Supplemental Material

Methods

MIA-induced osteoarthritis

Anesthetized (2-3% isoflurane in O_2) rats received a single intra-articular injection of MIA (1 mg/50µl) in sterile saline through the infrapatellar ligament of the left knee. Control rats received 50µl sterile saline.

Immunohistochemistry

Synovial sections from patients with OA (n=9), RA (n=8) or from PM controls (n=9) were prepared for immunohistochemistry[1] with an affinity-purified rabbit polyclonal antibody directed against human TRPV1 (SC-20813, Santa Cruz Biotechnology Inc). Sections were incubated with the TRPV1 antibody, then a biotinylated secondary antibody and peroxidase-labelled avidin-biotin complex (ABC) and visualised by the nickel enhanced diaminobenzidine method[2]. TRPV1 and macrophage (CD68) immunoreactivities in synovial sections from arthritic human (n=6) were colocalised by double immunofluorescence. A mouse monoclonal antibody directed against CD68 for human (clone KP1) was used and visualised using Texas Red-conjugated horse anti-mouse antibodies. Negative controls with primary antibodies omitted confirmed the specificity of detected immunoreactivities. Quantification was undertaken in a blinded fashion with computer-assisted image analysis as described previously[1]. TRPV1 fractional area was defined as the percentage synovial tissue section area (within 200 µm of the synovial surface) occupied by TRPV1 immunoreactivity.

MIA- and saline-treated rats were terminally anaesthetised, transcardially perfused and the lumbar spinal cord was removed, post-fixed and stored. 40 µm free-floating sections of L3-5 lumbar spinal cord were incubated with a polyclonal guinea-pig anti-TRPV1 antibody (1:500, Neuromics, Edina, MN, USA catalogue number GP14100, previously validated in spinal cord [3]) at 4°C for 4 hours and then 24 hours at room temperature. Sections were incubated with

Alexa 568 conjugated goat anti-guinea pig secondary antibody (1:300, Molecular Probes). Negative controls with primary antibody omitted were conducted. TRPV1 immunostaining was visualised with a Leica DMRB / DM4000 B fluorescence microscope and images were acquired using Openlab software (PerkinElmer). Quantification was performed with Velocity 5.5 software (Perkin Elmer, UK) using a minimum of five sections per rat. Images were flat-field corrected prior to quantification, using an intensity correction slide (Leica, Milton Keynes, UK). A region of interest analysis of the superficial dorsal horn was quantified by an observer blinded to the experimental groups.

LC-MS/MS analysis

Internal standard 15-HETE-d8 (10 µl of 7.6 µM) was added to each sample or blank sample (0.2 ml water), along with 10 µl of the antioxidant butylhydroxytoluene (BHT). Ethyl acetate: hexane (9:1 *v/v*) was used to extract 12-HETE from the knee joint tissue. Measurement of 12-HETE was performed using LC-MS/MS using a Shimadzu series 10AD VP LC system (Shimadzu, Columbia, MD, USA) and an Applied Biosystems MDS SCIEX 4000 Q-Trap hybrid triple-quadrupole–linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) in electrospray negative ion mode. Quantification was performed by an internal standardisation calibration method using Analyst 1.4.1 software (Applied Biosystems, Foster City, CA, USA). The limit of quantification was 0.05 pmol/g 12-HETE.

TaqMan quantitative real-time polymerase chain reaction (RT-PCR)

RNA extraction and cDNA synthesis: Sections (40 x 30µm sections per sample) were cut from OTC-embedded synovium by cryostat (Leica, CM1900). Samples were heated at 65°C in HPLC water to melt the OTC. Samples were centrifuged (10,000rpm) and the supernant discarded. For total RNA extraction synovial samples were homogenized in 1ml of ice cold Tri reagent (Sigma-Aldrich,UK) and RNA was purified and extracted according to the manufacturers' instructions. For cDNA synthesis, 500 ng of total RNA was reverse transcribed using AffinityScript (Invitrogen) in a total reaction volume of 25µl. Reactions were

incubated for 10 min at 25℃, then 60 min at 50℃ and the reaction terminated by incubation at 70℃ for 15 min. Due to low gene expression in synovia, cDNA synovial samples were pre-amplified for 16 cycles at 95℃ for 10min, then 95℃ for 15s, and 60℃ for 4min. Samples were stored at -20℃.

Taqman quantitative real time polymerase chain reaction: cDNA samples were amplified with Taqman® Fast Universal PCR Master Mix Kit (Applied biosystems,USA) and run in triplicate on a 96 well Optical Reaction Plate (Applied Biosystems, USA). Gene expression was quantified using the relative standard curve method based on TaqMan qRT-PCR. Thermocycler parameters were set as follows: 40 cycles of 95℃ for 20 s, 95℃ for 1s and 60℃ for 20s. Primers and probes were designed by primer 3.0 and synthesized by Eurofins (UK). Data are expressed as a ratio of target gene normalized by β-actin.

Primers and probes: Beta actin forward primer -5- AGCCATGTACGTAGCCATCCA-3, reverse primer -5- TCTCCGGAGTCCATCACAATG-3 , probe-5-TCTCCCTGTATGCCTCTGGTCGTACCAC-3; TRPV1 forward primer -5-TCAAAGACCCAGAGACAGGAAAG-3, reverse primer -5-CTGTCTTCCGGGCAACGT-3, probe-5-AAAAGCCAGCTCAATCTGCACAATGG-3.

Drugs and reagents

Sodium pentobarbital (Sigma, Dorset, U.K) was dissolved in 10% ethanol, 20% propylene glycol, 70% distilled H_20 to allow i.p. and i.v injection for the induction (50-60mg/kg) and maintenance (20mg/kg/hr) of anaesthesia respectively in joint afferent recording experiments. Mono-sodium iodoacetate (MIA; Sigma, Dorset, UK) was dissolved in sterile saline for intra-articular injection (1mg/50µl). JNJ-17203212 and capsaicin were obtained from Tocris (Bristol, UK) and for intra-arterial injection in knee joint afferent recording experiments were made up in 2.5 or 5% ethanol, 10% Tween 80 in saline. Human TRPV1 (SC-20813) polyclonal antibody was purchased from Santa Cruz Biotechnology Inc. (USA). Rat TRPV1

polyclonal guinea-pig antibodies were purchased from Neuromics, Edina, MN, USA (catalogue number GP14100). Biotinylated secondary antibodies, peroxidase-labelled ABC and fluorescein-conjugated ABC kits were from Vector Laboratories Ltd., Peterborough, UK. CD68 for human (clone KP1) and goat anti-rabbit horseradish peroxidase conjugate was from DakoCytomation, Ely, UK. For mass spectrometry (MS) acetonitrile, ammonium hydroxide, ethanol, ethyl acetate, hexane, formic acid and methanol were all purchased from Fisher Scientific (Loughborough, UK). All solvents were HPLC grade and far UV grade acetonitrile was also used. 12-hydroxyeicosatetraenoic acid (12-HETE) was purchased from Cambridge Bioscience (Cambridge, UK). HPLC grade water (ELGA Ltd. High Wycombe, UK) was used in all experiments.

Results

Effects of systemic JNJ-17203212 on core body temperature

Consistent with previous reports[4], systemic administration of JNJ-17203212 (10.6mg/1.05ml) significantly ($p<0.05$ at 0.5 hr) increased core body temperature (baseline = 37.40 ±0.17ºC; 0.5h = 38.63 ± 0.27 ºC, 2h = 38.10 ± 0.13ºC) compared to systemic vehicle-treated rats (baseline = 37.10 ±0.12ºC; 0.5h = 37.58 ± 0.11 ºC, 2h = 37.62 ± 0.09ºC).

S1: Effects of intra-articular injection of MIA versus saline on pain behaviour

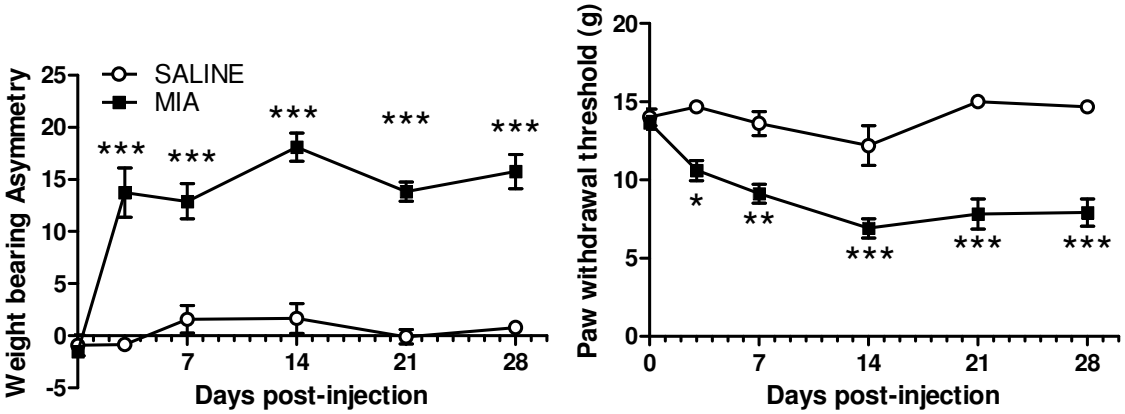


Figure S1: Representative sample of data showing that intra-articular injection of MIA produced increased weight-bearing asymmetry and decreased mechanical withdrawal thresholds on the ipsilateral hind limb compared to saline-treated rats (n= 52 rats and n=15 rats respectively). Analysis was performed using a 2-way ANOVA (weight bearing) with a Bonferroni's post-hoc test or Kruskall Wallis test (withdrawal thresholds), *$p<0.05$, **$p<0.01$, ***$p<0.001$.

S2: Effects of intra-articular and systemic JNJ-17203212 on allodynia

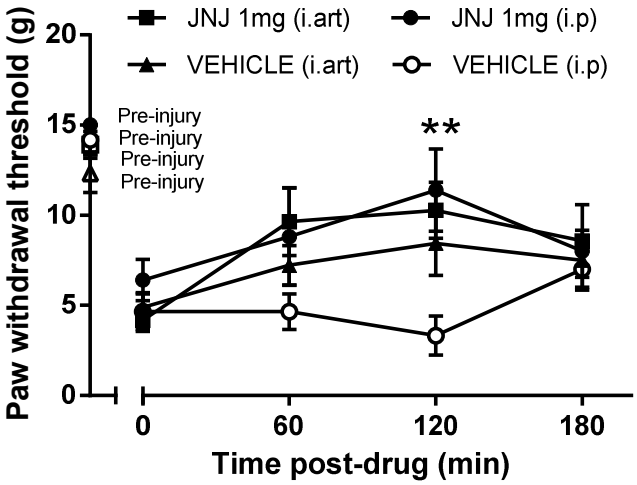


Figure S2. Systemic but not intra-articular administration of JNJ-17203212 significantly attenuated distal allodynia in rats 14 days post MIA injection (n=7/11 rats), compared to vehicle (3%Tween80, 0.5% EtOH in saline)-treated rats (n=6/9 rats). Data are presented as means ± SEM and analysis used a Mann Whitney test (allodynia). ***P* <0.01.

S3: Representative extracted ion chromatograms

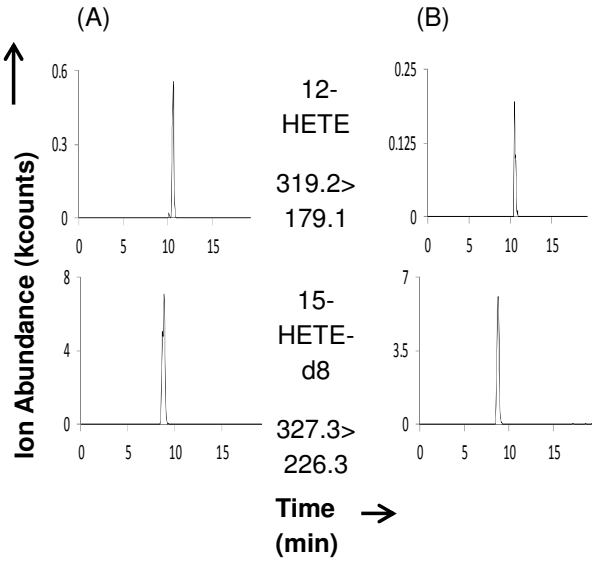


Figure S3: Representative selective ion chromatograms of 12-HETE and the internal standard 15-HETE-d8 in extracts of knee joint and DRG in (A) MIA vs (B) saline treatment.

S4: TRPV1 mRNA levels in synovium from MIA- and saline-treated rats

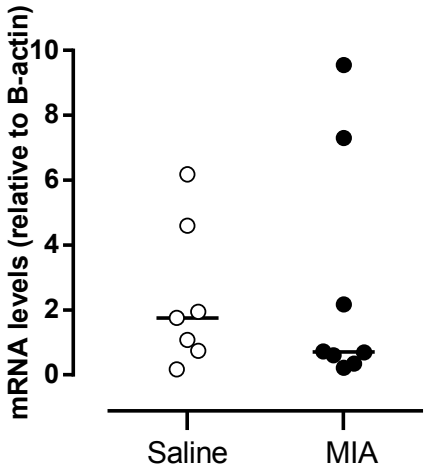


Figure S4: TRPV1 messenger RNA expression relative to beta-actin in synovium from saline- and MIA-treated (n=7/8) rats.

S5: Baseline mechanical thresholds of knee afferents in MIA- and saline-treated rats

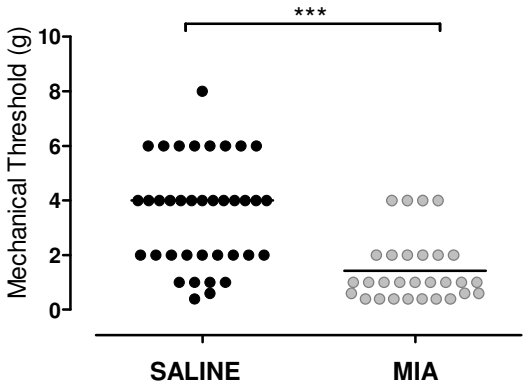
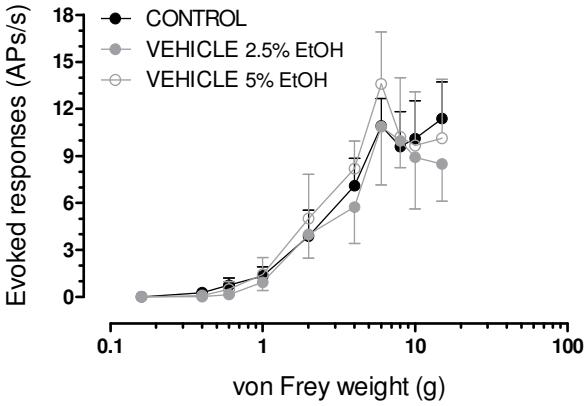


Figure S5: Baseline mechanical thresholds of joint afferents were significantly reduced in MIA rats compared to saline rats ($p<0.01$, n=30/37 MIA/saline respectively). Bars represent median values and analysis used Mann-Whitney test. ** $p<0.01$.

S6: Effects of vehicle on evoked responses of knee afferents

A. MIA-treated rats



B. Saline-treated rats

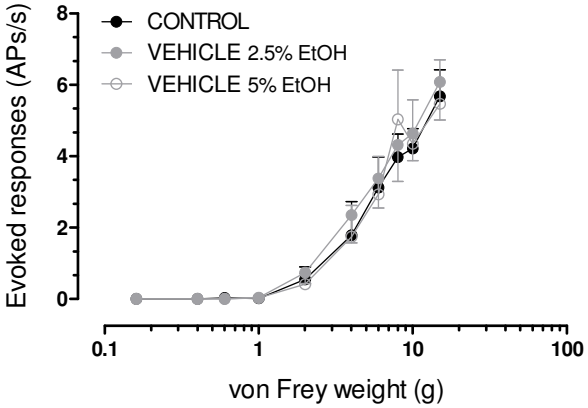


Figure S6: Peripheral vehicle(2.5 and 5% ethanol, 10% tween 80 in saline) administration had no significant effect on evoked responses of joint afferents in both MIA (A) and saline rats (B) (n=11/7 MIA/saline respectively). Data are displayed at means ± SEM. Analysis used two-way ANOVA with Bonferroni's post-hoc test.

S7: Effects of JNJ-17203212 on capsaicin evoked firing of knee joint afferents

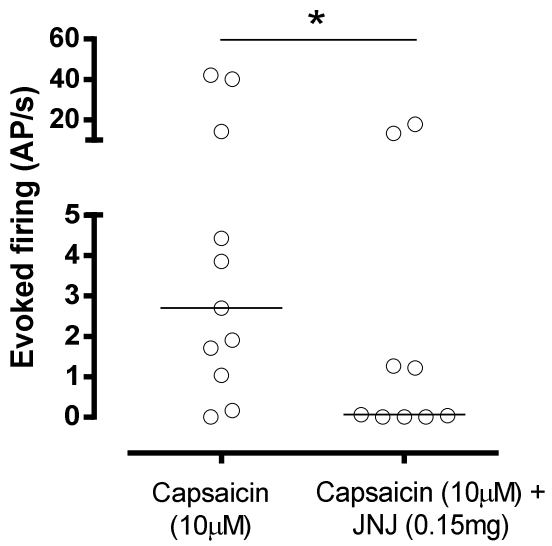


Figure S7. JNJ-17203212 significantly attenuated capsaicin-evoked firing of knee joint afferents, confirming antagonism and selectivity of JNJ-17203212 at TRPV1 at the level of the joint. Capsaicin (10μM/100μl) was administered close to the ipsilateral knee joint either alone (n=8) or 15 mins post-JNJ-17203212 (0.15mg/100μl; n=7). Data points are mean firing frequency (1 s bins) during 30 s post-capsaicin administration. Horizontal lines are medians, analysis used Mann Whitney, $^{*}p<0.05$.

References

1. Haywood L, McWilliams DF, Pearson CI, et al. Inflammation and angiogenesis in osteoarthritis. *Arthritis Rheum* 2003;**48**(8):2173-2177.
2. Shu SY, Ju G, Fan LZ. The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. *Neurosci Lett* 1988;**85**(2):169-171.
3. Yu SQ, Wang DH. Enhanced salt sensitivity following shRNA silencing of neuronal TRPV1 in rat spinal cord. *Acta Pharmacol Sin* 2011;**32**(6):845-852.
4. Swanson DM, Dubin AE, Shah C, et al. Identification and biological evaluation of 4-(3-trifluoromethylpyridin-2-yl)piperazine-1-carboxylic acid (5-trifluoromethylpyridin-2-yl)amide, a high affinity TRPV1 (VR1) vanilloid receptor antagonist. *J Med Chem* 2005;**48**(6):1857-1872.