as differences in sub-group analysis of WIN55,212-2–treated spinal cords. However, analysis with two-factor ANOVA with repeated measures revealed no significant differences in either of these factors ($P>0.05$) (Fig. 8C). To conclude this section, we found no evidence for functional CB2 receptors in the sham or neuropathic spinal cord using these assays.

DISCUSSION

Our results confirm that systemic delivery of the CB2 partial agonist GW405833 is able to reduce CCI-induced mechanical allodynia. However, although intrathecal delivery of the non-selective CB1/CB2 agonist WIN55,212-2 reverses allodynia in CCI, this was not the case for GW405833 and the CB2-selective full agonist JWH-133. Although the results of systemic delivery involving GW405833 match those previously reported in the CCI model (Hu et al., 2009) and others (LaBuda et al., 2005; Whiteside et al., 2005; Beltramo et al., 2006), our intrathecal results differ from those demonstrated for other models of neuropathic pain where significant reversal of mechanical allodynia has reported following intrathecal delivery (Romero-Sandoval et al., 2008; Yamamoto et al., 2008). One possible explanation for this is differences in drug administration. In particular, precise placement of the spinal injection is vitally important for determining the site of action of drugs delivered to the intrathecal space. Each of these studies describe drug administration directly between vertebrae L4/L5 or L5/L6; however, the site of the neuropathy in these models is in spinal cord segments centered around L5 in the rat and L4 in the mouse, which are up to several centimeters from the corresponding vertebrae (Gelderd and Chopin, 1977; Rigaud et al., 2008) and can only be confidently reached by catheterization (Wu et al., 2004) as we have performed in this study. The pharmacokinetic parameters of intrathecally delivered THC have been described in the mouse (Smith and Martin, 1992) and show that sequestration at the site of administration contributes to a limited rostral spread with these lipophilic compounds. This is a particularly relevant consideration for intrathecal administration in our case and indicates that caudally delivered lipophilic cannabinoids would have a limited rostral redistribution (and vice versa)

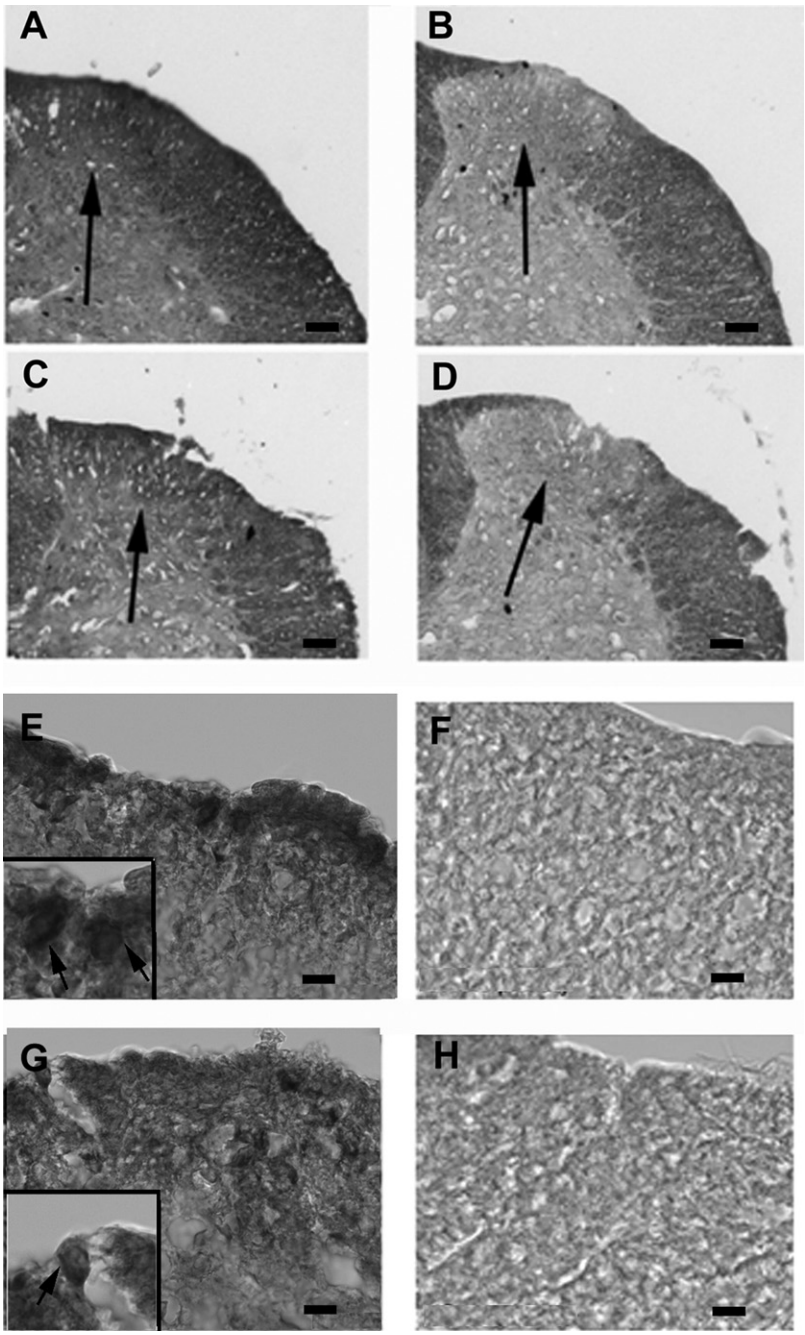

Fig. 6. CB2 receptor immunolabeling in the lumbar spinal cord of wild-type (A, B, E, F) and CB2 receptor knockout (C, D, G, H) mice. Immunolabeling is concentrated in the superficial dorsal horn of wild-type (A, E) and knockout mice (C, G), and is abolished upon omission of primary antibody (B, D, F, H). Receptor labeling in both animals is membrane bound (F, G insets). Scale bars: (A–D) 100 μM; (E–F) 20 μM.

and perhaps suggests an alternate site of action in these earlier works, such as the more proximal dorsal root ganglia (DRGs).

A major consideration for the proper use of ligands designed to target receptor subtypes is their selectivity relative to dose. We found that GW405833, while able to reduce mechanical allodynia when administered systemically at a dose of 30 mg/kg, is ineffective at doses below this, consistent with all previous reports of anti-allodynic activity in pain models (LaBuda et al., 2005; Whiteside et al., 2005; Beltramo et al., 2006; Hu et al., 2009). *In vivo* pharmacokinetic profiling by Valenzano et al. (2005) indicates that following i.p. delivery of 30 mg/kg GW405833 plasma levels reach almost 2 μM, and a high brain:plasma ratio of $>$5 could mean CNS levels approaching 10 μM. *In vitro* characterization by the same group revealed that this compound has a Ki of 3.6 nM at rat CB2 and a Ki of 273 nM at rat CB1 (Valenzano et al., 2005); thus, potential plasma and CNS concentrations of this compound at 30 mg/kg may well be in excess of the Ki of this

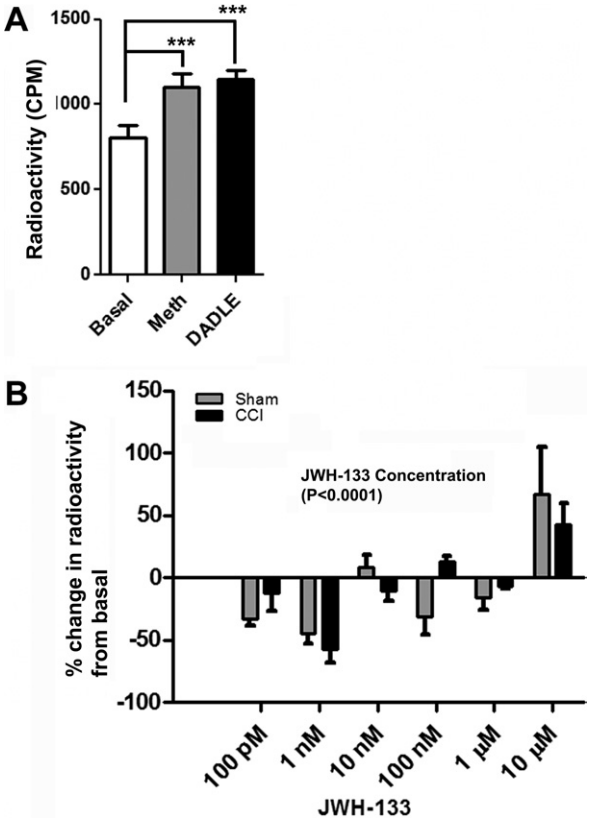

Fig. 7. [^{35}S]GTPγS membrane binding assays. Stimulation with 30 μM methanandamide or 10 μM DADLE increased [^{35}S]GTPγS binding in lumbar spinal cord homogenates (A) ($P<0.001$ vs. basal). Stimulation of spinal cord homogenates from sham- and CCI-treated rats with JWH-133 (B) is concentration, but not surgery dependent ($P<0.0001$ and $P>0.05$, respectively). Data expressed as mean % of basal binding±SEM. $n=4$ for all groups in (A); $n=5$ for all groups in (B).

compound for the CB1 receptor. The importance of the CB1 receptor in the pain pathway is well established and is highlighted by a recent study by Sain and colleagues (Sain et al., 2009), who found that the anti-nociceptive effects of the potent, high affinity CB1/CB2 agonist CP 55,940 are absent in CB1 knockout mice and not CB2 knockout mice, in a variety of acute and persistent pain models, including SNL.

Although we employed GW405833 and JWH-133, many previous intrathecal studies have employed JWH-015 (Romero-Sandoval et al., 2008; Gu et al., 2011; Paszcuk et al., 2011). However, this compound exhibits poor (27-fold) selectivity for CB2 over CB1 (Showalter et al., 1996). Another CB2 agonist, AM1241, has also been used previously (Curto-Reyes et al., 2010; Hsieh et al., 2011). But although this compound is highly selective for CB2 over CB1 (Ibrahim et al., 2003), it is associated with considerable non-cannabinoid receptor binding at pharmacologically relevant concentrations (Yao et al., 2008a). Although Yamamoto et al. (2008) employed JWH-133 in a mouse model and although JWH-133 has been estimated to be 200-fold selective for CB2 over CB1 in rats (Huffman et al., 1999), its binding selectivity mouse cannabinoid receptors has not, to our knowledge, been determined. We have been careful to limit the dose of CB2 agonists delivered to the the probable CB2-selective range. We set 30 μg as our highest dose, as potential CB1-mediated motor deficits with intrathecal JWH-133 at doses above 100 μg have been described (Yoon and Choi, 2003).

Previous studies that had suggested a role for CB2 in the central pain pathway have used different models of pain from our experiments, which may further help explain why our results differ. Previous work has employed spinal nerve lesions, as in the spinal nerve transection model (Romero-Sandoval et al., 2008) or a partial tight ligation of the sciatic nerve (PSL) (Racz et al., 2008; Yamamoto et al., 2008), which produce a more severe injury. Also, for ethical reasons, we employed acute administration of a postoperative analgesia. However, this analgesic regime had previously been shown to have no effect on the later development of mechanical allodynia in a model of neuropathic pain (Stewart and Martin, 2003), and the time course, degree of allodynia, and gliosis in our study corresponds with previous literature.

The neuropathy induced by SNL differs from that induced by CCI, which more closely models the chronic phase of neuropathic pain (Kim et al., 1997). Mechanical allodynia is considerably more severe in SNL and reaches a maximum as early as 4 days following surgery. By contrast, allodynia following CCI develops over up to 2 weeks and is less severe than in the SNL model (Kim et al., 1997). Therefore, the spinal neuropathy and induction of adaptive receptor expression changes are likely to be greater following SNL than CCI. Therefore, CB2 may be upregulated in the spinal cord in the SNL model in a different way than in the CCI model if CB2 is expressed in regenerating neurons, as suggested by Wotherspoon et al. (2005). This may also occur in the brain in some models of neural injury (Viscomi et al., 2009). The degree of Wallerian degeneration of spinal sensory afferents in models of neuropathic pain is associated with the extent of sympathetic sprouting into the DRG (Ramer et al., 1997), and there is evidence that regenerative sympathetic sprouting contributes substantially more to neuropathic pain in the SNL model than in the CCI model (Kim et al., 1997; Ramer and Bisby, 1999). Therefore, it may be theorized that Wallerian degeneration and axonal sprouting, and therefore induction of CB2 expression may greater in the ipsilateral spinal cord following SNL than following CCI. In support of this, Wotherspoon et al. (2005) found that CB2 immunolabeling was colocalized with growth-associated protein 43 (GAP-43) in the ipsilateral neuropathic spinal cord. By contrast GAP-43 is weakly expressed in the spinal cord following CCI more than a week after surgery, and reaches a relatively low peak only after 2–3 weeks (Cameron et al., 1991; Cameron et al., 1997).

The differences between the SNL model and CCI may therefore help to explain the differences between our results for CB2 immunolabeling compared with those obtained by Wotherspoon et al. (2005). However, we propose that there are also problems with respect to the reliability of results that can be obtained in immunolabeling using the CB2 antibody used in both studies.

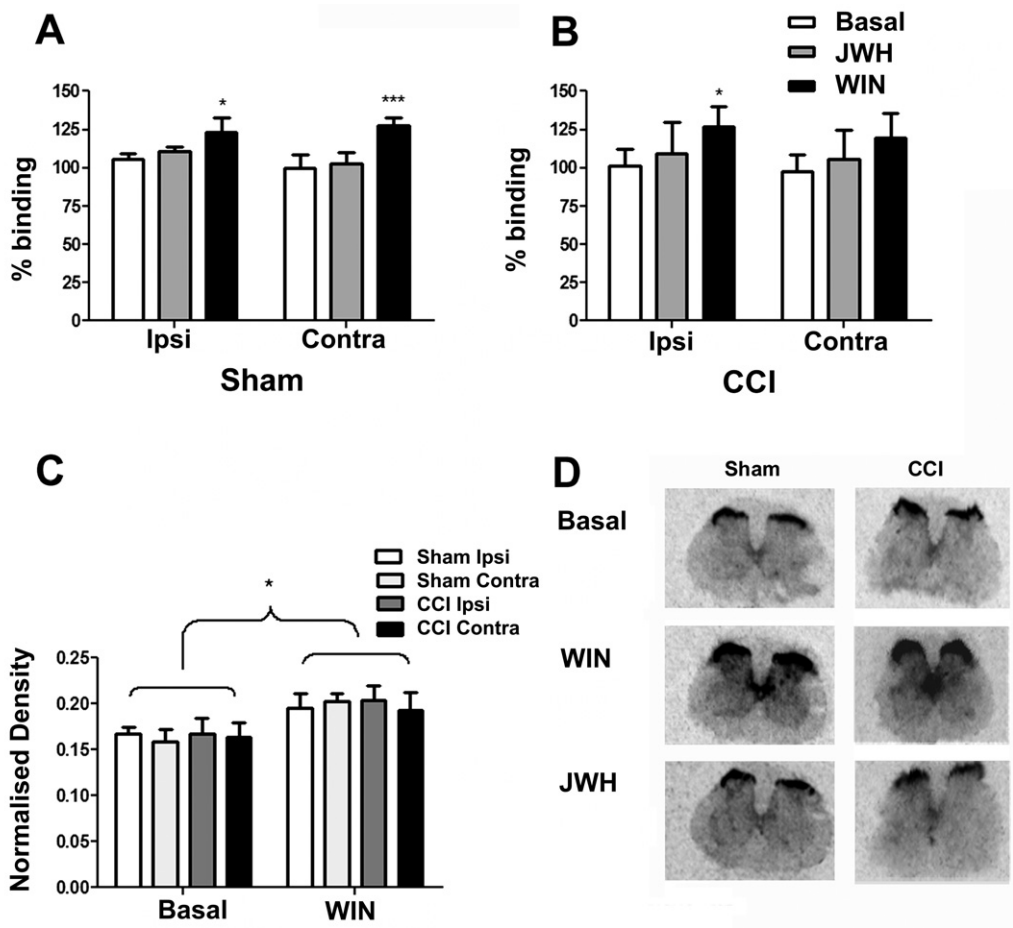

Fig. 8. [^{35}S]GTPγS autoradiography. Stimulation of lumbar spinal cords with 10 μM WIN55,212-2, but not 1 μM JWH-133, increases [^{35}S]GTPγS binding in the ipsilateral and contralateral dorsal horns of sham rats (A) and ipsilateral dorsal horns of CCI-treated rats (B) ($P<0.05$ sham and CCI ipsilateral and $P<0.001$ sham contralateral, vs. basal). WIN55,212-2 stimulation was comparable between groups (C). All data normalized to sham basal contralateral binding and expressed as a %±SEM. WIN55,212-2 resulted in increased [^{35}S]GTPγS binding in superficial and deep dorsal laminae and lamina X of both sham and CCI rats, whereas JWH-133 had no observable effect on binding in any laminae region (D). Left side is ipsilateral to surgery. $n=5$ for all groups.

CB2 immunolabeling not only appeared in CCI spinal cords, but equally in spinal cords taken from sham control rats. The immunolabeling was most intense in small unramified cell soma in the superficial dorsal horn in both wild-type rats and mice, but was observed equally in CB2 knockout mice.

The extent of CB2 expression in the CNS is contentious (Atwood and Mackie, 2010). Our results show that a commonly used CB2 antibody labels rat CB2 when overexpressed in CHO cells, but also labels cells in wild-type and knockout mouse spinal cord (Fig. 6). The epitope for this N-terminus–directed antibody is the peptide sequence from residues 20 through 33 from human CB2, and the knockout used in this study retains the first 25 amino acids of the CB2 receptor N-terminus. It is therefore possible that this antibody could be binding to a potential truncated peptide remnant of the CB2 receptor N-terminus. This is unlikely, as our results show that the immunolabeling in wild-type and knockout mice is most intense in cellular membranes (insets of Fig. 6). Even if the first 25 amino acids of the remaining N-terminus were transcribed, trafficking of this small cytosolic peptide would be very different than that of the membrane integral CB2 receptor, and a similar cellular staining pattern would be unlikely. We conclude that while this CB2 antibody can recognize overexpressed CB2 receptors in transfected cells, it is not likely specific for CB2 in native tissue sections and therefore cannot provide definitive evidence about CB2 expression in the spinal cord. These results demonstrate the insufficiency of relying on an immunizing peptide as a negative control for primary antibody specificity, highlighting the necessity of using appropriate knockout controls.

Irrespective of CB2 immunolabeling, our results using cannabinoid-stimulated GTPγS do not support the hypothesis that functional CB2 is expressed in the healthy or CCI-treated spinal cord at currently measurable levels. Consistent with our results for intrathecal delivery of cannabinoids, we were able to stimulate G protein activity in both spinal cord homogenates and sections using the CB1/CB2 non-selective agonist WIN55,212-2, but not with the CB2-selective agonist JWH-133, except at a concentration

far exceeding that specific for activation of the CB2 receptor. The distribution of WIN55,212-2-stimulated activity in spinal cords closely matched that previously described for cannabinoid binding sites in the spinal cords of wild-type but not CB1 knockout mice (Ibrahim et al., 2003) with intense activity in the outer laminae of the dorsal horn, extending through the gray matter into lamina X surrounding the central canal. This distribution also matches results described using CB1 immunohistochemistry (Lim et al., 2003). However, Lim et al. (2003) described an increase of CB1 immunolabeling following induction of CCI, whereas we did not find any evidence for changes in cannabinoid receptor stimulation following nerve injury. It is possible that any changes in functional cannabinoid receptor expression between the healthy and neuropathic spinal cord were below the level of detection using our methods. But it should also be noted that immunolabeling for CB1 receptors is as contentious and problematic as for CB2 receptors (Grimsey et al., 2008) and that Lim and colleagues did not include a description of controls using CB1 knockout tissue.

Conclusions

Consistent with previous literature, we have shown that the CB2-selective partial agonist GW405833 is anti-allodynic in the CCI model of neuropathic pain when delivered systemically. However, although in this study intrathecal delivery of the CB1/CB2 non-selective agonist WIN55,212-2 reversed mechanical allodynia in this model, intrathecal delivery of two different CB2-selective agonists did not. This is in contrast to previous studies, which have shown that intrathecal delivery of CB2 agonists can reverse allodynia in neuropathic pain models that involve relatively severe injury, such as SNL. We hypothesize that the choice of neuropathic pain model may be important both for expression of CB2 receptors in the spinal cord and for the efficacy of CB2 agonists. We also propose that conclusions about CB2 receptor protein expression in the spinal cord are not reliable with respect to at least one commonly used CB2 antibody. When we employed a cannabinoid-stimulated G protein coupled receptor activity assay, we again found that although the CB1/CB2 agonist WIN55,212-2 was able to induce activity, this was not true for the CB2-selective agonist JWH-133.

We hypothesize that subtle changes in cannula placement for the delivery of cannabinoids can have important effects on results and interpretation and that it cannot be ruled out that the DRG of the peripheral nervous system are the target for CB2-mediated anti-nociception in some studies that have employed intrathecal delivery. This can only be answered with more detailed knowledge of the distribution of cannabinoids upon intrathecal delivery. However, save for one study utilizing radiolabeled THC, the pharmacokinetics of intrathecally delivered cannabinoids are largely unstudied, such that the concentrations of novel cannabinoid ligands at potential sites of action are not known. This is important, because the CB2 agonists and antagonists used in studies to date are only selective for the CB2 receptor over CB1 in a concentration dependent manner; an important consideration for systemic delivery also.

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