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RESEARCH PAPER

Spinal administration of the monoacylglycerol lipase inhibitor JZL184 produces robust inhibitory effects on nociceptive processing and the development of central sensitization in the rat

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BACKGROUND AND PURPOSE

The cannabinoid receptor-mediated analgesic effects of 2-arachidonoylglycerol (2-AG) are limited by monoacylglycerol lipase (MAGL). 4-nitrophenyl 4-[bis (1,3-benzodioxol-5-yl) (hydroxy) methyl] piperidine-1-carboxylate (JZL184) is a potent inhibitor of MAGL in the mouse, though potency is reportedly reduced in the rat. Here we have assessed the effects of spinal inhibition of MAGL with JZL184 on nociceptive processing in rats.

EXPERIMENTAL APPROACH

In vivo spinal electrophysiological assays in anaesthetized rats were used to determine the effects of spinal administration of JZL184 on spinal nociceptive processing in the presence and absence of hindpaw inflammation. Contributions of CB_1 receptors to these effects was assessed with AM251. Inhibition of 2-oleoylglycerol hydrolytic activity and alterations of 2-AG in the spinal cord after JZL 184 were also assessed.

KEY RESULTS

Spinal JZL184 dose-dependently inhibited mechanically evoked responses of wide dynamic range (WDR) neurones in naïve anaesthetized rats, in part via the CB_1 receptor. A single spinal administration of JZL184 abolished inflammation-induced expansion of the receptive fields of spinal WDR neurones. However, neither spinal nor systemic JZL184 altered levels of 2-AG, or 2-oleoylglycerol hydrolytic activity in the spinal cord, although JZL184 displayed robust inhibition of MAGL when incubated with spinal cord tissue *in vitro*.

CONCLUSIONS AND IMPLICATIONS

JZL184 exerted robust anti-nociceptive effects at the level of the spinal cord *in vivo* and inhibited rat spinal cord MAGL activity *in vitro*. The discordance between *in vivo* and *in vitro* assays suggests that localized sites of action of JZL184 produce these profound functional inhibitory effects.

LINKED ARTICLES

This article is part of a themed section on Cannabinoids. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2012.167.issue-8

Abbreviations

2-AG, 2-arachidonoylglycerol; 2-OG, 2-oleoylglycerol; AEA, N-arachidonoylethanolamine (anandamide); AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl) pyrazole-3-carboxamide; DAGLα, diacylglycerol lipase α; FAAH, fatty acid amide hydrolase; JZL184, 4-nitrophenyl 4-[bis (1,3-benzodioxol-5-yl) (hydroxy) methyl] piperidine-1-carboxylate; MAFP, methyl arachidonylfluorophosphonate; MAGL, monoacylglycerol lipase; RF, receptive field; URB597, 3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate; WDR, wide dynamic range

Introduction

The roles of the cannabinoid CB_1 and CB_2 receptors and the endogenous ligand anandamide (AEA) in the modulation of nociceptive processing have been extensively studied (Pertwee, 2001; Guindon and Hohmann, 2009; receptor nomenclature follows Alexander *et al.*, 2011). In contrast, the modulation of nociceptive processing by the more abundant endocannabinoid 2-arachidonylglycerol (2-AG) has been less widely investigated.

2-AG is synthesized in response to high levels of neuronal activity by diacylglycerol lipase (DAGLα), and reduces neuronal excitation via actions at CB_1 receptors (Tanimura *et al.*, 2010). Signalling is rapidly terminated via enzymic hydrolysis of 2-AG by monoacylglycerol lipase (MAGL) (Dinh *et al.*, 2002). In the superficial laminae of the spinal cord, CB_1 receptor expression is pre-synaptic on glutamatergic terminals (Nyilas *et al.*, 2009) and on around 20% of GABAergic interneurones in the dorsal horn (Hegyi *et al.*, 2009), while DAGLα is expressed on post-synaptic membranes (Nyilas *et al.*, 2009). The complementary location of the 2-AG signalling machinery in nociceptive regions of the spinal cord suggests that it may have an important role in the modulation of nociceptive processing. Enhancing 2-AG signalling in the spinal cord is thus an attractive, mechanism-based approach for the development of analgesics. However, progress in this area has been hindered by the paucity of selective MAGL inhibitors.

Recent reports identify 4-nitrophenyl 4-[bis (1,3-benzodioxol-5-yl) (hydroxy) methyl] piperidine-1-carboxylate (JZL184) as a potent inhibitor of MAGL in mice, with high selectivity over other serine hydrolases and lipases (Long *et al.*, 2009a). Indeed, systemic JZL184 (8 $mg \cdot kg^{-1}$) administration in mice produced a fivefold elevation of brain levels of 2-AG, with minimal inhibition of fatty acid amide hydrolase (FAAH), an enzyme associated primarily with AEA hydrolysis. A higher dose of JZL184 (16 $mg \cdot kg^{-1}$) produced significant anti-nociceptive effects in models of acute pain and neuropathic pain in mice (Kinsey *et al.*, 2009). However, hypomotility and inhibition of FAAH (>50%) were also reported with this dose, although levels of AEA were not elevated (Kinsey *et al.*, 2009). JZL184 displays a 10-fold lower potency in the rat (Long *et al.*, 2009b), yet local administration in the rat hindpaw attenuated inflammatory nociceptive processing (Spradley *et al.*, 2010; Guindon *et al.*, 2011), via both CB_1 and CB_2 receptor-mediated mechanisms. Administration of JZL184 via this route also raised local levels of 2-AG in the hindpaw Guindon *et al.* (2011) . The potential contribution of a spinal site of action of MAGL inhibitors to the reported anti-nociceptive effects of this compound has yet to be explored.

The aim of the present study was to investigate whether inhibition of MAGL at the level of the spinal cord contributed to the inhibitory effects of JZL184 in models of acute and inflammatory pain in the rat. To further consolidate the evidence that the actions of JZL184 are mediated via inhibition of MAGL, we also measured levels of 2-AG and enzymic activity in the spinal cord. Here we report a robust inhibition of acute and inflammation-driven spinal nociceptive processing by JZL184, but were unable to detect concomitant changes in 2-AG levels or in *ex vivo* hydrolysis of 2-oleoylglycerol (2-OG) in the spinal cord. The contribution of species and tissue differences in the effects of JZL184 on MAGL activity *in vitro* were then investigated to further analyse the mechanisms underlying these findings.

Methods

Animals

All animal care and experimental procedures were in accordance with the Animals (Scientific Procedures) Act 1986 and International Association for the Study of Pain guidelines. Eighty-nine male Sprague Dawley rats (225–300 g) and three male C57BL/6 mice (25–35 g) were purchased from Charles River, Margate, UK. Animals were group housed with *ad libitum* access to food and water. The results of all studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010).

In vivo *electrophysiological studies*

Methods were based on those of Elmes *et al.* (2004). Rats were anaesthetized with 2–3% isoflurane in 66% N_2O/33% O_2; a tracheal cannula was inserted and they were placed in a stereotaxic frame. A laminectomy was performed to expose segments L4–L5 of the spinal cord. On completion of surgery, isoflurane levels were reduced to 1–1.5%, which maintained a state of complete areflexia. Extracellular single-unit recordings from one lamina V–VI wide dynamic range (WDR) neurone per animal were performed. Action potentials were digitized and analysed using a CED micro1401 interface and Spike 2 data acquisition software (Cambridge Electronic Design, Cambridge, UK).

Electrical and mechanically evoked responses of WDR neurones were recorded. Mechanical stimuli were applied using von Frey hairs (calibrated to 8, 10, 15, 26 and 60 g). von Frey hairs were applied for 10 s each, at 10 s intervals, recording action potentials in bins of 1 s. Trains of mechanical stimuli (8–60 g) were repeated at 10 min intervals. Responses were deemed stable when variance in responses for each filament was less than 10% for three separate sets of stimuli.

JZL184 in naïve rats. JZL184 and AM251 were stored as ethanol stock solutions. Ethanol was evaporated prior to dilution into vehicle (3% Tween 80 in 0.9% physiological saline solution) on the day of use. Drugs were administered topically onto the spinal cord via a Hamilton syringe. Effects of spinal administration of 25, 50 and 100 μg in 50 μL JZL184 (0.96, 1.92 and 3.85 mM, respectively, n = 5–8 per dose), or three equivalent doses of 50 μL vehicle (n = 8) on mechanically evoked responses of WDR neurones were studied at 60 min intervals. Doses were based on pilot studies and earlier reports of local administrations of this compound (Spradley *et al.*, 2010**;** Guindon *et al.*, 2011). In a separate group of rats, the CB_1 receptor antagonist AM251 (1 μg in 50 μL, 3.6 μM) was given 30 min prior to spinal administration of either JZL184 (100 μg in 50 μL, n = 7) or vehicle (50 μL, n = 6). The dose of AM251 employed was based on earlier reports (Ibrahim *et al.*, 2005; Jhaveri *et al.*, 2006).

JZL184 in carrageenan-inflamed rats. Effects of spinal administration of JZL184 (100 μg in 50 μL) on carrageenan-induced receptive field (RF) expansion of WDR neurones were studied. The RF size of the neurone was mapped using 8 and 26 g von Frey filaments as previously described (Torsney and Fitzgerald, 2003). Once stable responses were obtained, rats received an intraplantar injection of 100 μL of a 2% carrageenan solution into the ipsilateral hindpaw. On the basis of the time course of the effects of spinal JZL184 and the profile of the carrageenan response, JZL184 (100 μg, n = 6) or vehicle (n = 6) was given spinally at 50 min after carrageenan injection. The RF size of WDR neurones in response to mechanical stimuli was recorded until 180 min after carrageenan. Paw circumference, as a marker of the inflammatory response, was recorded at 60 min intervals.

Effects of systemic JZL184 on food intake in rats

Feeding studies were similar to those previously reported in mice (Wiley *et al.*, 2005). Rats received 40 $mg \cdot kg^{-1}$ JZL184 (dissolved in a vehicle of 4:1 polyethylene glycol 400:Tween 80 at a concentration of 40 $mg \cdot mL^{-1}$, and administered i.p. in a volume of 1 $mL \cdot kg^{-1}$, n = 5) or vehicle (n = 5), and were then fasted for 3 h. At this time point, 20 g of standard rat chow was supplied, and food intake over a 60 min period was assessed. Behavioural measurements were conducted at 4 h post-drug, based on an earlier report (Long *et al.*, 2009a). To reduce the effects of handling stress on levels of lipids, rats were stunned and decapitated, and the lumbar region of the spinal cord were collected for analysis 2 h following behavioural assessment. Given that JZL184 is an irreversible inhibitor, the effects of JZL184 are long lasting; a single systemic administration of 16 $mg \cdot kg^{-1}$ produced >80 % inhibition of MAGL for 24 h in mice (Long *et al.*, 2009a).

Tissue collections

To determine the effects of spinal JZL184 on endocannabinoid levels, a separate group of rats was surgically prepared as described for electrophysiological studies. Rats received a single spinal dose of either 50 μL of JZL184 (100 μg, n = 16) or vehicle (50 μL 3% Tween 80 in 0.9% physiological saline solution, n = 16). Mechanical stimulation of the hindpaw was performed as described above, and rats were killed 40 min after drug administration (based on timing of maximal inhibitory effect on neuronal responses) via anaesthetic overdose. The lumbar enlargement of the spinal cord was excised, split into ipsi- and contralateral halves, and snap frozen in liquid nitrogen and stored at –80°C.

To determine whether carrageenan-induced inflammation altered the spinal endocannabinoid receptor system, rats received either an intraplantar injection of 100 μL 2% carrageenan solution (n = 6) or 100 μL saline (n = 6). At 3 h, rats were killed and spinal cord tissue was collected as above.

For *in vitro* investigation of the effects of JZL184 on monoacylglycerol hydrolysis, male Sprague Dawley rats (n = 3) and C57BL/6 mice (n = 3) were killed and whole brain and spinal cords were rapidly dissected out. Brains were hemisected along the sagittal plane, and spinal cords (thoracic and lumbar regions) remained whole. Tissues were preserved and stored as described above.

Measurement of endocannabinoids and N-acylethanolamines

2-AG and AEA were quantified in JZL184- (n = 11), and vehicle-treated (n = 11) spinal cord samples based on a liquid chromatography-tandem mass spectrometry as described previously (Richardson *et al.*, 2007; Sagar *et al.*, 2010). Briefly, samples were homogenized and extracted with ethyl acetate: hexane (9:1 v/v), supernatants, evaporated to dryness and reconstituted in acetonitrile : water (1:1) before analysis. HPLC analysis used a Shimadzu series 10AD VP system (Shimadzu, Columbia, MD, USA) and a Phenomenex Luna C18 column (150 × 2.0 mm, 3 μm particle size) with a gradient mobile phase (water/methanol/acetonitrile). Mass spectrometry was performed on an ABI MDS SCIEX 4000 QTRAP hybrid triple quadrupole-linear ion trap instrument (Applied Biosystems, Foster City, CA, USA) in electrospray positive mode. Quantification was performed using Analyst 1.4.1 using an internal standard method, with deuterated AEA (AEA-d_8) and 2AG (2AG-d_8) as internal standards (Cayman Europe, Tallinn, Estonia).

Protein and gene expression

DAGLα and MAGL gene and protein expression in spinal cord tissue from carrageenan- (n = 3), and saline-treated rats (n = 3) were probed relative to β-actin via Taqman qRT-PCR and Western blotting respectively. Methods were as previously described (Sagar *et al.*, 2010). Primer sequences were: DAGLα: forward primer ACCTGCGGCATCGGTTAG, reverse primer CTTTGTCCGGTGCAACAG, probe CAGC TGGTCCCGCCGTCTAAAAGTG; MAGL: forward primer TGCCATCTCCATCCTAGCAG, reverse primer CAAGGATAT GTTTGGCAGGA, probe ATCCGGAATCTGCATCGACTTTGA. Goat anti-DAGLα (Abcam, 1:1000, Cambridge, UK), rabbit anti-MAGL (Cayman, 1:200 dilution, Ann Arbor, MI, USA) (Chanda *et al.*, 2010) and mouse anti-β-actin (Sigma, 1:5000, St Louis, MO, USA) primary antibodies were utilized. Secondary antibodies were IRDye® conjugated donkey polyclonal anti-goat, or goat polyclonal anti-rabbit, and goat polyclonal anti-mouse IgG (Li-Cor® Biosciences, 1:10000 dilution, Lincoln, NE, USA) as appropriate. Scanning and densitometric analysis of blots were performed using a Li-Cor® ODYSSEY infrared imaging system and software.

Measurement of MAGL and FAAH activity

Homogenates from spinal cords were separated into membrane and cytosolic fractions by centrifugation (2 × 20 000× *g*, 30 min). MAGL activity was determined in cytosolic fractions via incubation of diluted homogenates with 2-oleoyl-[^{3}H]-glycerol ([^{3}H]-2-OG, American Radiolabelled Chemicals, St Louis, MO, USA). Monoacylglycerols share synthetic and catabolic pathways and, although these lipids have varying effects *in vivo*, the current evidence for monoacylglycerol turnover suggests that the catabolic pathway does not discriminate between different acyl sidechains *in vitro*. 2-AG, 2-LG and 2-OG have equal affinity at rat brain cytosol MAGL (Ghafouri *et al.*, 2004), and this is further supported by the observation that 2-OG and 2-AG are hydrolyzed by MAGL with approximately the same catalytic efficiency (Ho and Hillard, 2005). 2-OG has the advantage that, unlike 2-AG, it is not a substrate for arachidonic acid-biotransforming enzymes, such as cyclooxygenases and lipoxygenases, which may reduce the concentrations of 2-AG and hence lead to false estimates of MAGL activity. For these reasons, 2-OG has been used as a substrate for the determination of MAGL activity *in vitro* (see Dinh *et al.*, 2002; Hohmann *et al.*, 2005; Vandevoorde *et al.*, 2005; Björklund *et al.*, 2010). Therefore, [^{3}H]-2-OG was selected for use in this study and incubated with cytosolic fractions at 37°C for 30 min, followed by termination of the reaction with 300 µL activated charcoal (10% w/v) in 0.5 M HCl. [^{3}H]-glycerol in the supernatant layer following a 13 000× *g*, 5 min centrifugation was quantified by liquid scintillation counting. A 30 min pre-incubation with 1 µM MAFP was utilized to quantify non-enzymatic hydrolysis of substrate, which was not different from tissue blanks. For *in vitro* assays of JZL184 potency, varying concentrations of JZL184 were substituted for MAFP. The duration of the pre-incubation and incubation periods was based on pilot experiments, which replicated published reports (Long *et al.*, 2009a; Björklund *et al.*, 2010). FAAH activity in the membrane fraction was measured in the presence of 5 µM *N*-arachidonoyl-[^{3}H]-ethanolamine ([^{3}H]-AEA, American Radiolabelled Chemicals) in a similar fashion, quantifying liberated [^{3}H]-ethanolamine by liquid scintillation counting. URB597 (1 µM) was utilized to determine non-FAAH AEA hydrolysis, which was not different from tissue blanks. MAFP, URB597 and JZL184 were dissolved and diluted in ethanol to the required concentrations.

Data analysis

Mean maximal effects of JZL184 on neuronal responses were compared with baseline data via repeated measures two-way analysis of variance, with Bonferroni *post hoc* test. Mean maximal inhibitory effects, as a percentage change from baseline response, were compared with time-matched vehicle data via Kruskal–Wallis test with Dunn's multiple comparison *post hoc* test. RF size was quantified using region of interest analysis in ImageJ (NIH open software with Mac biophotonics plug-ins, Bethesda, MD, USA) and expressed as a percentage change compared with baseline. Effects of JZL184 compared with vehicle on the RF size were analysed with a Mann–Whitney *U*-test.

For enzyme assays, hydrolytic rates were compared via Mann–Whitney *U*-tests. Concentration-inhibition data were analysed via non-linear regression curves in GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA).

In all other experiments, statistical comparisons between two groups were made with a Mann–Whitney *U*-test, and between three groups with a Kruskal–Wallis test and Dunn's multiple comparison *post hoc* tests.

Materials

4-Nitrophenyl 4-[bis (1,3-benzodioxol-5-yl) (hydroxy) methyl] piperidine-1-carboxylate (JZL184) was a kind gift from Dr Jonathan Long of the Scripps Research Institute, San Diego, CA, USA. 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-(1-piperidyl) pyrazole-3-carboxamide (AM251) and

Table 1

Depth and electrical properties of dorsal horn WDR neurones used in these studies (mean ± SEM)

Treatment group	Depth (µm)	C-fibre latency (ms)	C-fibre threshold (mA)
Vehicle (50 µL) *n* = 8	856 ± 82	173 ± 15	1.1 ± 0.04
JZL184 (25, 50, 100 µg) *n* = 5–8	776 ± 81	198 ± 13	1.4 ± 0.21
AM251 (1µg) + Vehicle (50 µL) *n* = 6	842 ± 85	187 ± 26	1.2 ± 0.04
AM251 (1µg) + JZL184 (100µg) *n* = 8	797 ± 72	219 ± 14	1.3 ± 0.06
Carrageenan + Vehicle (50 µL) *n* = 6	756 ± 67	187 ± 22	1.0 ± 0.05
Carrageenan + JZL184 (100µg) *n* = 6	717 ± 39	178 ± 23	1.2 ± 0.08

Mean depth and electrical characterization of all WDR neurons used in this study. Values are baseline responses, recorded prior to addition of any drugs, following electrical stimulation by a train of 16 transcutaneous electrical stimulations (0.5 Hz, 2 ms width) threefold above the C-fibre threshold.

methylarachidonylfluorophosphonate (MAFP) were obtained from Tocris Biosciences, Bristol, UK. 3′-(aminocarbonyl)[1,1′-biphenyl]-3-yl)-cyclohexylcarbamate (URB597) was obtained from Cayman Chemical, Ann Arbor, MI, USA.

Results

Effects of spinal JZL184 on responses of WDR neurones in naïve rats

The characteristics of the WDR neurones recorded in this study (Table 1) are consistent with those previously reported by ourselves and others. Spinal JZL184 produced a time- and dose-related reduction in 26 and 60 g-evoked firing of WDR neurones in the spinal cord (Figure 1A). A typical rate recording showing the extent of this inhibition following the 100 μg dose can be seen in Figure 1C. Low weight-evoked responses of WDR neurones (8–15 g stimuli) were not significantly inhibited by JZL184, compared with baseline evoked responses (Figure 1A). Repeated spinal administration of vehicle did not alter mechanically evoked responses of WDR neurones, compared with baseline (Figure 1B).

The contribution of CB_1 receptors to the JZL184-mediated inhibition of noxious mechanically evoked responses of WDR neurones in the dorsal horn of the spinal cord was assessed. Spinal administration of AM251 (1 μg) did not significantly alter evoked responses of WDR neurones *per se*, but when combined with JZL184 (100 μg), 26 and 60 g-evoked responses of WDR were significantly higher than in the presence of JZL184 alone, and did not significantly differ to responses of WDR neurones in vehicle-treated rats (Figure 1B). By contrast, AM251 did not significantly reverse the inhibitory effects of JZL184 on the 15 g-evoked responses of WDR neurones.

Effects of spinal JZL184 on inflammation-induced expansion of neuronal receptive fields

The next series of experiments investigated whether spinal administration of JZL184 altered nociceptive processing driven by an inflammatory stimulus. As levels of spinal endocannabinoids are altered in pain states (Sagar *et al.*, 2009), we first needed to establish the effects of carrageenan inflammation on the spinal endocannabinoid system. The carrageenan model of inflammatory pain did not alter levels of DAGLα and MAGL mRNA (Figure 2A,B) or protein (Figure 2C,D) in the spinal cord, compared with saline-treated rats. Furthermore, rates of *ex vivo* 2-OG hydrolysis in spinal cord homogenates from carrageenan-treated rats were equivalent to those in saline-treated rats (Figure 2E). On this basis, the carrageenan model of hindpaw inflammation was considered suitable for our study.

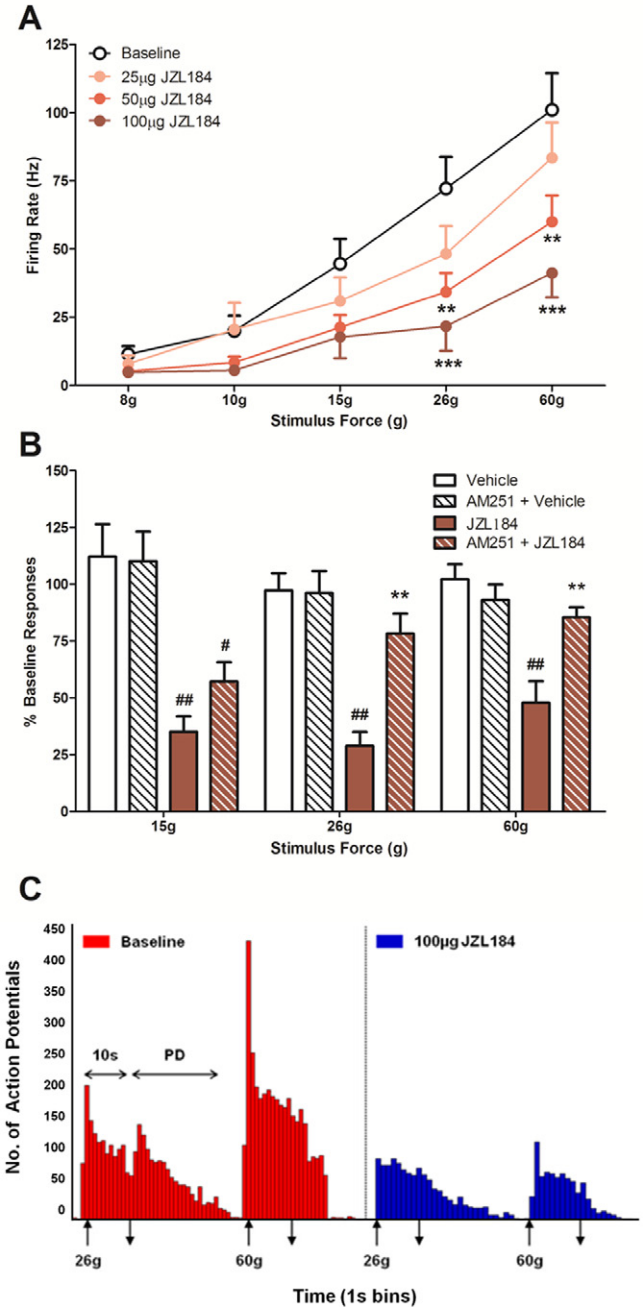

Figure 1

Effects of spinal administration of 25, 50 and 100 μg JZL184 (n = 5–8) or vehicle (n = 8) on mechanically evoked responses of dorsal horn WDR neurones in naïve rats. (A) Mean firing rates (Hz ± SEM) at baseline (30 min), and mean maximal inhibition following each dose of JZL184. Data were analysed with repeated measures two-way ANOVA with Bonferroni *post hoc* test. $^{**}P < 0.01$, $^{***}P < 0.001$ versus baseline. (B) Effects of CB_1 receptor antagonism on JZL184-mediated inhibition of noxious, mechanically evoked, WDR responses. AM251 (1 μg in 50 μL) was administered before vehicle (50 μL, n = 6), or JZL184 (100 μg in 50 μL, n = 6). Data are expressed as mean maximal % of baseline (±SEM). Vehicle data were time matched to the maximal effect of JZL184 (30–40 min). Statistical analyses were performed separately for each stimulus force, via Kruskal–Wallis test with Dunn's multiple comparison *post hoc* test. $\#P < 0.05$, $\#\#P < 0.01$ versus combined vehicle data. Comparisons between AM251+JZL184 and JZL184 alone made via Mann–Whitney U-tests $^{**}P < 0.01$ versus JZL184 alone. (C) Typical rate recordings of 26 and 60 g-evoked responses of a single WDR neurone at baseline (red bars) and 40 min after administration of 100 μg JZL184 (blue bars). Stimuli applied (up arrow) and removed (down arrow) after 10 s. PD indicates post-discharge firing period.

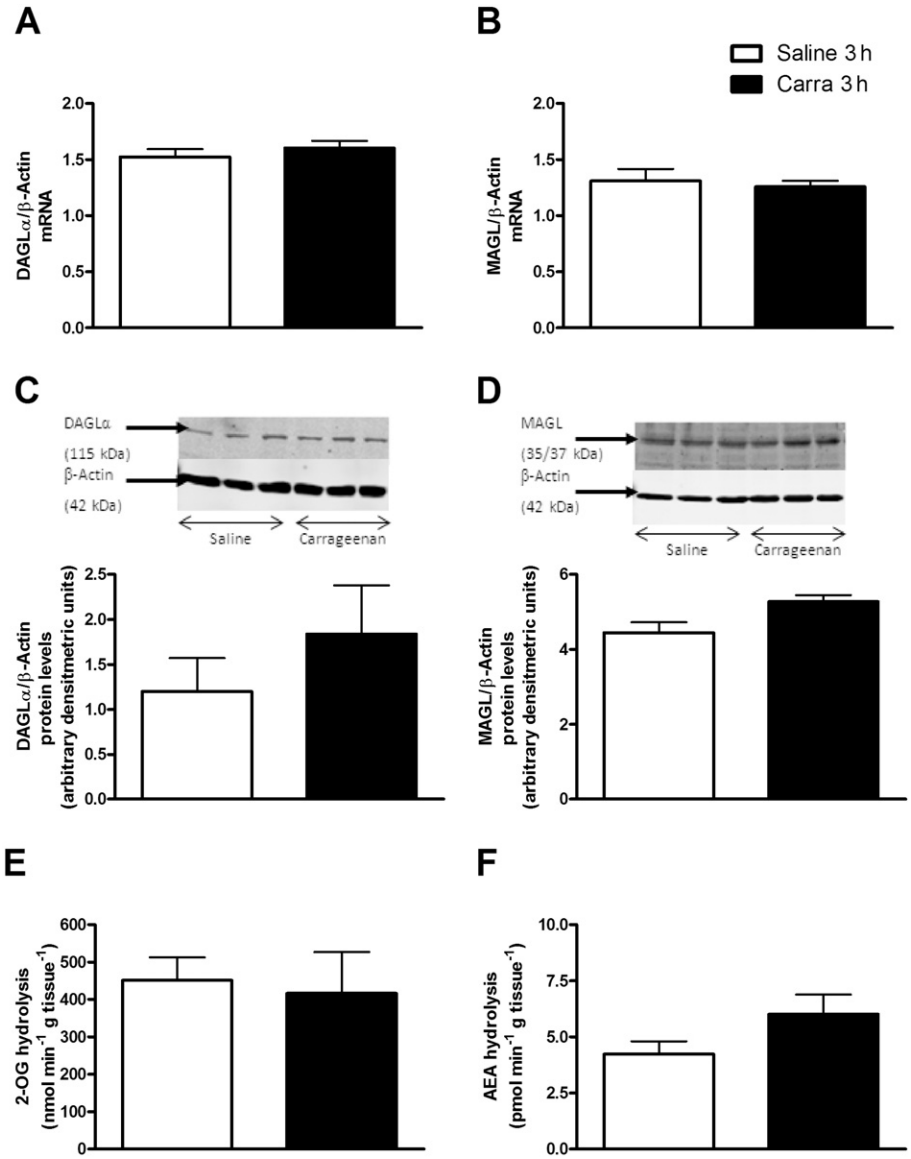

Figure 2

Effects of carrageenan-induced inflammation on components of the endocannabinoid system in the ipsilateral lumbar spinal cord. Tissue was collected 3 h after intraplantar injection of carrageenan. Carrageenan-induced inflammation (Carra) did not significantly alter spinal DAGLα (A) or MAGL (B) mRNA, or protein expression (C and D, respectively), or MAGL (E) or FAAH (F) activity.

Carrageenan-induced hindpaw inflammation induced an increase in the RF size of WDR neurones to 8 and 26 g mechanical stimuli (Figure 3A,B). Spinal administration of 100 μg JZL184 at 50 min after carrageenan injection significantly inhibited the carrageenan-induced expansion of the hindpaw RFs (Figure 3A,B), without affecting hindpaw swelling, an index of local inflammation (Figure 3C).

Are the effects of JZL184 on spinal nociceptive processing associated with elevations in levels of 2-AG in naïve rats?

Levels of 2-AG and AEA were measured in the ipsilateral and contralateral lumbar enlargement of the spinal cord. As spinal administration of 100 μg JZL184 had robust inhibitory effects on neuronal responses at 40 min post-drug treatment, this dose and time point were employed for these studies. JZL184 treatment did not alter levels of 2-AG or AEA in the ipsilateral or contralateral spinal cord (Figure 4A,B). In light of published data, this result was surprising, but we have since replicated it several times, including with batches of JZL184 obtained from different sources (data not shown). The data in Figure 4A,B are collated from two of these separate experiments.

To further investigate the apparent lack of effect of spinally administered JZL184 on levels of 2-AG, we then measured levels of MAGL and FAAH activity via assay of 2-OG and AEA hydrolysis in the spinal cord homogenates from these rats. Spinal JZL184 pretreatment did not alter *ex vivo* 2-OG

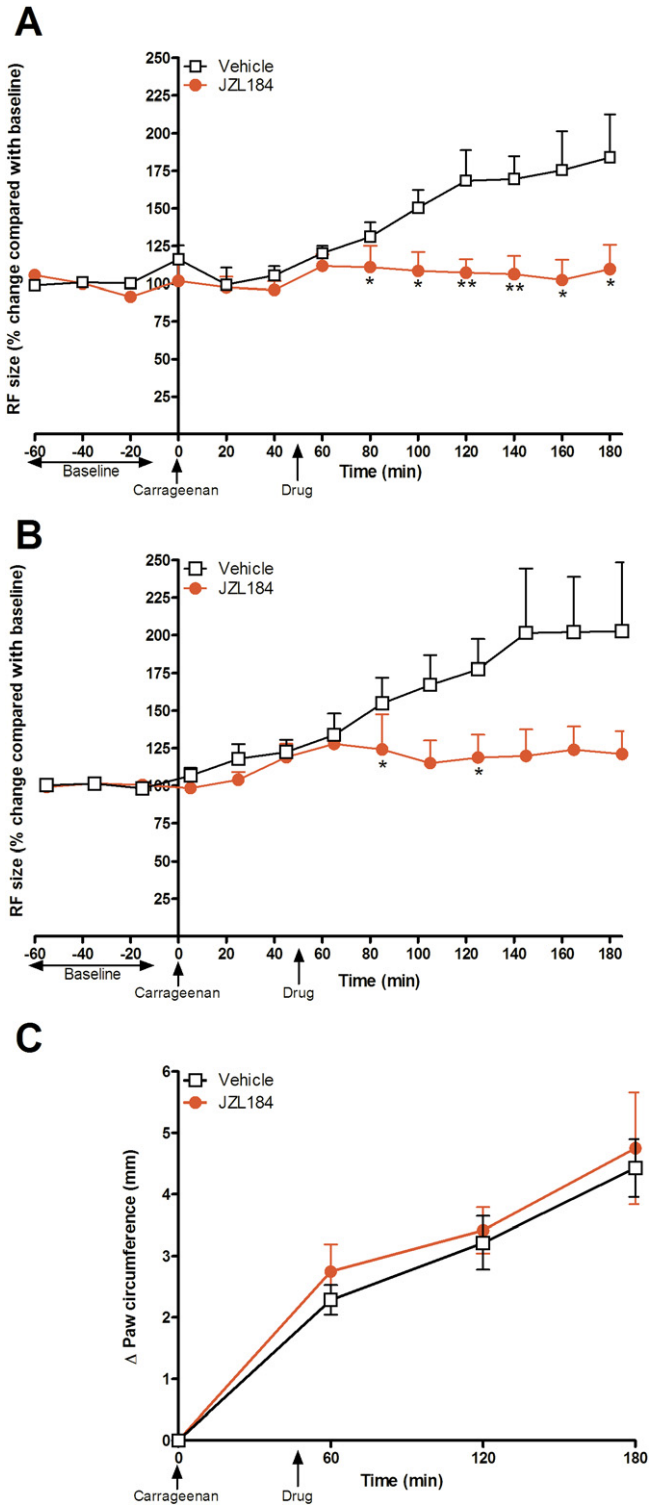

Figure 3

Effects of spinal JZL184 on carrageenan-induced expansion of WDR receptive fields (mean ± SEM). JZL184 (100 μg) abolished carrageenan-induced expansion of 8 g- (A) and 26 g-evoked (B) WDR receptive fields. Vehicle (n = 6) or 100 μg JZL184 (n = 6) were administered spinally at 50 min after carrageenan injection. (C) Change in ipsilateral hindpaw circumference (mm) following intraplantar injection of carrageenan. *P < 0.05, **P < 0.001 versus vehicle-treated rats; Mann–Whitney U-test.

(Figure 4C) nor AEA hydrolysis when compared with vehicle treatment (Figure 4D). Mean calculated 2-OG hydrolytic rates were 287 ± 15 and 298 ± 12 nmol·min^{-1}·g·tissue^{-1} for vehicle- and JZL184-treated rats respectively. Mean AEA hydrolytic rates were 6.1 ± 0.5 and 5.4 ± 0.3 pmol·min^{-1}·g·tissue^{-1} for vehicle- and JZL184-treated rats respectively. There was no effect of laterality in either case.

Effects of systemic JZL184 on feeding behaviour and the spinal cord endocannabinoid system

The lack of concordance between the effects of spinal JZL184 treatment on neuronal responses, and the lack of effect on MAGL activity and levels of 2-AG, was surprising. In light of this, we investigated whether systemic administration of JZL184 was able to produce inhibition of MAGL activity in the spinal cord of the rat. To confirm a physiological effect of systemically administered JZL184, feeding behaviour was utilized as a CB_1 receptor-mediated endpoint in these experiments. Consistent with the purported role of the endocannabinoids in feeding behaviour (Di Marzo, 2011), i.p. administration of 40 mg·kg^{-1} JZL184 significantly increased feeding behaviour, compared with vehicle treatment (Figure 5A). Despite this physiological effect, 2-OG hydrolysis rates in spinal cord homogenates were unchanged after treatment with JZL184, compared with those after vehicle treatment. This may reflect the effects of anaesthesia on MAGL activity in the rat. Further studies are currently in process to clarify this point.

Collectively, these data clearly demonstrated that neither spinal nor systemic administration of JZL184 altered hydrolytic rates of monoacylglycerols in the lumbar spinal cord of rats, although these routes of administration produced functional effects on either pain processing or food intake.

In vitro *inhibition of 2-OG hydrolysis by JZL184*

Previous *in vitro* assays of JZL184-mediated inhibition of MAGL have focussed on the brain, where JZL184 has been reported to be 10-fold less potent in rat, as distinct from mouse, cell membranes (Long *et al.*, 2009b). To date, effects of JZL184 in the spinal cord are unknown. An analysis of rat spinal cord preparations revealed similar profiles of inhibition by JZL184, with pIC_{50} values of 6.7 ± 0.1 and 7.1 ± 0.1 calculated for membrane and cytosolic fractions respectively (Figure 5C). Comparison of JZL184-mediated inhibition of 2-OG hydrolysis in rat and mouse brain membrane fractions revealed pIC_{50} values of 6.5 ± 0.1 and 7.5 ± 0.1 for rat and mouse brain membranes respectively (data not shown). These data confirm a nanomolar potency of the compound at rodent MAGL *in vitro*, and a 10-fold reduction of potency in rat versus mouse brain membranes, replicating the data of Long *et al.* (2009b).

Discussion

Here we report for the first time that spinal administration of the MAGL inhibitor JZL184 attenuated nociceptive responses in both naïve rats and a model of inflammatory pain. In naïve

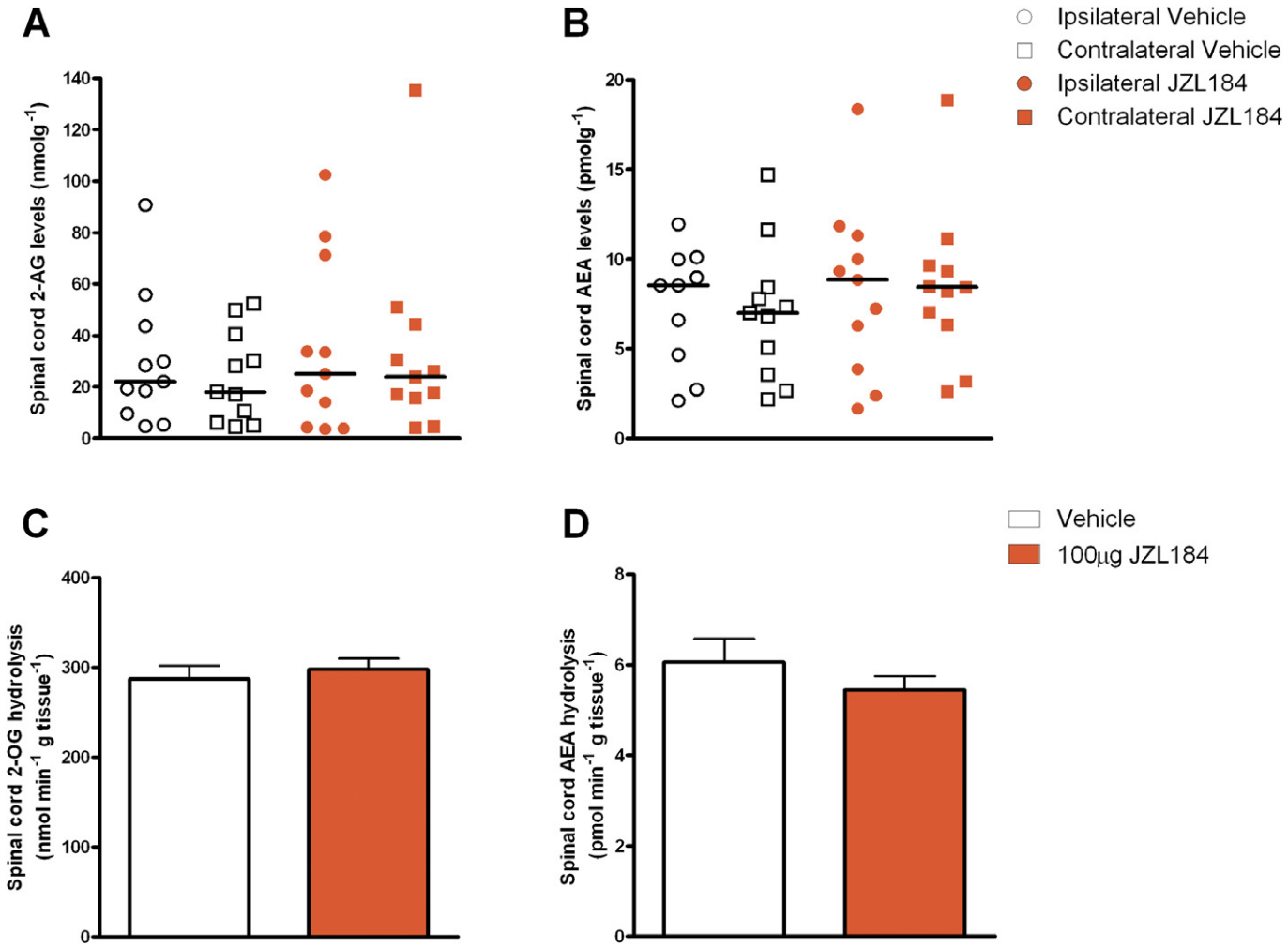

Figure 4

Effects of spinal JZL184 on spinal cord levels of 2-AG (A) and AEA (B) and the activities of their respective catalytic enzymes (C,D). Spinal JZL184 did not significantly elevate spinal cord levels of 2-AG (A) or AEA (B) ($n = 11$), nor did it significantly inhibit 2-OG (C) or AEA (D) hydrolysis in spinal cord homogenates ($n = 5$). Rats received a spinal administration of either vehicle (50 μL) or JZL184 (100 μg in 50 μL), and tissue was collected for analysis 40 min later.

rats, JZL184 significantly inhibited noxious, mechanically evoked, responses of WDR neurones, via a mechanism involving the CB_1 receptor. In carrageenan-inflamed rats, spinal administration of JZL184 ablated the expansion of the receptive fields of the spinal WDR neurones, suggesting that JZL184 can attenuate mechanisms underlying the development of central sensitization associated with hindpaw inflammation. These robust effects of JZL184 on pain responses were not associated with any detectable elevation of 2-AG in the spinal cord, as would be expected with an inhibitor of MAGL. Furthermore, spinal administration of JZL184 did not alter the *ex vivo* rate of monoacylglycerol hydrolysis in the spinal cord, indicating that MAGL activity was not globally attenuated.

The ability of spinally administered JZL184 to inhibit nociceptive neurotransmission, in a CB_1 receptor antagonist-sensitive manner, is in accordance with the purported role of 2-AG signalling at CB_1 receptors in a negative feedback loop in spinal nociceptive pathways (Nyilas *et al.*, 2009). The enzymic machinery of 2-AG signalling is expressed in the dorsal horn of the spinal cord, and DAGLα and CB_1 receptors are expressed in complementary positions for inhibitory control of nociceptive input (Nyilas *et al.*, 2009). Consistent with this action, activation of spinal CB_1 receptors is robustly anti-nociceptive in naïve animals (Kelly and Chapman, 2001) and spinal cannabinoids are anti-allodynic in inflammatory pain states (Martin *et al.*, 1999) and can block induction of spinal hyper-excitability (Nackley *et al.*, 2004). It is noteworthy that, under conditions of intense noxious stimulation, activation of CB_1 receptors on GABAergic interneurones disinhibits spinal excitatory neurones and produces heterosynaptic pain sensitization (Pernia-Andrade *et al.*, 2009). Interestingly, the authors report that these pro-nociceptive effects of CB_1 receptor activation are not associated with less severe models of inflammatory pain or neuropathic pain, which is likely to account for the differences in findings with our current study, which employs a less severe model of inflammatory pain. Blockade of spinal CB_1 receptors selec-

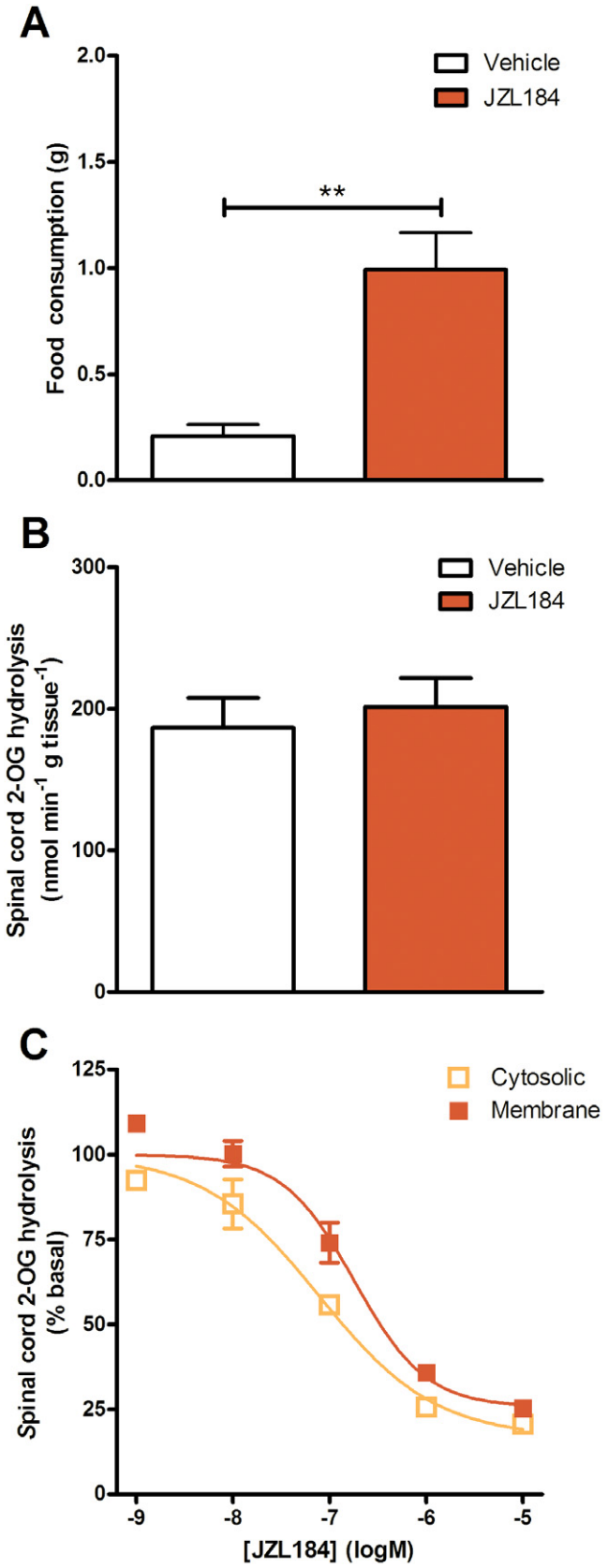

Figure 5

Effects of systemic administration of JZL184 on food intake and spinal cord 2-OG hydrolysis, and *ex vivo* analysis of MAGL inhibition by JZL184. (A) Effects of i.p. injection of vehicle (1 mL·kg^{-1} 4:1 PEG 400:Tween 80, $n = 5$) or JZL184 (40 mg·kg^{-1}, $n = 5$) on food intake (g) over 60 min following 180 min fasting. Data represent mean food consumed in g ± SEM. $^{**}P < 0.01$ versus vehicle-treated rats; Mann–Whitney *U*-test. (B) 2-OG hydrolytic activity present in spinal cord homogenates taken from rats 6 h after i.p. injection of vehicle or JZL184 (mean ± SEM). (C) *In vitro* concentration-dependent inhibition of 2-OG hydrolysis. Rat spinal cord homogenates were pre-incubated with varying concentrations of JZL184 for 30 min prior to assay. Data are means ± SEM of 2-OG hydrolysis expressed as % of basal ($n = 3$ rats).

tively facilitates nociceptive responses of dorsal horn neurones (Chapman, 1999), indicating the presence of endocannabinoid tone. 2-AG is the most likely candidate, as accumulation of spinal 2-AG, but not AEA, has been demonstrated following induction of stress-induced analgesia (Suplita *et al.*, 2006). JZL184 enhanced 2-AG-mediated retrograde synaptic transmission in rat CNS tissue (Pan *et al.*, 2009) and we propose that this mechanism underlies the anti-nociceptive effects of spinal JZL184 reported here.

Two lines of evidence, elevation of 2-AG and decreased rates of 2-OG hydrolysis, were investigated to confirm the contribution of MAGL inhibition to the anti-nociceptive effects of spinal JZL184. We were unable to provide evidence that spinal JZL184 can alter levels of spinal 2-AG or rates of 2-OG hydrolysis, when analysing the whole lumbar enlargement. The basis for this finding may reflect the inherent problems of quantifying localized changes in bioactive lipids in tissue. It is feasible that levels of 2-AG are elevated in discrete areas of the spinal cord and that these changes are small relative to the background levels of 2-AG present in the dorsal horn of the spinal cord. This notion is consistent with the report by Guindon *et al.* (2011) that effects of JZL184 on levels of 2-AG were only detectable following dissection of small areas of hindpaw tissue. Large *post mortem* alterations in neural 2-AG levels have also been reported (Sugiura *et al.*, 2001), and these changes may be exacerbated by hypoxia (Nomura *et al.*, 2011). Thus, killing animals via anaesthetic overdose may also mask effects. It is noteworthy that inhibitors of FAAH produce detectable elevations in total hindpaw (Jhaveri *et al.*, 2006), and spinal cord levels of AEA (Okine *et al.*, 2012), without recourse to further subdivision of tissue. However, in neural tissue basal levels of AEA are far lower compared with 2-AG (e.g. Richardson *et al.*, 2007), and thus small changes in levels are more readily discriminated and quantified.

Given that our study is the first to investigate the effect of JZL184 on nervous tissue in the rat, we went on to further evaluate the importance of the route of administration of JZL184 in this species. We demonstrated that systemic administration of JZL184 significantly stimulated feeding behaviour, a well established cannabinoid-receptor mediated effect (Matias *et al.*, 2006), but did not alter spinal MAGL activities. Because neither spinal nor systemic JZL184 altered spinal MAGL activities and, given the reported differences in the potencies of JZL184 between mice and rats, we compared

the effects of *in vitro* incubation of JZL184 on MAGL activity in rat and mouse CNS tissue. We have replicated the reported effects of JZL184 on mouse brain membranes (Long *et al.*, 2009a), and also report nanomolar potency of JZL184 at spinal MAGL in the rat. These data indicate that JZL184 is a potent inhibitor of MAGL in the rat CNS and suggest that this mechanism may underlie the observed functional effects of this compound.

The efficacy of spinal JZL184 in selectively inhibiting nociceptive neurotransmission and disrupting a correlate of central sensitisation provides strong evidence that manipulation of 2-AG signalling is effective at alleviating the spinal hyper-excitability underlying pain states. Our data also indicate that highly localized changes in 2-AG may be sufficient to produce significant effects on physiological responses.

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Conflict of interest

The authors state no conflict of interest.

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