Osteoarthritis and Cartilage 18 (2010) 1536–1543

Osteoarthritis and Cartilage

Paradoxical effects of the cannabinoid CB_2 receptor agonist GW405833 on rat osteoarthritic knee joint pain

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ARTICLE INFO

Article history:
Received 3 February 2010
Accepted 10 September 2010

Keywords:
Joint pain
Osteoarthritis
Cannabinoid CB_2 receptor
Electrophysiological recordings
Animal model
Transient receptor potential vanilloid channel-1
Primary joint afferents

SUMMARY

Objective: The present study examined whether local administration of the cannabinoid-2 (CB_2) receptor agonist GW405833 could modulate joint nociception in control rat knee joints and in an animal model of osteoarthritis (OA).

Method: OA was induced in male Wistar rats by intra-articular injection of sodium monoiodo-acetate with a recovery period of 14 days. Immunohistochemistry was used to evaluate the expression of CB_2 and transient receptor potential vanilloid channel-1 (TRPV1) receptors in the dorsal root ganglion (DRG) and synovial membrane of sham- and sodium mono-iodoacetate (MIA)-treated animals. Electrophysiological recordings were made from knee joint primary afferents in response to rotation of the joint both before and following close intra-arterial injection of different doses of GW405833. The effect of intra-articular GW405833 on joint pain perception was determined by hindlimb incapacitance. An *in vitro* neuronal release assay was used to see if GW405833 caused release of an inflammatory neuropeptide (calcitonin gene-related peptide – CGRP).

Results: CB_2 and TRPV1 receptors were co-localized in DRG neurons and synoviocytes in both sham- and MIA-treated animals. Local application of the GW405833 significantly reduced joint afferent firing rate by up to 31% in control knees. In OA knee joints, however, GW405833 had a pronounced sensitising effect on joint mechanoreceptors. Co-administration of GW405833 with the CB_2 receptor antagonist AM630 or pre-administration of the TRPV1 ion channel antagonist SB366791 attenuated the sensitising effect of GW405833. In the pain studies, intra-articular injection of GW405833 into OA knees augmented hindlimb incapacitance, but had no effect on pain behaviour in saline-injected control joints. GW405833 evoked increased CGRP release *via* a TRPV1 channel-dependent mechanism.

Conclusion: These data indicate that GW405833 reduces the mechanosensitivity of afferent nerve fibres in control joints but causes nociceptive responses in OA joints. The observed pro-nociceptive effect of GW405833 appears to involve TRPV1 receptors.

Introduction

Osteoarthritis (OA) is the most common form of arthritis and is characterized by extensive remodelling of subchondral bone and permanent destruction of articular cartilage leading to joint pain. Currently, no disease modifying drugs are available; therefore, treatment of OA is primarily restricted to analgesics which often have limited efficacy and hazardous side-effects. An established animal model of OA pain involves the intra-articular injection of the glycolysis inhibitor sodium mono-iodoacetate (MIA) which disrupts cartilage metabolism, leading to OA like lesions[1–5], nerve sensitization and joint pain[6,7].

A family of agents which have shown great promise for the treatment of chronic pain are cannabinoids. Cannabinoid agonists suppress nociceptive transmission and inhibit pain related behaviour in different models of arthritis pain[7–10]. Two cannabinoid receptors, cannabinoid receptor 1 (CB_1 receptor) and cannabinoid receptor 2 (CB_2 receptor), have been cloned and characterized. The CB_1 receptor is present in the central and peripheral nervous system while the CB_2 receptor is predominantly associated with the immune system[11,12].

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1063-4584/$ – see front matter
doi:10.1016/j.joca.2010.09.005

The potential anti-inflammatory and analgesic effects of CB_2 specific cannabinoid agonists have been tested in various pain models[13–18]. Current cannabinoid pain therapies are frequently limited by central nervous system (CNS)-mediated side-effects; however, selective CB_2 receptor agonists have been shown to be devoid of such effects[14,18,19]. When applied systemically, the CB_2 receptor agonist GW405833 partially reversed the inflammation and hyperalgesia found in multiple acute inflammatory models[20,21].

In addition to being agonist ligands at cannabinoid receptors, several cannabinoids are also activators of the transient receptor potential vanilloid channel-1 (TRPV1)[22–25]. TRPV1 is expressed on nociceptive afferent neurones throughout the periphery and has been demonstrated to play a critical role in the induction of thermal hyperalgesia in inflammatory pain models[26–28]. Several studies have shown that the TRPV1 channel is involved in mediating the anti-nociceptive effect of CB_1 agonists[22,24,27–29]. Thus, certain cannabinoids may act as dual cannabinoid–vanilloid mediators, particularly under conditions of inflammatory hyperalgesia.

This study examined whether local application of the CB_2 receptor agonist GW405833 can reduce nociceptive activity of afferent nerve fibres and pain behaviour in control and MIA-treated rat knee joints. Since the pro-nociceptive neurotransmitter calcitonin gene-related peptide (CGRP) modulates joint mechanonociception centrally[30] and CGRP knockout arthritic mice do not develop secondary hyperalgesia[31], we looked at the release of CGRP from spinal cord homogenates in order to examine GW405833 activity at native TRPV1 receptors[32]. Activation of the receptor was measured in both the basal and stimulated/phosphorylated states to simulate the normal and diseased conditions respectively.

Methods

Animals

Experiments were performed on 217 male Wistar rats (250–450 g). The animal handling and surgical procedures outlined in this study all adhered to the Canadian Council guidelines for the care and use of experimental animals which also review animal ethics.

MIA model of OA

Fifty-three rats were deeply anaesthetised with 2% isoflurane in 100% O_2 (1 L/min). To induce OA, 50 μl of 3 mg sodium MIA in 0.9% saline was injected into the joint cavity through the patellar ligament. Animals were allowed to recover for 14 days which has consistently been shown to cause severe end-stage OA in this species[1,4,5]. In a further 42 animals, 50 μl of saline was injected into the knee joint and this cohort served as a sham control group.

Immunohistochemistry

CB_2 and TRPV1 expressions were evaluated in DRGs and synovial membrane from sham-injected ($n = 4$) and MIA-injected ($n = 4$) male Wistar rats. Animals were anaesthetised and perfused transcardially with saline followed by 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), pH 7.4. DRG (L4–L6) and synovial membrane were dissected, post-fixed in the 4% PFA/PBS solution for 3 h at room temperature, and then transferred to a 30% sucrose/PBS solution and maintained overnight at 4°C. Tissue then was mounted in M1 embedding media (Thermo Shandon, Pittsburgh, PA, USA) and stored at −80°C. Sections 12–14 μm were cut from fixed tissue on a cryostat and mounted serially onto Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA). After equilibration to room temperature, the sections were blocked for endogenous proteins for 1 h with 1× Dako Tris-buffered saline with Tween 20 wash buffer (Dako, Carpinteria, CA, USA) containing 1% BSA, 0.1% triton X100 and 10% normal serum of the host species of the second antibody. Sections were incubated overnight at 4°C with a cocktail of primary antibody of rabbit anti-CB_2 (gift from Dr. Ken Mackie, Indiana University, Bloomington, IN) and either guinea pig anti-TRPV1 for synovium (Neuromics, GP14100, Edina, MN, USA) or goat anti-TRPV1 for DRG (Santa Cruz, SC-12498, Santa Cruz, CA, USA), followed by several washes the next morning with 1× wash buffer. Negative staining was confirmed using a blocking peptide (CB_2) or omission of the primary antibody (TRPV1). The sections then were incubated for 1 h at room temperature with a cocktail of goat anti-guinea pig Alexa 594 and goat anti-rabbit Alexa 488 for synovium and a cocktail of donkey anti-goat Alexa 594 and donkey anti-rabbit 488 for the DRG. All secondary antibodies were from Molecular Probes (Invitrogen, Carlsbad, CA, USA). Following several washes with 1× wash buffer, the slides were cover-slipped with fluorescent mounting media and stored in the dark at 4°C for later analysis. Labeling was visualized on a Leica fluorescence microscope (Bannockburn, IL, USA) equipped with the appropriate filter blocks. Merged images were a composite of single-labeled files using a feature of Spot software (Diagnostic Instruments, Sterling Heights, MI, USA). For DRG, cell profiles were outlined and immunohistochemical staining (staining intensity) was recorded and analyzed using Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

Surgical procedures

The detailed methods for the surgical procedure have been described previously[7]. Rats were deeply anaesthetized with urethane (25% stock solution; 2 g/kg, i.p.). The left jugular vein was cannulated so that the muscle relaxant gallamine triethiodide (50 mg/kg) could be injected to eliminate neural interference arising from the hindlimb musculature. The right saphenous artery was cannulated below the knee joint to permit local close intra-arterial injection of drugs to the knee joint. Rotational torque was applied to the knee joint by placing the right hindpaw into a shoe-like holder which was connected to a force transducer and a torque meter.

Extracellular electrophysiological recording

The technique used for recording afferent activity from articular nerve fibres in the rat knee joint has been described previously[33,34]. The saphenous nerve was isolated in the inguinal region and cut centrally to prevent the generation of spinally-mediated reflexes. Neural strands were then placed over a platinum electrode to record single afferent fibre activity. The indifferent electrode was a silver wire electrode placed in the muscle tissue of the ipsilateral hindlimb. The receptive field of the fibres was identified by the elicitation of a response to gentle probing of the knee joint with a glass rod. The mechanical threshold of each recorded joint afferent was determined by a gradual increase of the torque applied to the joint until the fibre starts eliciting action potentials. The mechanosensitivity of articular afferents was measured in response to outward non-noxious rotation and noxious hyper-rotation of the knee joint. Since no differences were observed between the two movements in respect to the effect of the applied drugs, the data for both rotation movements were pooled for statistical analysis. The amount of force applied during the joint rotation ranged between 15 and 25 mNm for the non-noxious movement and between 35 and 45 mNm for the noxious movement. To establish a control baseline level of activity for each fibre, three movement cycles of the knee to discrete torque levels were performed at the beginning of the experiment and the mean afferent firing rate was taken as

baseline activity. Percentage changes in firing rate compared to baseline were calculated after close intra-arterial injection of either vehicle or GW405833 (10^{-12} mol; 10^{-10} mol; 10^{-8} mol; 100 μl bolus). In a separate group of animals the CB_2 receptor antagonist AM630 (10^{-8} mol; 100 μl bolus) or the TRPV1 receptor antagonist SB366791 (500 μg/kg i.p.) was administered 30 min prior to the GW405833 to confirm cannabinoid and/or TRPV1 receptor involvement in joint mechanosensitivity. Doses of GW405833 were chosen based on our previous experience with cannabinoid agonists[7]. The conduction velocity of the nerve fibres was determined by electrically stimulating their receptive field with a pair of bipolar, silver wire electrodes. Afferents with conduction velocities below 2 m/s were classified as unmyelinated type IV fibres, whereas afferents with conduction velocities above 2 m/s were classified as thinly myelinated type III fibres.

Weight-bearing assessment

On three consecutive days prior to behavioural testing, rats were regularly handled and gradually habituated to the test equipment. Hindlimb weight bearing was determined using an incapacitance tester (Linton, Norfolk, UK) consisting of a dual channel weight averager. The force exerted by each hindlimb (measured in grams) was averaged over a 5 s period. Each data point is the mean of three readings. The percent weight distribution onto the treated (ipsilateral) hindlimb was calculated. For the MIA animals, weight distribution was measured between treated (intra-articular injection of GW405833: 10^{-6} mol; 100 μl bolus) and contralateral non-treated hindlimbs. For the antagonist studies, either the TRPV1 antagonists SB366791 (500 μg/kg i.p.) or the CB_2 receptor antagonist AM630 (10^{-8} mol; l00 μl bolus) was injected 30 min prior to administration of GW405833.

CGRP release assay

Adult male rats were sacrificed by exposure to CO_2 followed by cervical dislocation. The lumbar portion (L1–L6) of the spinal cord was dissected out and homogenised in 15 ml of ice-cold Krebs buffer per cord. The homogenate was diluted to 5 mg/ml and plated into a 96-well filter plate (100 μl/well). Phosphorylation of the TRPV1 receptor was achieved by activation of protein kinase C through addition of a low concentration (10 nM) of phorbol 12,13-dibutyrate (PDBu). Buffer was removed by vacuum filtration and buffer with or without PDBu and/or antagonist was added (100 μl/well) and the plate incubated at 37°C for 10 min. Agonists with or without PDBu and/or antagonist were added (100 μl/well) and the plate incubated for a further 10 min at 37°C. Buffer was then transferred into the immunoplate (SpiBio, Montigny Le Bretonneux, France). Anti-CGRP tracer was added to all wells (100 μl/well) and the plate covered with plastic film and incubated at 4°C for 16–20 h. After the incubation period the plate was washed with wash buffer (300 μl/well) and Ellman's reagent added (200 μl/well). The plate was left at room temperature to develop for 45 min and absorbance read at 405 nm. Data were expressed as a percentage of 300 nM capsaicin control and curves were fitted using the 4 parameter Hill equation.

Drugs and reagents

Sodium MIA, GW405833, AM630, SB366791 were obtained from Tocris Bioscience (Missouri, USA; Bristol, UK); gallamine triethiodide, PDBu, capsaicin, DMSO, cremophor and urethane were obtained from Sigma–Aldrich (Ontario, Canada; Poole, UK). All reagents were dissolved in vehicle solution (2% DMSO, 1% cremophor, 0.9% saline) and aliquots of the drug were kept frozen (−20°C) in Eppendorf vials until required. The pH of all solutions was determined to exclude acidity as a sensitising factor on afferent nerve fibres. All solutions were found to have a neutral pH (pH 7.4) before injection. For the CGRP release assays, drugs were dissolved at 10 mM in 100% DMSO and then diluted in Krebs buffer. Gallamine triethiodide was made fresh on the day of experimentation and dissolved in 0.9% saline.

Statistics

All data were normally distributed and expressed as means with 95% confidence interval (CI – lower, upper limits) for "*n*" observations. The effect of administered drugs between animal groups was analyzed by either one- or two-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons post-test and an unpaired Student's *t* test. The time course and dose-dependency of GW405833 were tested by one-way ANOVA with individual points being compared to control using a one-sample *t* test. The maximum response produced by GW405833 maximal efficacy (E_{max}) was calculated from dose–response curves. All differences were considered statistically significant at a P value less than 0.05.

Results

Colocalization of CB_2 and TRPV1 receptors in DRG and synovium of control and MIA-treated animals

Consistent with previous results, CB_2 antibody staining was observed in small, medium and large diameter neurons in the DRG of both sham- and MIA-treated animals [Fig. 1(A); Supplementary Fig. 1(A)]; whereas TRPV1 antibody labeling was primarily restricted to small- and medium-sided cells in both groups of animals [Fig. 1(B); Supplementary Fig. 1(B)]. In addition, TRPV1 staining was evident in approximately 25% of CB_2-positively stained cells in DRGs from saline- and MIA-treated rats [Fig. 1(C); Supplementary Fig. 1(C)].

In sham- and MIA-treated animals, both CB_2 and TRPV1 antibody labeling was found in cells of the synovial membrane [Fig. 1(D) and (E); Supplementary Fig. 1(D) and (E)]. Examination of the individual antibody-labeled tissue sections and merged images indicated that there were many instances of synovial cells double–labeled with CB_2 and TRPV1 antibodies [Fig. 1(F); Supplementary Fig. 1(F)]. While a quantitative assessment was not conducted in these studies, there were no apparent differences in the pattern or level of expression of CB_2 and TRPV1 receptors in synovium or DRG from MIA- and sham-treated knee joints at 14 days after injection.

Neuronal characteristics

Between 1–3 afferent fibres were examined per animal such that a total of 154 units (77 units in control knee joints; 77 units in MIA-treated knee joints) were recorded in this study. The electrophysiological characteristics of these fibres are summarized in Table I. The basal firing rate of afferents in the control joint was 30.8 (CI: 27.5–34.0) during non-noxious movement and 76.5 (CI: 71.6–81.4) during noxious movement. The basal firing rate of afferents in the MIA joint was 49.0 (CI: 45.7–52.2) during non-noxious movement and 100.0 (CI: 95.2–104.8) during noxious movement. Compared to saline-injected control joints, the average firing rate of afferents in the MIA-treated knee joint was significantly higher during both joint rotations ($P < 0.0001$ unpaired Student's *t* test). All units tested could be activated by local intra-arterial injection of KCl (0.4 mM, 0.1 ml) at the end of the experiment confirming that administered reagents reached mechanosensory nerve endings throughout the experiment.

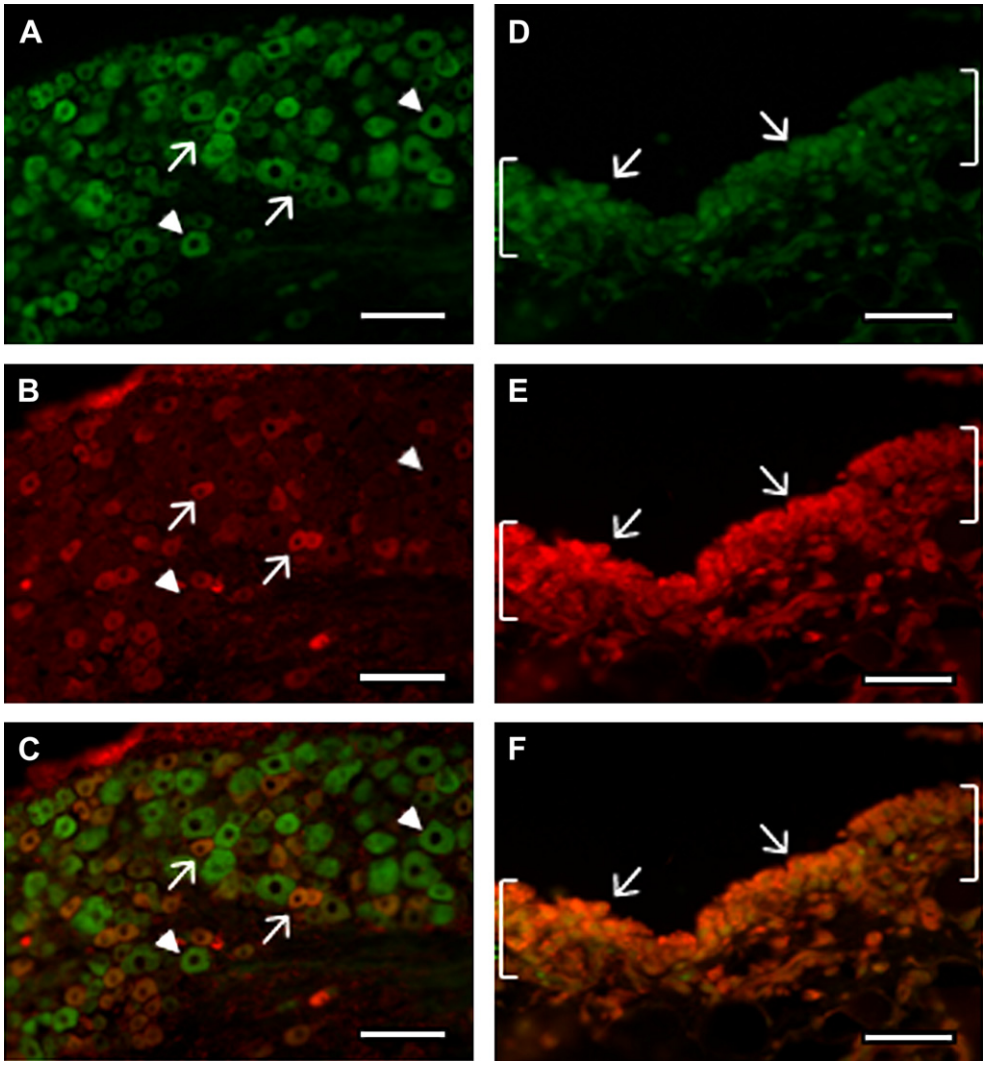

Fig. 1. Colocalization of CB_2 and TRPV1 antibodies in sham-injected control rat DRG cells (A–C) and synovial membrane (D–F). CB_2 (green: A) and TRPV1 (red: B) antibody staining is evident in the lumbar DRG cells. CB_2 staining is evident in small, medium and large diameter DGR cells, whereas TRPV1 expression is primarily in small- and medium-sized cells. Merged images (C) indicate colocalization of CB_2 and TRPV1 in small to medium-sized cells (open arrows). Examples of large diameter DRG neurons positively labeled with CB_2 only are also shown (arrow heads). Scale bars = 100 μm. In the synovial membrane, CB_2 (green: D) and TRPV1 (red: E) antibody staining is evident. Merged images (F) indicate colocalization of CB_2 and TRPV1 antibody labeling in synoviocytes (open arrows). Scale bars = 50 μm.

Effect of GW405833 in control knee joints

With rotation of control knee joints, injection of GW405833 induced a significant suppression of mechanosensory nerve activity. A specimen recording showing the inhibitory effect of GW405833 on afferent firing rate is shown in Fig. 2(A). Application of vehicle had no significant effect on mechanosensitivity. The desensitizing effect of GW405833 was found to be dose-dependent across the dose range 10^{-12}–10^{-8} mol, [$P < 0.0001$; one-way ANOVA; Fig. 2(B)]. The E_{max} of GW405833 was −26.5% (CI: −33.3 to −19.7). Compared to the vehicle, the desensitizing effect of GW405833 was statistically significant [$P < 0.0001$ two-way ANOVA; Fig. 2(B); $n = 22$–27].

Table I
Proportion and range of electrophysiological characteristics of thinly myelinated type III and unmyelinated type IV fibres recorded in control and osteoarthritis (OA) knee joints. M_T = mechanical threshold; E_T = electrical threshold; CV = conduction velocity

		M_T (mNm)	E_T (V)	CV (m/s)	% of fibres
Control joints	Type III	5–34	6.5–9	2.6–7.4	39
	Type IV	7–45	5.5–10.5	0.4–2.4	61
OA joints	Type III	3–16	6–10	2.9–23.4	28
	Type IV	3–22	6.5–12	0.6–2.4	72

Effect of GW405833 in MIA knee joints

In MIA joints, GW405833 caused a significant sensitization of recorded fibres while application of vehicle had no significant effect on joint mechanosensitivity. A specimen recording showing the excitatory effect of GW405833 on afferent nerve activity in MIA joints is shown in Fig. 3(A). The sensitising effect of GW405833 was found to be dose-dependent across the dose range 10^{-12}–10^{-8} mol [$P < 0.0001$; one-way ANOVA; Fig. 3(B)] with an E_{max} of 34.9% (CI: 24.7–45.1). Compared to vehicle, this sensitising effect was statistically significant (two-way ANOVA $P < 0.0001$; $n = 24$–28).

Response characteristics of type III and type IV fibres

In a subgroup of recorded fibres, the response properties of type III vs type IV afferents were compared. No differences in mechanical

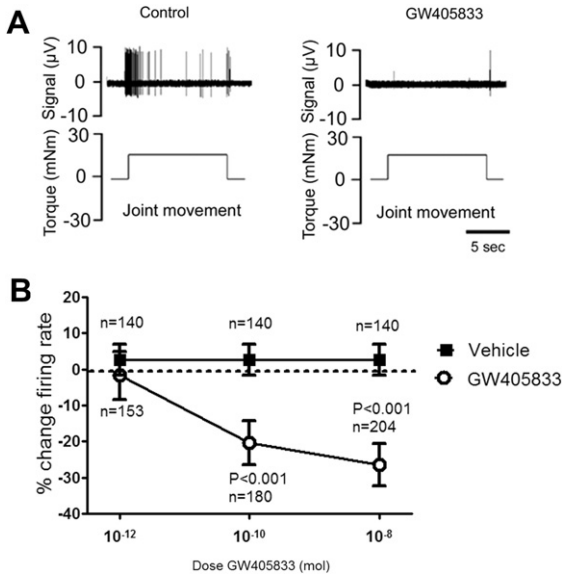

Fig. 2. Effect of GW405833 on control knee joints. (A) Specimen recording of a single unit during rotation of a control knee joint before and after close intra-arterial application of GW405833. GW405833 significantly reduced the firing rate of joint afferent nerve fibres. (B) The effect of GW405833 on knee joint afferent nerve mechanosensitivity in response to rotation of saline-injected knee joints. The desensitizing effect of GW405833 was dose-dependent and significantly different from the effect of vehicle control treatment [$P < 0.0001$ by two-way ANOVA ($n = 22–27$ fibres)]. Values are the mean of all time points with 95% CIs.

threshold, voltage threshold or baseline firing frequency were found; however, the inhibitory effect of GW405833 in control animals was mediated mainly by activation of type III fibres since the inhibitory effect of type IV fibres is comparatively smaller (unpaired Student's *t* test; $P = 0.005$; Table II). The pro-nociceptive effect of GW405833 in MIA animals was mediated by activation of type IV fibres since type III fibres were not sensitized by GW405833 (unpaired Student's *t* test; $P = 0.007$; Table II).

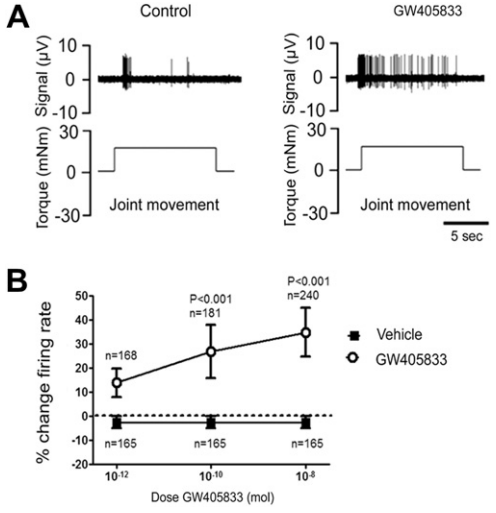

Fig. 3. Effect of GW405833 on osteoarthritis (OA) joints. (A) Specimen recording of a single unit during rotation of an OA knee joint before and after close intra-arterial application of GW405833. GW405833 significantly sensitized the firing rate of joint afferent nerve fibres. (B) The effect of GW405833 on knee joint afferent nerve mechanosensitivity in response to rotation of OA knee joints. The sensitising effect of GW405833 was dose-dependent and significantly different from the effect of vehicle control treatment [$P < 0.0001$ by two-way ANOVA ($n = 24–28$ fibres)]. Values are the mean of all time points with 95% CIs.

Table II
Response characteristics of type III and type IV fibres after GW405833 application. In control joints the desensitizing effect of GW405833 was significantly higher in type III fibres compared to type IV fibres. In MIA joints the sensitising effect was mediated *via* type IV fibres. Differences between type III and type IV fibres were assessed using an unpaired Student's *t* test. [Data are means with 95% CIs (lower limit, upper limit)]

		% change firing rate	*N*	*P* value
Control joints	Type III	−61.6 (−79.9, −43.3)	30	
	Type IV	−29.5 (−42.61, −16.37)	67	(0.005)
MIA joints	Type III	2.3 (−8.4, 13.1)	40	
	Type IV	27.4 (9.0, 45.4)	85	(0.007)

Effect of selective CB_2 receptor antagonism on GW405833 responses in control knee joints

Co-administration of the selective CB_2 receptor antagonist AM630 reduced the desensitizing effect of the highest dose of GW405833 [$P < 0.0001$ one-way ANOVA with Bonferroni's multiple comparison test; $n = 20$; Fig. 4(A)]. AM630 given alone had no significant effect on afferent firing frequency in control knees.

Effect of selective CB_2 and TRPV1 receptor antagonism on GW405833 responses in MIA knee joints

The CB_2 receptor antagonist AM630 significantly blocked the sensitising effect of GW405833 [$P < 0.0001$; one-way ANOVA with Bonferroni's multiple comparison test; $n = 17$; Fig. 5(B)]. Pre-administration of the TRPV1 receptor antagonist SB366791 before GW405833 also inhibited the sensitising effect of the cannabinoid during joint rotation [$P < 0.0001$; one-way ANOVA with Bonferroni's multiple comparison test; $n = 26$; Fig. 4(B)]. AM630 and SB366791 given alone had no significant effect on afferent firing frequency in MIA knees.

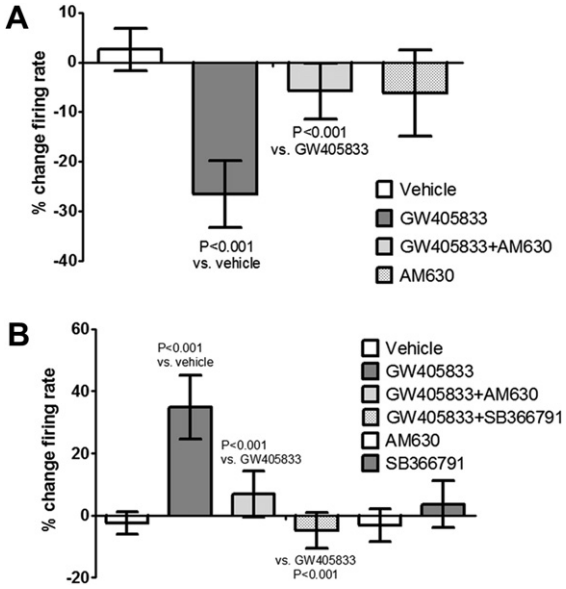

Fig. 4. (A) Effect of co-administration of the CB_2 receptor antagonist AM630 with GW405833 (10^{-8} mol) on control (saline-injected) knee joints. The CB_2 antagonist AM630 significantly reduced the desensitizing effect of GW405833; one-way ANOVA with Bonferroni's multiple comparison test. (B) In the OA joint GW405833 causes significant sensitization compared to the vehicle. This GW405833-mediated sensitization was blocked by co-administration of AM630 or by pre-administration of the TRPV1 receptor antagonist SB366791; one-way ANOVA with Bonferroni' s multiple comparison test. When administered alone, AM630 or SB366791 had no significant effect on joint mechanosensitivity. Values are the mean of all time points with 95% CIs of 12–28 fibres.

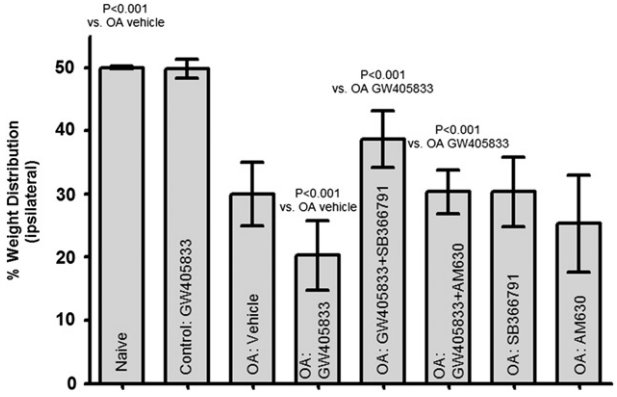

Fig. 5. Effect of intra-articular injection of GW405833 (10^{-6} mol) on hindpaw weight distribution in control and OA joints, compared to vehicle injection. In control joints, GW405833 had no significant effect on weight distribution compared to naive rats. In OA rats, following GW405833 injection, body weight was redistributed onto the non-injected contralateral hindlimb while co-administration of the CB_2 receptor antagonist AM630 or pre-administration of the TRPV1 receptor antagonist SB366791 attenuated this GW405833 induced weight-bearing deficit. The algesic effect of GW405833 was maximal 60 min after injection. Bonferroni's multiple comparison test; $n = 8$ animals/group. Data are means with 95% CIs.

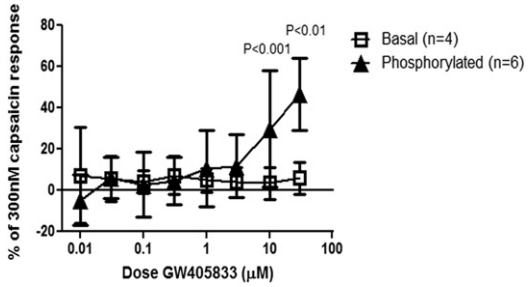

Fig. 6. Concentration–response curves for the effect of GW405833 on CGRP release from adult rat spinal cord. GW405833 assayed alone under basal (open squares) conditions of the TRPV1 channel no CGRP release was observed. After phosphorylation of the TRPV1 channel (solid triangles) CGRP release was significantly increased (two-way ANOVA $P < 0.001$). Each data point represents mean CGRP release with 95% CIs expressed as a percent of capsaicin and buffer controls. $n = 3$.

Effect of GW405833 on hindlimb weight distribution in control and MIA knee joints

The induction of OA caused a significant reduction in the amount of weight being borne on the ipsilateral hindlimb compared to naïve rats ($P = 0.001$; one-way ANOVA with Bonferroni's multiple comparison test; $n = 8$; Fig. 5). While naive rats distribute their body weight evenly between the two hindlimbs, in the MIA rat the weight placed on the ipsilateral hindlimb was reduced to about 30%. Intra-articular injection of GW405833 (10^{-6} mol) into the MIA-treated joint caused a further shift in weight distribution such that only 20% of the animal's body weight was being placed on the ipsilateral hindlimb. The shift in weight distribution reached a maximal level of incapacitance at the 60 min time point ($P < 0.001$; one-way ANOVA with Bonferroni's multiple comparison test; $n = 8$; Fig. 5). This observation indicates that GW405833 caused an enhanced pain response in the MIA joint. Injection of the vehicle had no effect on weight distribution. In saline-injected control rat knees, intra-articular injection of GW405833 had no significant effect on hindlimb weight distribution ($P = 0.67$ one-way ANOVA; $n = 6$; Fig. 5).

Effect of CB_2 and TRPV1 blockade on hindlimb weight distribution

Intra-articular co-administration of the CB_2 antagonist AM630 (10^{-8} mol) completely blocked the GW405833-mediated pain response at the 60 min time point ($P < 0.001$; one-way ANOVA with Bonferroni's multiple comparison test; $n = 8$; Fig. 5). Furthermore, intraperitoneal injection of the TRPV1 channel antagonist SB366791 prior to intra-articular injection of GW405833 blocked the pain response to the CB_2 agonist ($P < 0.001$; one-way ANOVA with Bonferroni's multiple comparison test; $n = 8$). Administration of AM630 or SB366791 by itself had no significant effect on joint incapacitance, compared to vehicle. These behavioural data are consistent with the electrophysiological results, confirming that GW405833 causes a painful response in the MIA joint and that both the CB_2 receptor and the TRPV1 channel are involved in mediating this effect.

In vitro CGRP release – effect of phosphorylation of the TRPV1 receptor

The effect of GW405833 was assessed *in vitro* under both basal (non-phosphorylated) and stimulated (phosphorylated) conditions. With the TRPV1 receptor in its non-phosphorylated state, GW405833 had no effect on CGRP release up to a concentration of 30 μM (Fig. 6). However, with TRPV1 in the phosphorylated state, GW405833 evoked a significant release of CGRP with an EC_{50} of 9 μM and E_{max} of 60% (compared to a maximally effective concentration of 0.3 μM capsaicin). These data suggest that under phosphorylated conditions GW405833 can act as an agonist at the TRPV1 receptor.

Discussion

Several studies confirm the presence of functionally active CB_2 receptors on immunocytes, primary afferent nerves, dorsal root ganglia and in the spinal cord[35–37]. Recently, CB_2 receptor expression has been demonstrated in the synovium of OA patients, supporting a potential role for CB_2-selective ligands in the treatment of arthritis pain[36–38]. In the present study, we discovered that CB_2 receptors are present in the knee joints of rats and that local application of the CB_2 receptor agonist GW405833 inhibited nociceptive responses in non-arthritic knee joints. In the MIA joint, however, GW405833 was no longer anti-nociceptive, but surprisingly led to sensitization of joint mechanosensitive nerve fibres. These electrophysiological findings were corroborated in a behavioural test of joint pain which showed an algesic effect of GW405833 after induction of OA whereas the drug had no effect on weight bearing in control animals. The paradoxical finding that GW405833 is anti-nociceptive in control joints but pro-nociceptive in MIA knees indicates that the modulatory role of this particular cannabinoid in pain transmission is dictated by the pathophysiological status of the organ. Similar observations have been reported in other studies where the CB_2 receptor agonist JWH133 produced an increase in synovial blood flow when applied locally in normal knee joints but failed to elicit any significant vasomotor changes in inflamed knee joints[39]. The algesic effect of GW405833 highlighted here is in contrast to other studies showing an analgesic effect of GW405833 in models of acute inflammatory pain[16,21,40–42]. It should be noted, however, that in the aforementioned studies GW405833 was administered systemically in very high doses and could therefore be having spinal or even supraspinal sites of action[43].

The mechanisms responsible for the reported anti-nociceptive effects of CB_2 agonists are still unknown. In the present study, it is unlikely that GW405833 is inhibiting mechanosensitivity in normal joints by activating CB_2 receptors on immunocytes thereby decreasing the release of pro-inflammatory mediators since there are negligible amounts of circulating inflammatory cells under control conditions. It is more likely that the anti-nociceptive effect of GW405833 reported here represents a direct inhibition of

mechanotransduction in joint primary afferent nerve fibres. Closer analysis of the relative contribution of different mechanosensitive nerve fibre subtypes to this phenomenon revealed that type III and type IV afferents produced a differential response to GW405833. The observed reduced mechanosensitivity in control joints was mainly mediated *via* thinly myelinated type III afferents, whereas the pro-nociceptive effect of GW405833 in MIA joint was mediated mainly by type IV afferents. Interestingly, these electrophysiological findings correspond with our histological data which showed that CB_2 receptors on large and medium-sized neurones (type III fibres) occur in isolation whereas CB_2 receptors on small sized neurones (type IV fibres) often co-localize with the pro-nociceptive TRPV1 channel. It has been shown that unmyelinated type IV fibres originate from small diameter somata and myelinated type III fibres originate from medium and large diameter somata[44]. This finding is similar to a previous study showing TRPV1 and CB_2 receptor co-localisation in a subpopulation of human sensory neurones[25]. Thus in MIA joints, GW405833 may promote joint pain by interacting with TRPV1 channels on type IV joint afferents. Indeed in this study, pre-treatment of MIA joints with the TRPV1 antagonist SB366791 blocked the pro-nociceptive and hyperalgesic effects of GW405833. Evidence elsewhere found that CB_2 receptor activation can modulate TRPV1 sensitivity[25] and that the TRPV1 channel is essential for the functional activity of CB_2 receptor agonists in modulating synovial blood flow[39].

An additional explanation as to why GW405833 reduced mechanosensitivity in control joints but increased nociceptive responses when administered to MIA joints may be related to whether the TRPV1 channel exists in its phosphorylated (sensitized) or dephosphorylated (desensitized) state[45,46]. In an *in vitro* assay, we found that GW405833 caused the release of the pro-nociceptive neuropeptide CGRP from spinal cord neurones but only when neuronal TRPV1 receptors were in the phosphorylated state. Thus, GW405833 may be acting as a weak partial agonist at the phosphorylated TRPV1 receptor leading to the secondary release of CGRP which further sensitises joint nociceptors causing pain. Inflammation-induced phosphorylation of TRPV1[47,48] as well as an up-regulation of the channel[49] could increase the probability of GW405833 binding to TRPV1 in MIA animals such that the pro-nociceptive, TRPV1-mediated actions of GW405833 in MIA knees supersede its anti-nociceptive CB_2 receptor-mediated effects as seen in normal joints.

Conclusion

In this study it was found that local administration of the CB_2 receptor agonist GW405833 significantly suppressed joint nociceptive transmission in normal joints by reducing primary afferent nerve activity. Conversely in MIA joints, GW405833 appears to bind to sensitized TRPV1 receptors on type IV joint afferents leading to the secondary release of CGRP which further sensitises joint afferents causing hyperalgesia. Whether this process holds true for other CB_2 receptor agonists requires additional investigation.

Author contributions

Experimental design and intellectual input: NS, JJMcD, MPJ, AJM, ESN.

Data collection and analysis: NS, CZ, JJMcD, AJM, LMB, DLH.

Data interpretation and manuscript writing: NS, JJMcD, AJM, MPJ, ESN.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

The technical assistance of Chantelle Reid is gratefully acknowledged. This work was supported by an Industry Partnership Programme grant from the Canadian Arthritis Network (CAN) and Eli Lilly & Co, USA. JJMcD is the recipient of an Alberta Heritage Foundation for Medical Research (AHFMR) Senior Scholar award and an Arthritis Society of Canada Investigator award. NS received postdoctoral fellowship funding from CAN and AHFMR.

Supplementary data

The supplementary data associated with this article can be found in the on-line version at doi:10.1016/j.joca.2010.09.005.

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