Inhibition of P2X7 receptors by Lu AF27139 diminishes colonic hypersensitivity and CNS prostanoid levels in a rat model of visceral pain

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Abstract

Visceral pain is a prominent feature of various gastrointestinal diseases. The P2X7 receptor is expressed by multiple cell types including dorsal root ganglion satellite glial cells, macrophages and spinal microglia, all of which have been implicated in nociceptive sensitization. We have used the selective and CNS penetrant P2X7 receptor antagonist Lu AF27139 to explore this receptor's role in distinct rat models of inflammatory and visceral hypersensitivity. Rats injected with CFA in the hindpaw displayed a marked reduction in hindpaw mechanical threshold, which was dose-dependently reversed by Lu AF27139 (3–30 mg/kg, p.o.). In rats injected with TNBS in the proximal colon, the colorectal distension threshold measured distally was significantly lower than sham treated rats at 7 days post-injection ($P < 0.001$), indicative of a marked central sensitization. Colonic hypersensitivity was also reversed by Lu AF27139 (10–100 mg/kg) and by the κ-opioid receptor agonist U-50,488H (3 mg/kg, s.c.). Moreover, both Lu AF27139 and U-50,488H prevented a TNBS-induced increase in spinal and brain levels of PGE2 and LTB4, as well as an increase in brain levels of PGF2α and TXB2. Lu AF27139 was well tolerated as revealed by a lack of significant effect on rotarod motor function and coordination at all doses tested up to 300 mg/kg. Thus, P2X7 receptor antagonism is efficacious in a rat model of visceral pain, via a mechanism which potentially involves attenuation of microglial function within spinal and/or supraspinal pain circuits, albeit a peripheral site of action cannot be excluded.

Introduction

Visceral hypersensitivity is a cardinal feature of gastrointestinal diseases such as ulcerative colitis, Crohn's disease and irritable bowel syndrome. Pain driven by sensitized visceral afferents has important temporal and spatial qualities distinct from that driven by sensitized cutaneous afferents, and currently no drugs have been approved for the treatment of visceral pain conditions leaving a huge unmet need for this population of patients.

Pathophysiological changes occurring within central pain circuits are common to a number of pain conditions of differing aetiology and have also been linked to visceral/abdominal pain. This potentially includes a role for functional microglia [1, 2, 3] which can release various pro-inflammatory mediators including cytokines, chemokines and prostanoids, all of which can modulate neuronal activity within the spinal dorsal horn [4]. Recruitment of the ATP-gated purinoceptors P2X7 has also been described [5, 6, 7, 8]. In turn, this has driven the development of a number of small molecule antagonists of P2X7 receptors that are capable of alleviating pain and inflammation in preclinical models [9, 10, 11, 12, although see 13]. Although visceral pain states have not been as well validated in this regard using small molecule P2X7 receptor antagonists, intrathecal injection of the P2X7 receptor antagonist A438079 has been shown to reverse the associated tactile and thermal hypersensitivity in a TNFR1/R2 -/- model of chronic arthritis, induced by CFA joint injection and supplemented with intracolonic mustard oil irrigation [14].

The P2X7 receptor is also expressed within the periphery on a variety of non-neuronal cells including immunocompetent, and satellite glial cells [15, 16, 17]. It is also localised within cells of the intestine

where it has been shown to be regulated by inflammation and contribute to visceral hypersensitivity [18, 19, 20]. Although P2X7 receptor polymorphisms have been investigated in patients with Crohn's disease, their contribution to disease has proven inconclusive [21]. Nevertheless, an increase in the number of mast cells expressing P2X7 receptors in the colons of mice with colitis and Crohn's patients has been reported [19].

Together, these observations suggest that modulation of P2X7 receptor activity could be a worthwhile strategy to follow for the treatment of clinical conditions involving visceral/abdominal pain. Indeed, the orally active P2X7 receptor antagonist AZD9056 has recently been shown to produce a marked improvement in pain and general well-being in Crohn's patients, despite a lack of change in peripheral inflammatory biomarkers [22]. Here, we have used a well characterized model of visceral pain in rats induced by intracolonic irrigation of the hapten, trinitrobenzene sulfonic acid (TNBS) to investigate the functional role of P2X7 receptors in visceral pain via administration of the novel and highly selective antagonist Lu AF27139 [23, 24].

Materials And Methods

Experimental animals

Animals were obtained from either Charles River (Germany) or Janvier (France) and housed as described within the appropriate experimental method sections. All animal procedures were performed in accordance with Danish legislation (Law no. 474 of May 15th, 2014 and Order no. 88 of January 30, 2013) regulating experiments on animals, which is in compliance with the European Directive 2010/63/EU with specific protocols approved by the Danish Council for Animal Experiments. All in vivo experiments were performed according to ARRIVE Essential 10 guidelines, with pain-related experiments also aligned with the Ethical Guidelines of the International Association for the Study of Pain [25].

CFA-induced inflammatory hyperalgesia

Male Sprague Dawley rats (Charles River) weighing 250–300 g on the day of drug administration were used. They were housed in Makrolon III cages (20 x 14 x 18 cm or 20 x 40 x 18 cm; in groups of 3–5 per cage according to weight) containing wood-chip bedding material (3 x 1 x 4 mm) in an air-conditioned building with controlled environmental parameters (relative humidity 55 ± 15%, temperature 20 ± 2°C, and light from 06.00 to 18.00 hrs). Food and water were available *ad libitum*. The rats were allowed to habituate to the housing facilities for at least one week prior to being assigned to behavioural experiments whereupon they were randomly distributed across treatment groups.

Individual rats received a subcutaneous injection of CFA (50% in phosphate buffered saline, 100 µl total volume, Sigma Aldrich) into the plantar surface of the hindpaw. All rats were then immediately returned to their home cage. Prior to this (pre-CFA baseline response), and then again 24 hrs post CFA injection (post-CFA baseline response), hindpaw pressure thresholds were measured to obtain an index of evoked

mechanical hyperalgesia [26]. To do this, the investigator gently restrained the rat prior to the application of progressively increasing mechanical pressure to the mid hindpaw region using an electronic version of the Randall-Selitto device (IITC, U.S.A.). The point at which the rat attempted to make a reflex hindpaw withdrawal which in some instances was followed by vocalization was recorded as the paw pressure threshold (g). After approximately 20 sec a second measurement was taken from an adjacent region of the hindpaw. Thereafter, CFA inflamed rats were randomly assigned to receive oral administration of Lu AF27139 or vehicle with the investigator blinded to treatment and the paw pressure threshold measured again 120 min later. At the end of the CFA experiment rats were sacrificed by cervical dislocation and blood and brain removed for exposure analysis.

Rotarod test for motor function and coordination

Male Sprague Dawley rats (Charles River) with body weight 200–250 g and housed under the same conditions as CFA rats were initially trained on an accelerating rotarod (Ugo Basile SRL, Gemonio, VA, Italy) over a 3 day period [24]. On the first day to enable the rats to become accustomed to being on the rod, three trials of low constant speed (5–10 rpm, 5 min duration each) were used. Subsequently, on the second day, a first constant speed trial (10 rpm, 5 min duration) was then followed by a trial consisting of three 5 min accelerations during which all rats had to stay on until a speed of 20 rpm was attained; rats were allowed to fall from the rod twice before the trial was completed. Finally, on the third training day the same protocol as that used on training day 2 was used with the exception that no speed limit was used during the second trial and with rats allowed to fall from the rod only once before it was completed. On the day of drug testing, having been randomly assigned to treatment groups rats were removed from their home cage and injected with drug or vehicle (and the experimenter blinded to treatment), and then returned to their home cage according to the required drug pre-treatment time. They were then subjected to a trial consisting of 3 x 5 min accelerations during which the speed of the rod was gradually increased from 0 rpm until the rat fell from the rod or the individual 5 min acceleration was completed. Data were analysed by expressing the mean of the 3 accelerations for each rat as a % of the corresponding baseline response obtained the preceding day during the acceleration trial, with these values then used to calculate the group mean for each treatment.

TNBS model of visceral hypersensitivity

The TNBS (Sigma-Aldrich, France) model was performed under contract by ANS Biotech (Riom, France) as described previously [27]. Sixty-six male Sprague-Dawley rats (Janvier), weighing 390–450 g on the day of surgery were used. They were housed 3 per cage (cages type III H, with each rat identified by tail markings) in a room with controlled environmental parameters (relative humidity 45–65%, temperature 20–24°C, and light from 06.30–18.30 hrs). Rats had free access to tap water and were fed *ad libitum* with pelleted complete diet (irradiated A04 C-10, Safe, France). The rats were allowed to habituate to the housing facilities for at least 5 days prior to being enrolled into the TNBS experiment as highlighted in Fig. 1, whereupon they were randomly distributed across treatment groups.

To enable injection of TNBS, rats were fasted overnight and then the following day were anaesthetized with a mixture of xylazine 10 mg/kg and ketamine 60 mg/kg, i.p. Thereafter, TNBS (50 mg/kg, 1 ml/kg) was injected into the proximal part of the colon (1 cm from the caecum) using a 26G syringe (Terumo, China). After surgery, animals were returned to their home cages in a regulated environment, and fed *ad libitum* until D_{-1} (animals will be fasted 24 hrs before distention). "Naïve" animals (rats without surgery) were kept under the same housing conditions.

Seven days (D_7) after TNBS injection, colonic sensitivity was assessed in animals fasted overnight by measuring the intracolonic pressure required to induce a behavioural response during colonic distension. Colonic distensions were performed after gently inserting a 5 cm balloon (Protex, Sagani Rubber industries, Japan) into the colon of awake animals, 10 cm from the anus with the catheter (Centracath 30, Vygon, France) taped to the base of the tail. Animals were allowed to acclimate for 30 min after insertion of the balloon. At this time, colonic pressure was gradually increased in increments of 5 mmHg, starting at 5 mmHg up to 75 mmHg (the cut off) or until pain-like behaviour was observed. Pain-like behaviour was characterized by an elevation of the hind part of the animal body and a clearly visible abdominal contraction corresponding to a severe cramp [28, 29]. This assessment was then repeated four times at 60 min, 80 min, 100 min and 120 min after drug or vehicle administration which was administered with the experimenter blinded to treatment.

Subsequently, compound efficacy was measured as a % of the distention threshold in treated and naïve rats according to the equation;

$$\text{Activity\% treated} = \frac{\text{Distension threshold treated} - \text{Distension threshold TNBS}}{\text{Distension threshold naive} - \text{Distension threshold TNBS}} X\ 100/1$$

where;

Distention threshold treated	**Arithmetic mean of the values for the "treated" group (animals undergoing TNBS surgery on D_{-7} and treated with the test products or with the reference compound)**
Distention threshold TNBS	Arithmetic mean of the values for the "TNBS" group (animals undergoing TNBS surgery on D_{-7} and treated with the vehicle alone)
Distention threshold naïve	Arithmetic mean of the values for the "naïve" group (animals not undergoing surgery and treated with the vehicle alone)

All CFA and TNBS experiments were performed according to the Ethical Guidelines of the International Association for the Study of Pain and the Danish Committee for Experiments on Animals.

Tissue and sample collection from TNBS rats

At the end of the TNBS study (within 30 min after the end of the test for each rat), cerebro-spinal fluid (CSF) samples were collected (n = 11 group) for use in another study. Briefly, rats were anaesthetized (xylazine 10 mg/kg i.p., ketamine 60 mg/kg i.p.) and approximatively 100 μl of CSF was collected by intra-cisterna magna puncture and immediately frozen at -80°C. After CSF collection, plasma samples were collected from all animals (n = 11 per group). Blood was collected by intra-cardiac puncture into K_2EDTA collection tubes. Samples were then gently mixed and placed on ice and centrifuged within 30 min of collection at 3500 g for 15 min at 4°C. Resultant plasma was collected and immediately frozen at -80°C.

Finally, after blood collection, the brain and spinal cord were collected from each rat (n = 8 per group). Each hemisphere of the brain was weighed, frozen immediately in tubes and stored at -80°C. Spinal cord was also weighed and immediately frozen at -80°C. The brain was homogenized in 3X w:v and spinal cord in 4X w:v in a MSD lysis buffer (MesoScale Discovery, Rockville, MD). The homogenates were spun down and the supernatant was aliquoted and frozen at -80°C until analysis.

Measurement of cytokines and cathepsin S

TNFα (range 0.72–793 pg/ml), IL-6 (range 13.8–8550 pg/ml) and IL-1β (range 6.92–8100 pg/ml) were measured in the spinal cord using a custom rat cytokine assay as per the manufacturer's instructions (N45IA-1; MesoScale Discovery, Rockville, MD). Cathepsin S (range 15.6–1000 pg/ml) was measured using a rat cathepsin S(CTSS) ELISA kit (CUSABIO, Wuhan, Hubei Province, China).

Measurement of bioactive lipids

The bioactive lipids [prostaglandins E2 (PGE2), D2 (PGD2) and F2α (PGF2α); thromboxane B2 (TXB2); leukotriene B4 (LTB4) and the endocannabinoid 2-arachidonoyl glycerol (2-AG)] were analysed simultaneously using an LC-MS/MS system consisting of a triple quadrupole mass spectrometer operated in multiple reaction monitoring mode (TSQ Quantum, Thermo Scientific, San Jose, CA) as described by Gandhi et al [30]. The spray voltage was set at 2000 kV in negative mode and 4500 kV in positive mode, vaporizer temperature was 450°C, cycle time of 0.3 sec, capillary temperature was set at 325°C, sheath gas and AUX gas pressures were set at 40 and 20 psi respectively.

Chromatographic separation was performed on an Acquity UPLC H-class system from Waters (Milford, MA). Analytes were resolved on a Kinetex C18 column (Phenomenex, Torrance, CA). In order to achieve shorter run times, a linear gradient with 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was employed. To achieve efficient resolution, the LC flow was set at 0.45 min and the gradient used was 30% B at 0 min, held constant up to 3 min, ramped to 90% B at 3.65 min, held constant up to 4.8 min and returned to 30% B at 5 min for the next injection. Total run time was 5 min. Injection volume of samples was 20 μl. Column temperature was set at 20°C.

Lu AF27139 exposure and estimation of fraction unbound in plasma and brain

Upon completion of CFA and rotarod experiments 3 animals from each Lu AF27139 treatment group were euthanized from the individual experiments enabling plasma and brain tissues to be collected. As mentioned above brain, plasma and spinal cords were also extracted in the TNBS experiment. Rats were deeply anesthetized and cardiac blood collected into K_3EDTA tubes which were then centrifuged to obtain plasma samples which were stored at − 20°C. Immediately thereafter rats were decapitated and the brains harvested and stored frozen at − 20°C until ready for bioanalysis.

Fraction unbound (f_u) of Lu AF27139 was determined ex vivo for plasma and brain tissues with known in vivo total compound concentrations utilizing an equilibrium dialysis method. Specifically, brain tissues were homogenized in 3x w/v in homogenization buffer (50:30:20 Water:Isopropanol:DMSO) respectively. Lu AF27139 (10 µM) stock solution in DMSO was added to the tissue homogenates or plasma collected from vehicle treated rats as the built in vitro control), and were dialyzed (in triplicate) for 2.5 hrs against a semipermeable membrane (MW cutoff 2000 Da) using a HTDialysis apparatus (Gales Ferry, CT) containing 96-well teflon blocks for 2.5 hrs in an incubator maintained at 37°C with 5% CO_2. A 150 µl 0.01 M phosphate buffer in buffer compartment and 150 µl brain homogenate or plasma in tissue compartment was placed prior to start of equilibration. Following equilibration,120 µl buffer samples fortified with 20 µl blank tissue homogenate and 20 µl tissue samples fortified with 120 µl blank buffer were collected, and protein precipitation was carried out adding 150 µl ice cold acetonitrile containing 100 ng/ml internal standard. The supernatants were harvested following centrifugation and analyzed using the LC-MS/MS as described in section 2.11.2. Fraction unbound was calculated by dividing the peak area response (peak area of analyte/peak area of internal standard) in the buffer compartment by the peak area response in the tissue compartment followed by correcting the dilution factor using the equation suggested by [31].

In vitro fraction unbound determination in either mouse or rat brain and plasma was performed similarly as to the ex vivo method detailed above except plasma or brain homogenates were spiked with 10 µM compound stock in DMSO, a slight modification to a method previously described by [32].

General method for LC-MS/MS analysis of Lu AF-27139 used for all exposure studies

The LC system consisted of a Waters UPLC (Ultra Performance Liquid Chromatography) system (Waters, Milford, MA). Chromatographic separation was performed on a Kinetex™ C18 column (100 × 2.1 mm, 2.6 µm) fitted with a KrudKatcher Ultra HPLC In-Line Filter (0.5 µm × 0.1 mm ID) (Phenomenex, Torrance, CA). The LC mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Gradient elution was performed with 5% B for first 0.2 min, increased to 50% B at 0.67 min, then ramped to 95% B at 0.83 min and held constant for up to 2 min before column re-equilibration at 5% B for an additional 1.0 min for a total run time of 3 min. Column temperature was maintained at 40°C, and auto-sampler temperature was 4°C. Injection volume was 10 µl. Lu AF27139 eluted at 1.35 min and the internal standard at 1.41 min respectively.

Mass spectrometric analysis was performed on a Thermo TSQ Quantum Triple Quadrupole system. Specifically, the mass spectrometer was tuned in positive ESI mode for Lu AF27139 and the internal standard. MRM transitions were acquired for Lu AF27139 by monitoring the precursor ion at 498.1 m/z and fragment ions at 358.0 m/z and 411.0 m/z with an optimized tube lens of 135 and collision energies of 30 and 20 psi respectively. The internal standard was monitored at 552.32 m/z as the precursor ion and 203.0 m/z for the fragment ion with an optimized tube lens of 130 and collision energy of 50 psi.

Drugs

Lu AF27139 was obtained from the Department of Medicinal Chemistry, Lundbeck A/S, Denmark and prepared according to [23]. It was then dissolved in 20% hydroxypropyl-β-cyclodextrin (Kleptose®, Roquette, France and Sigma-Aldrich, Denmark for TNBS and CFA experiments respectively) in water for injection (Aguettant, France). Diclofenac was obtained from Sigma-Aldrich, Denmark (#D6899) and dissolved in in 0.9% NaCl. The (-) enantiomer of U-50,488H was purchased from Sigma-Aldrich, France (#U111) and dissolved in 0.9% NaCl saline (Aguettant, France). Morphine hydrochloride was obtained from Nomeco (Denmark) and dissolved in 0.9% NaCl saline. Lu AF27139 and diclofenac were administered p.o. in a dosing volume of 5–10 ml/kg. U-50,488H and morphine were administered s.c. in a dosing volume of 5 ml/kg. Drug doses are expressed as mg weight free base per kg body weight. Unless stated otherwise drugs were pre-administered using times expected to coincide with maximal effects on behaviour (60–120 min for Lu AF27139, 60 min for both diclofenac and U-50,488H, and 30 min for morphine). Prior to experiments the rats were randomly distributed into treatment groups. The investigator was blinded to drug treatment for evaluation of effects on behaviour.

Data analysis

Unless specified otherwise all data are presented as mean ± standard deviation (S.D.). Statistical analysis was performed with either SigmaPlot 11.2.0.5 (Systat Software Inc., Chicago, IL, USA) or GraphPad Prism (v8). All experiments were analysed using either one or two way analysis of variance (ANOVA). When the *F* value was significant this was followed by Tukey's test. $P < 0.05$ was considered to be statistically significant.

Results

Lu AF27139 attenuates CFA-induced mechanical hyperalgesia

The putative anti-hyperalgesic efficacy of Lu AF27139 was assessed separately in two rat pain models with distinct inflammatory-related pathophysiology. In the first model, injection of complete Freund's adjuvant in the rat hindpaw was used to produce a persistent inflammatory hyperalgesia that is associated with increased activity within peripheral and central pain circuits [33, 34]. Accordingly, hindpaw injection of CFA produced a robust inflammatory hyperalgesia as indicated by the reduction in paw pressure threshold to mechanical stimulation 24 hrs later (mean ± S.E.M., 300 ± 5 g *vs* 113 ± 3 g pre-

CFA, P < 0.001, Students *t*-test) as shown in Fig. 2. This mechanical hyperalgesia was significantly reversed by Lu AF27139 treatment (F(4,45) = 30.69, P < 0.0001). Notably, the efficacy obtained with the 30 mg/kg dose of Lu AF27139 approached that obtained with the NSAID diclofenac. Measurement of plasma and brain exposure in CFA rats as presented in Table 1 confirmed that all doses provided free unbound concentrations in each compartment sufficient to functionally engage P2X7 receptors [24].

Table 1

Exposure of Lu AF27139 in CFA, TNBS and rotarod experiments. Free plasma and brain concentrations (C_u; nM) of Lu AF27139 in rat were determined by the formulae ($Ct*f_u$) where Ct is the total tissue (plasma or brain; ng/g) drug concentration and f_u is the fraction unbound in these tissues as determined by ex vivo equilibrium dialysis. The $f_{u,\ plasma}$ is 0.02 ± 0 and $f_{u,\ brain}$ is 0.09 ± 0.03. In TNBS rats, spinal cord exposure is expressed as a total drug concentration (Ct) with $C_{t\ brain}$ provided for comparison.

		Dose (mg/kg, p.o.)				
Model	**Parameter**	3	10	30	100	300
CFA	$C_{u\ plasma}$	130 ± 10.4	334 ± 87.4	707 ± 260.1		
	$C_{u\ brain}$	111 ± 44.4	94 ± 11.2	368 ± 62.2		
TNBS	$C_{u\ plasma}$		293 ± 24	699 ± 94.2	1687 ± 132.2	
	$C_{u\ brain}$		53 ± 8.7	206 ± 28.3	700 ± 108.3	
	$C_{t\ brain}$		402 ± 62.3	1553 ± 213.6	5278 ± 816.8	
	$C_{t\ spinal\ cord}$		639 ± 163.5	2442 ± 1909	6280 ± 991	
Rotarod	$C_{u\ plasma}$			771 ± 67.1	2154 ± 155.4	2791 ± 173.8
	$C_{u\ brain}$			255 ± 30.6	1232 ± 124.7	2817 ± 350.5

Lu AF27139 has no effect on motor function and coordination

Prior to assessing the analgesic efficacy of Lu AF27139 in a resource intensive rat model of visceral hyperalgesia we wanted to confirm that over the dose range to be tested that it would not act via indiscriminate effects on motor function. At this time we had not tested Lu AF27139 in an exploratory motility assay wherein we have recently reported that distance travelled by naïve rats is unaffected by Lu

AF27139 when administered orally from 10–100 mg/kg [24]. In the current study, administration of Lu AF27139 in doses ranging from 30–300 mg/kg was shown to have no significant effect on motor function and coordination when compared to vehicle treatment when assessed either as time to fall from the rotarod (Fig. 3a) or rotarod speed (Fig. 3b). Table 1 confirms that free unbound concentrations in brain at all 3 doses were well in excess of those required for full P2X7 receptor target engagement. In contrast, the μ-opioid receptor agonist morphine administered at a typical analgesic dose markedly impaired motor function [$(F4,35) = 7.312$, $P = 0.0002$ and $(F4,35) = 9.917$, $P < 0.0001$ for time to fall and rotarod speed respectively].

TNBS-induced visceral hypersensitivity is reversed by Lu AF27139

Next, colonic hypersensitivity was assessed seven days after injection of TNBS into the proximal part of the colon. The colonic distension threshold (CDT) was measured from the distal colon to interrogate facets of central pathophysiology, and was performed four times at 60, 80, 100 and 120 min after drug or vehicle administration. Two way RM ANOVA revealed that treatment with Lu AF27139 partially reversed this colonic hypersensitivity throughout the duration of the experiment $(F5,60) = 42.2$, $P < 0.0001$) as depicted in Fig. 4a-d. The most pronounced effect of treatment was observed at 80 min after injection (Fig. 4b), where both the 30 and 100 mg/kg doses significantly increased the TNBS-mediated reduction in CDT compared with vehicle ($P < 0.01$ and $P < 0.001$ respectively). This is consistent with the measured Cmax for Lu AF27139 after oral administration in rats (Hopper et al., 2021). Notably, for all doses tested, the free unbound plasma concentrations were well in excess of that observed for the 3 mg/kg MED dose in the CFA study (Table 1). Administration of U-50,488H (3 mg/kg, s.c.) produced a more substantial increase in the CDT throughout the duration of the experiment compared with Lu AF27139 (Fig. 4a-d). However, it should be noted that the dose of U-50,488H used here as an assay control, produces a marked diuresis (> 500% *vs* vehicle) over a 6 h period when administered to naïve rats (data not shown).

Effect of P2X7 receptor inhibition on cytokine and cathepsin S levels in spinal cord and brain

Cytokines are elevated in inflamed peripheral tissues [5] and have been shown to be modestly but significantly elevated in the spinal cord in models of neuropathic pain [35, 36]. Therefore, we measured the levels of cathepsin S and the inflammatory cytokines TNFα, IL-1β and IL-6 in spinal cord (T11 to L6) and brain tissue of TNBS sensitized rats. In the spinal cord of naïve animals, the level of TNFa (1 pg/mL) was barely above the assay detection limit, and remained unchanged in either brain or spinal cord of Lu AF27139 treated TNBS rats. A similar pattern was observed for IL-1β which displayed a low baseline level in spinal cord (11.6 pg/ml), whilst in the brain approximately 40% of samples were below the detection limit. Again, these levels were unchanged in the spinal cord of TNBS rats and after Lu AF27139 treatment (range from 10.8–14.2 pg/ml). In contrast, although IL-6 was detectable in spinal cord and brain homogenates of naïve rats (37–61 pg/mL and 118–139 pg/ml respectively), these levels were unaltered

by TNBS or Lu AF27139 treatment. Similarly, cathepsin S levels in spinal cord were detectable at baseline (11.6 pg/ml), albeit there was no observable effect of treatments between groups (range from 10.8–14.2 pg/ml).

Effect of P2X7 receptor inhibition on levels of bioactive lipids in spinal cord and brain

We also analysed the levels of 6 bioactive lipids (PGE2, PGD2, PGF2α, LTB4, TBX2, 2-AG) within the spinal cord and brain of naïve rats and rats with TNBS-induced visceral hypersensitivity using a novel mass-spectrometry method. Analysis of spinal cord samples revealed that PGE2 and LTB4 were significantly increased in TNBS treated rats [$(F5,34) = 7.007$, $P = 0.0001$) and ($F5,41) = 12.44$, $P < 0.0001$) respectively] compared with vehicle treatment as shown in Fig. 5, and that these effects could be significantly prevented by Lu AF27139 and by the assay control U-50,488H.

Within brain tissue, the effect of TNBS on lipid levels was even more profound with PGE2, PGF2α, LTB4 and TBX2, all significantly increased [$(F5,38) = 7.967$, $P < 0.0001$), ($F5,42) = 6.856$, $P < 0.0001$, ($F5,42) = 4.134$, $P = 0.0038$), ($F5,42) = 6.375$, $P = 0.0002$ respectively] compared with vehicle treatment (Fig. 6). Again, administration of both Lu AF27139 and U-50,488H prevented the increase in each of these lipids within the brain of TNBS treated rats. Notably, Lu AF27139 treatment prevented the TNBS-induced increase in spinal cord and brain lipids at all doses tested from 10 mg/kg up to 100 mg/kg. Although a significant effect on brain 2-AG levels occurred [$(F5,42) = 2.621$, $P = 0.0377$), post treatment analysis failed to show a difference between any of the groups.

Discussion

The current data show that Lu AF27139 can diminish nociceptive responses in rat pain models associated with varying levels of peripheral and central inflammatory pathophysiology. Notably, in a model of visceral hyperalgesia induced by colonic administration of TNBS, the Lu AF27139-mediated attenuation of distally-measured nociceptive responses indicates that central pain circuits are the likely target for Lu AF27139 in this model. A correlative increase in levels of spinal and brain prostanoids after TNBS was also prevented by Lu AF27139, further confirming resolution of key markers linked to central pain mechanisms. Accordingly, the data suggest that P2X7 receptor antagonism is a viable option for the therapeutic management of visceral pain conditions that exhibit a predominantly CNS-mediated pathophysiology.

Lu A27139 efficacy in CFA and TNBS rats

The CFA model of hindpaw inflammatory pain produces an overt peripheral oedema linked to release of pro-inflammatory molecules. A corresponding nociceptor sensitization with increase in primary afferent activity then facilitates upstream sensitizing events within the sensory ganglia, where the neuronal cell bodies are enveloped by satellite glial cells. A complex interplay between these cell types has been reported to promote behavioural hyperalgesia via a process that involves increased structural and

neurochemical coupling, achieved in part by the neuronal release of ATP and inflammatory cytokines [37, 38]. Satellite glial cells in turn express a variety of purine receptor subtypes including P2X7 receptors, the activation of which leads to the further release of nociceptive signaling molecules [38]. Consistent with these observations, at free plasma concentrations sufficient to inhibit peripherally expressed P2X7 receptors Lu AF27139 dose-dependently reversed hindpaw mechanical thresholds in CFA-inflamed rats [23]. Whilst increased neuronal-glial coupling is a potentially attractive mechanism targeted by Lu AF27139, the experimental timeframe followed here after CFA injection will have produced a subsequent recruitment of central sensitizing mechanisms that will include increased functioning of spinal microglia [33, 34]. Notably, Lu AF27139 possesses excellent CNS penetrability properties and achieved brain concentrations in CFA rats sufficient to engage central P2X7 receptors; similar to those reported to inhibit LPS-primed BzATP-induced 1L-1β release from rat primary microglia [23]. Further experiments involving e.g. intrathecal injection of Lu AF27139 or siRNA would be required to address any potential central site of analgesic action more comprehensively in the CFA model.

As part of the ongoing clinical development of Lu AF27139 we wanted to explore the efficacy of Lu AF27139 in visceral pain as there are currently no approved clinical treatments in this range of indications. Again, increased DRG neuronal coupling has been reported to contribute to sensitization of visceral afferent input [39, 40]. Furthermore, P2X7 receptor expression within the intestinal epithelium and surrounding connective tissue is known to be upregulated after TNBS injection [41], and this can be blocked by systemic administration of the P2X7 receptor antagonist brilliant blue G (BBG). Similar observations using BBG have been reported in rats with dextran sodium sulphate-induced colitis via a mechanism that involves attenuation of pro-inflammatory cytokine function [42]. Thus, we choose to explore efficacy of Lu AF27139 in a rat model of colitis where confounds of local colonic inflammation induced by TNBS irrigation of the proximal colon [43] are minimized by measuring hypersensitivity to distension in the distal colon. Accordingly, nociceptive hypersensitivity reflects a central rather than peripherally-mediated pathophysiology [27]. This is an important consideration given that only the 30 and 100 mg/kg doses of Lu AF27139 increased the colonic distention threshold after TNBS. In contrast, all doses (10, 30 and 100 mg/kg) provided plasma concentrations in TNBS rats which exceeded that required to occupy peripheral P2X7 receptors (Table 1). Accordingly, our data indicate that Lu AF27139 analgesic efficacy is mediated via central target engagement in TNBS rats, and aligns with the ability of intrathecally administered BBG to reduce visceral hyperalgesia in a rat model of TNBS-induced pancreatic pain [44].

Translation of efficacy from pre-clinical pain models remains a considerable challenge, supporting the need to establish a biomarker link between efficacy and target engagement. Previously we identified attenuation of aberrant microglial function and prostanoid release within the CNS as a potential mechanism of action for Lu AF27139 in neuropathic pain [24]. Multiple lines of evidence also exist to support a similar function in visceral pain [45]. Both the increased visceromotor response to colorectal distension with accompanying increase in spinal microglia functioning in TNBS sensitized rats is blocked by the central administration of minocycline [1, 3]. Similarly, visceral hyperalgesia induced by chronic stress in rats can be reversed by minocycline [2]. Moreover, reports of a correlation in spinal microglial

P2X7 receptor expression with pancreatitis-induced pain resolving upon treatment with BBG and siRNA [44] provide a compelling argument for central P2X7 receptor involvement in visceral pain.

The P2X7 receptor regulates multiple microglial signalling pathways including IL-1β processing and release, cathepsin S release, stimulation of acid sphingomyelinase activity, phospholipase activity and subsequent prostaglandin and endocannabinoid synthesis [46, 47, 48, 49, 50, 51, 52, 53, 54]. Thus, we sought to investigate if any of the identified sequelae of microglial P2X7 receptor activation were elevated in the spinal cord of TNBS treated rats or modulated by Lu AF27139. Our data revealed increases in brain and spinal cord PGE2 and LTB4 levels in TNBS rats, with PGF2α and TXB2 also increased, albeit only in the brain. These elevated bioactive lipids in spinal cord and brain were clearly blocked by Lu AF27139, although it is noteworthy that the minimal effective dose was at least 3 fold lower than that required to affect visceral hypersensitivity *per se*. We do not have a simple explanation for this discrepancy other than to speculate that (i) resolution of altered lipid levels by Lu AF27139 is not in itself sufficient to impact on hypersensitivity (ii) additional pro-nociceptive mediators are recruited by P2X7 receptor activation at the higher brain exposure levels linked to Lu AF27139 effects on behaviour. The lack of change in cytokine or cathepsin S levels in the brain or spinal cord of TNBS rats suggests that they are not contributing directly to centrally-mediated colonic hypersensitivity in this model, similar to what we have previously reported in neuropathic pian [24]. While these findings are at odds with those published elsewhere typically the protein levels (eg IL-1β) measured were very low, or only transcript levels were reported [55]. However, we cannot exclude that the lack of change in the various lipids measured herein might be a consequence of the whole lumbar spinal cord being used for bioanalysis purposes, as this might potentially mask any specific effects of increased TNBS-mediated afferent input on these molecules within dorsal horn pain circuits. Furthermore, our data do not address a putative sensitizing role of these molecules within the periphery [56].

The reference κ-opioid receptor agonist U-50,488H [57] also effectively prevented TNBS-induced hypersensitivity and the associated increase in the same bioactive lipids. The localisation of κ-opioid receptors to pre- and post-synaptic neurones within the spinal cord, with minimal glial expression [58, 59], indicates that such modulation is regulated by multiple cell types at the circuit level. Moreover, Lu AF27139 did not reverse the TNBS-induced hypersensitivity as robustly as U-50,488H, albeit it should be noted that the U-50,488H dose tested produces marked diuretic effects in rats (data not shown), [60]. In contrast, we never observed any adverse effects with Lu AF27139. This included a complete lack of effect on motor function and coordination when tested in the rotarod test at doses efficacious in TNBS rats, a finding which is consistent with a lack of effect on exploratory locomotor activity over the same dose range [24]. Thus, Lu AF27139 appears to possess a benign side effect profile which augers well for P2X7 receptor block putatively translating as an effective therapeutic strategy into humans.

Conclusions

Polymorphisms in the P2X7 receptor have been reported to regulate sensitivity to pain in patients after mastectomy, and in patients with osteoarthritis and diabetic neuropathy [61, 62]. Although a human

genetic link to visceral pain has not yet been demonstrated, we believe the data presented here for Lu AF27139 in TNBS rats is sufficiently far reaching in terms of effects on symptoms and neurochemical mediators linked to P2X7 receptors to merit further investigation in relation to visceral pain.

Declarations

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Author contributions

Roland Staal: Conceptualization, Validation, Writing – Original Draft, Writing – Review & Editing, Visualization, Supervision, Project administration **Adarsh Gandhi:** Methodology, Investigation **Hua Zhou:** Methodology, Investigation **Manuel Cajina:** Methodology, Investigation **Anne-Marie Jacobsen:** Methodology, Investigation, Formal analysis, Writing – Original Draft **Sara Hestehave:** Methodology, Investigation **Allen Hopper:** Conceptualization, Writing – Review & Editing, Supervision **Suresh Poda:** Methodology, Formal analysis **Gamini Chandrasena:** Formal analysis **Stevin H. Zorn:** Conceptualization, Visualization, Project administration, Writing - Review & Editing **Brian Campbell:** Project administration **Märta Segerdahl:** Conceptualization **Thomas Möller:** Conceptualization **Gordon Munro:** Conceptualization, Validation, Writing – Original Draft, Writing – Review & Editing, Visualization, Supervision.

Availability of data and material

Raw data files are available upon reasonable request from MindImmune.

Ethics approval

Experiments were performed in accordance with Danish legislation (Law no. 474 of May 15th, 2014 and Order no. 88 of January 30, 2013) regulating experiments on animals, which is in compliance with the European Directive 2010/63/EU with specific protocols approved by the Danish Council for Animal Experiments.

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

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Figures

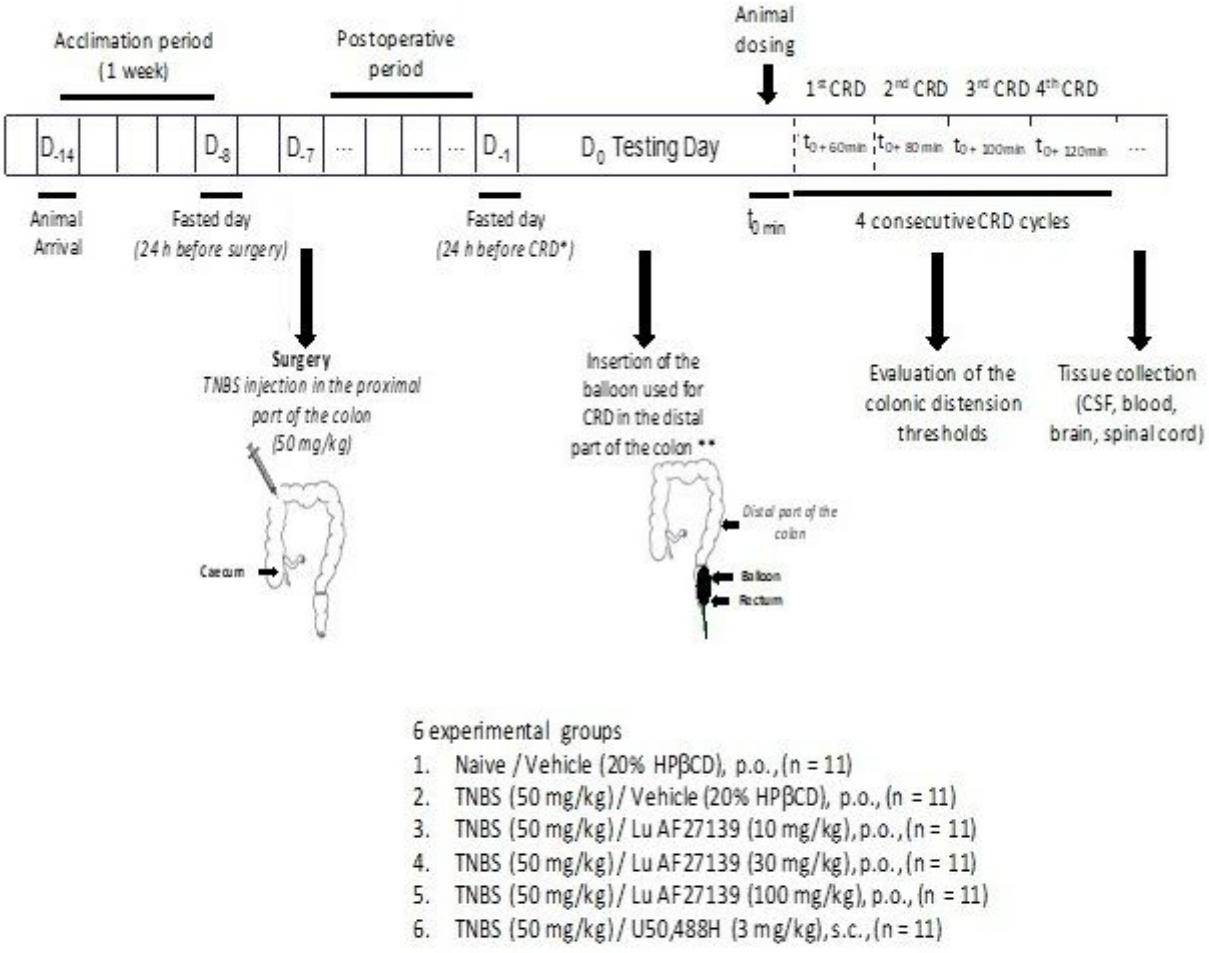

Figure 1

Figure 1

Schematic showing the study plan used for assessing efficacy of Lu AF27139 in the TNBS model of colonic hypersensitivity

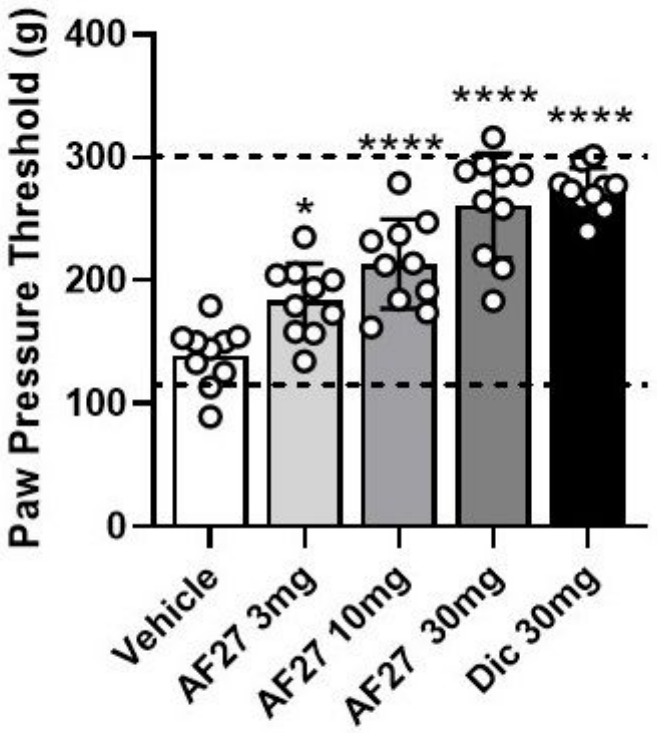

Figure 2

Figure 2

Lu AF27139 reverses mechanical hypersensitivity in CFA-inflamed rats. The paw pressure threshold (g) was measured in rats prior to injection of CFA (50%, 100 µl) into the hindpaw. Subsequently, measurement of the paw pressure threshold again 24 h later (dashed line) provided an index of the presence of mechanical hyperalgesia. Rats were then dosed with Vehicle, Lu AF27139 (3, 10, 30 mg/kg, p.o.) or diclofenac (30 mg/kg, p.o.). The post treatment paw pressure threshold was measured 120 and 60 min later. Data are expressed as mean ± S.D. All groups n=10 rats. *P<0.05, ****P<0.01 *vs* Veh (one way ANOVA followed by Tukey's)

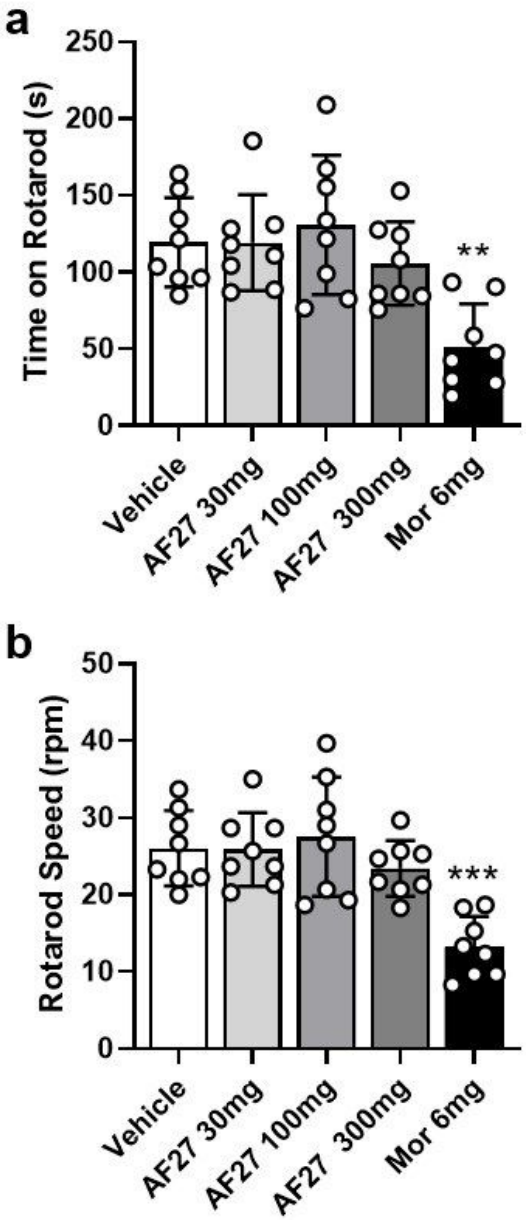

Figure 3

Figure 3

Lu AF27139 does not impair motor function and coordination in naïve rats. Normal, uninjured rats were administered either Lu AF27139 (30-300 mg/kg, p.o.), morphine (6 mg/kg, s.c.) or vehicle immediately after a baseline response had been obtained. Thereafter, the effects on motor performance represented as a) time on rotarod (s), (b) rotarod speed (rpm) was determined 2 hrs later for Lu AF27139 treated, and

30 min later for morphine treated groups. Data are presented as mean ± S.D. All groups n=8. **P<0.01, ***P<0.001 *vs.* Veh (one way ANOVA followed by Tukey's)

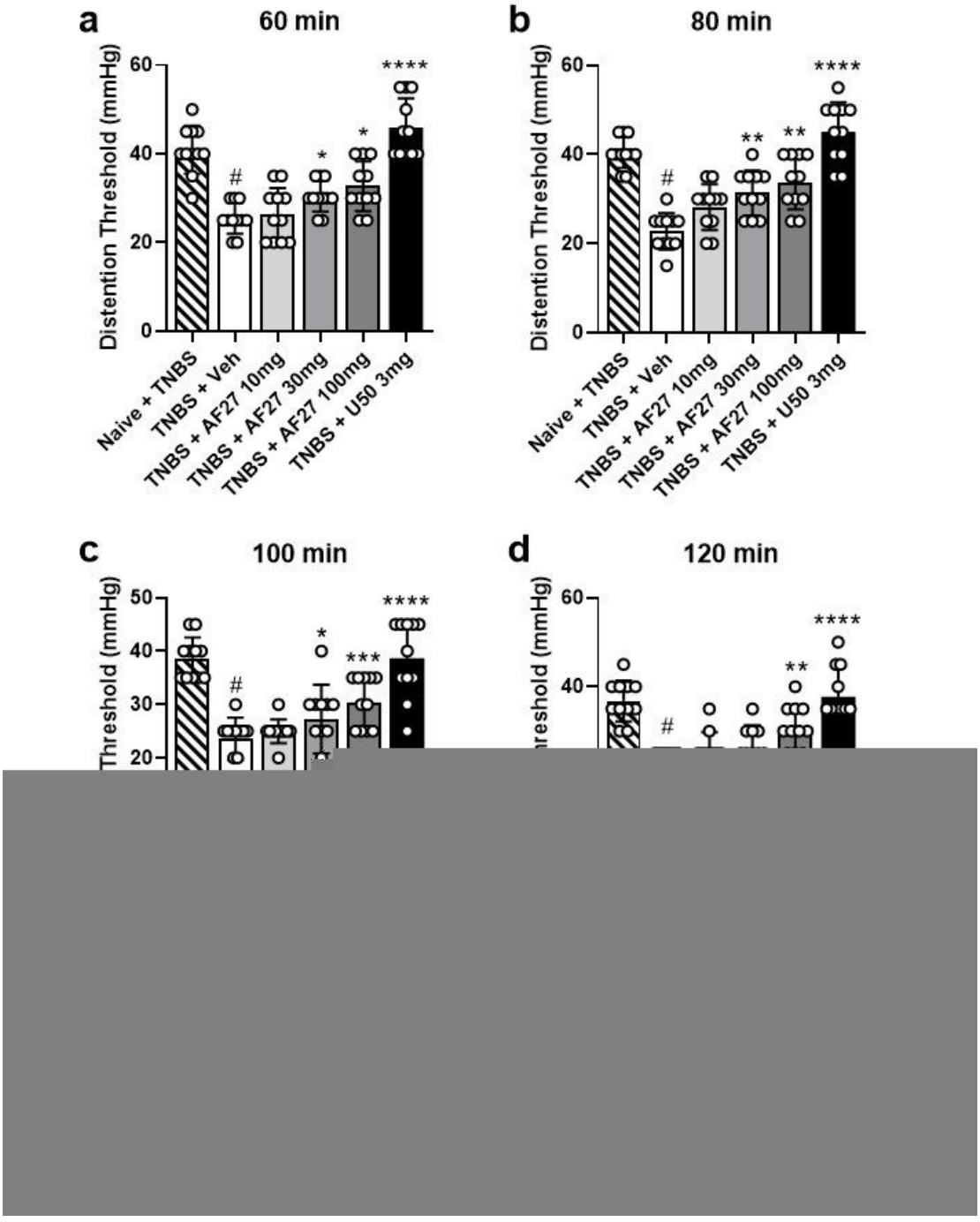

Figure 4

Lu AF27139 reverses visceral hypersensitivity in TNBS sensitized rats. All rats were injected with TNBS (50 mg/kg) in the proximal part of the colon seven days prior to insertion of an inflatable balloon into the

distal part of the colon to enable measurement of the colorectal distension threshold as an index of visceral hypersensitivity. With the exception of naïve animals, rats were then dosed with Vehicle, Lu AF27139 (10, 30, 100 mg/kg, p.o.) or U-50,488H (3 mg/kg, s.c.) at time=0 min, and then the colorectal distension threshold (mmHg) was measured at a) 60 min b) 80 min c) 100 min and d) 120 min later. Data are expressed as mean ± S.D. All groups n=11. $^{\#}$P<0.0001 *vs* naïve, *P<0.05, **P<0.01, ***P<0.01, ****P<0.0001 *vs* Veh at corresponding timepoint (Two way RM ANOVA followed by Tukey's)

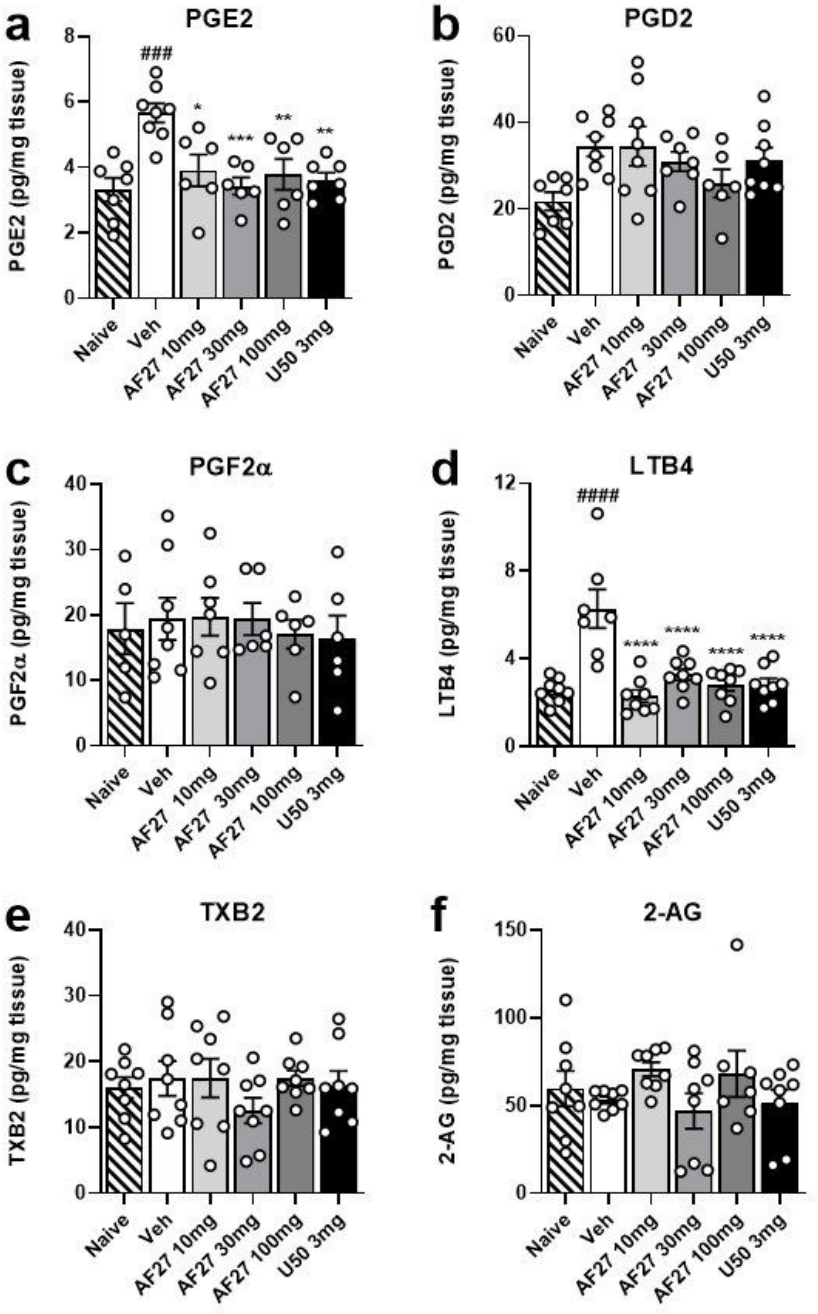

Figure 5

Figure 5

Levels of bioactive lipids in the spinal cord of TNBS sensitized rats. All rats were injected with TNBS (50 mg/kg) in the proximal part of the colon seven days prior to the experimental day (Day 7). Thereafter, with the exception of naïve animals, rats were administered either vehicle, Lu AF27139 (10, 30 or 100 mg/kg, p.o) or U-50,488H (3 mg/kg, s.c.) one hour prior to assessment of colonic hypersensitivity. Tissues were collected within 30 min of the last test, processed and analysed for a) PGE2, b) PGD2, c) PGF2a, d) LTB4, e) TXB2, f) 2-AG as described in Materials and Methods. Data are expressed as mean ± S.D. $^{###}$P<0.001, $^{####}$P<0.0001 *vs* Naïve, *P<0.05, **P<0.01, ***P<0.01, ****P<0.0001 *vs* Veh (One Way ANOVA followed by Tukey's)

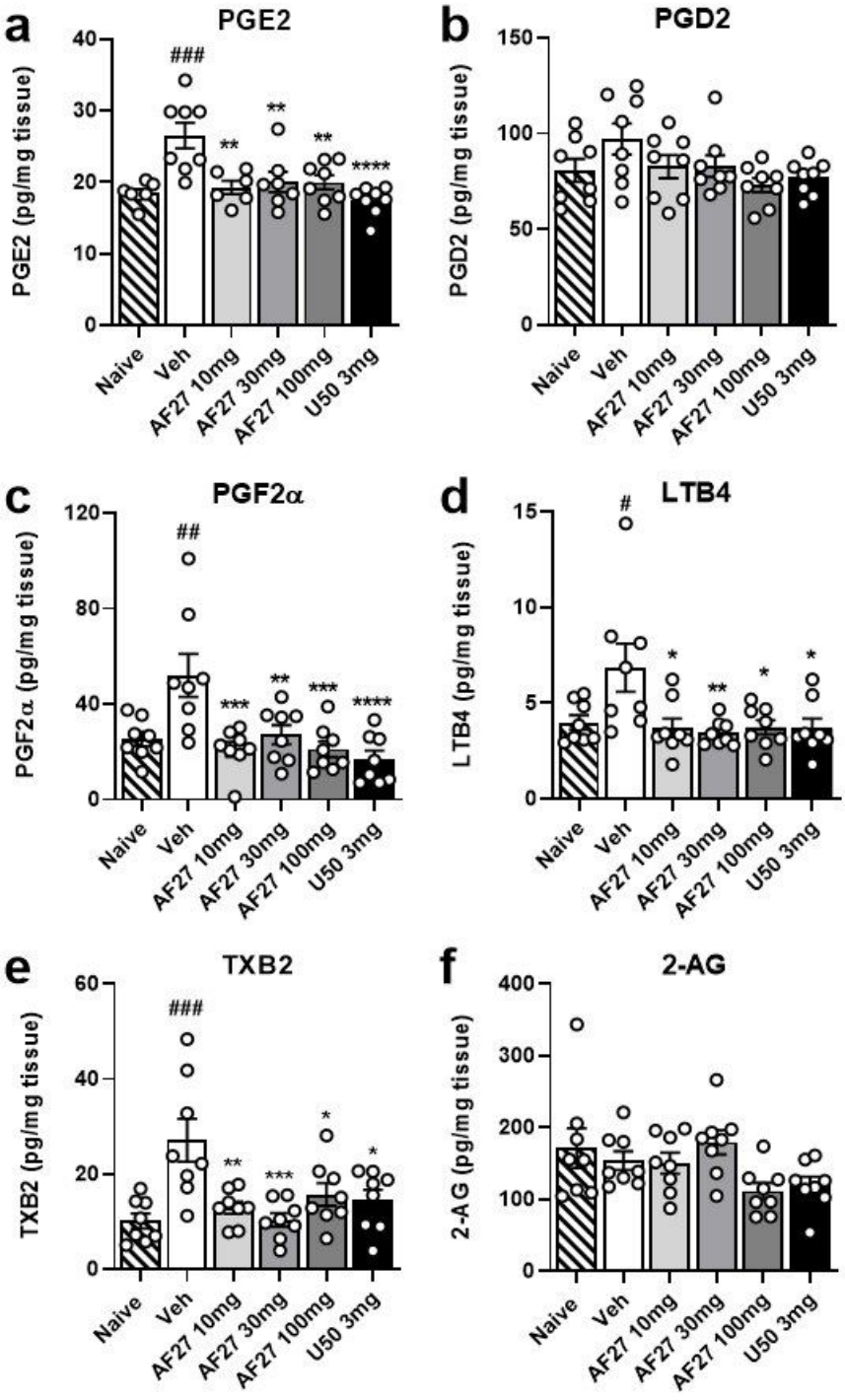

Figure 6

Figure 6

Levels of bioactive lipids in the brain of TNBS sensitized rats. All rats were injected with TNBS (50 mg/kg) in the proximal part of the colon seven days prior to the experimental day (Day 7). Thereafter, with the exception of naïve animals, rats were administered either vehicle, Lu AF27139 (10, 30 or 100 mg/kg, p.o) or U-50,488H (3 mg/kg, s.c.) one hour prior to assessment of colonic hypersensitivity. Tissues were collected within 30 min of the last test, processed and analysed for a) PGE2, b) PGD2, c) PGF2a, d)

LTB4, e) TXB2, f) 2-AG as described in Materials and Methods. Data are expressed as mean ± S.D. $^{\#}P<0.05$,$^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs* Naïve, *P<0.05, **P<0.01, ***P<0.01, ****P<0.0001 *vs* Veh (One Way ANOVA followed by Tukey's)