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RESEARCH PAPER

Inhibiting fatty acid amide hydrolase normalizes endotoxin-induced enhanced gastrointestinal motility in mice

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BACKGROUND AND PURPOSE

Gastrointestinal (GI) motility is regulated in part by fatty acid ethanolamides (FAEs), including the endocannabinoid (EC) anandamide (AEA). The actions of FAEs are terminated by fatty acid amide hydrolase (FAAH). We investigated the actions of the novel FAAH inhibitor AM3506 on normal and enhanced GI motility.

EXPERIMENTAL APPROACH

We examined the effect of AM3506 on electrically-evoked contractility *in vitro* and GI transit and colonic faecal output *in vivo*, in normal and FAAH-deficient mice treated with saline or LPS (100 $\mu g \cdot kg^{-1}$, i.p.), in the presence and absence of cannabinoid (CB) receptor antagonists. mRNA expression was measured by quantitative real time-PCR, EC levels by liquid chromatography-MS and FAAH activity by the conversion of [^{3}H]-AEA to [^{3}H]-ethanolamine in intestinal extracts. FAAH expression was examined by immunohistochemistry.

KEY RESULTS

FAAH was dominantly expressed in the enteric nervous system; its mRNA levels were higher in the ileum than the colon. LPS enhanced ileal contractility in the absence of overt inflammation. AM3506 reversed the enhanced electrically-evoked contractions of the ileum through CB_1 and CB_2 receptors. LPS increased the rate of upper GI transit and faecal output. AM3506 normalized the enhanced GI transit through CB_1 and CB_2 receptors and faecal output through CB_1 receptors. LPS did not increase GI transit in FAAH-deficient mice.

CONCLUSIONS AND IMPLICATIONS

Inhibiting FAAH normalizes various parameters of GI dysmotility in intestinal pathophysiology. Inhibition of FAAH represents a new approach to the treatment of disordered intestinal motility.

Abbreviations

2-AG, 2-arachidonoylglycerol; AEA, anandamide; AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl) -1-(2,4-dichlorophenyl)-4-methyl-1*H*- pyrazole-3-carboxamide; AM630, 6-iodo-2-methyl-1-[2-(4-morpholinyl) ethyl]-1*H*-indol-3-yl](4-methoxyphenyl) methanone; CB, cannabinoid; CB_1 receptor, cannabinoid receptor type 1; CB_2 receptor, cannabinoid receptor type 2; EC, endocannabinoid; EFS, electrical field stimulation; ENS, enteric nervous system; FAAH, fatty acid amide hydrolase; FAE, fatty acid ethanolamides; GI, gastrointestinal; JWH133, (6a*R*,10a*R*)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[*b*, *d*] pyran; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; TTX, tetrodotoxin; WIN55,212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4- morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate

Introduction

The endocannabinoid (EC) system consists of the cannabinoids (CBs) receptor type 1 and 2 (CB_1 and CB_2 receptors), endogenous CB ligands, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and the enzymes involved in their biosynthesis and degradation (Di Marzo, 2009; Izzo and Sharkey, 2010). This system is involved in regulating gastrointestinal (GI) function (Duncan *et al.*, 2005; Storr and Sharkey, 2007; Izzo and Camilleri, 2008; Izzo and Sharkey, 2010). ECs activate CB_1 and CB_2 receptors in the enteric nervous system (ENS), smooth muscle and epithelium (Duncan *et al.*, 2005; Wright *et al.*, 2005, 2008; Izzo and Camilleri, 2008; Izzo and Sharkey, 2010). In the ENS, ECs reduce ACh release, thereby inhibiting contractility and peristalsis (Aviello *et al.*, 2008; Izzo and Sharkey, 2010). In the intestinal epithelium, CB_1 receptor activation regulates cell proliferation and promotes healing (Wright *et al.*, 2005; Cianchi *et al.*, 2008).

ECs are rapidly degraded (Puffenbarger, 2005; Ahn *et al.*, 2008). Fatty acid amide hydrolase (FAAH) catalyses the hydrolysis of AEA, as well as palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) (Cravatt and Lichtman, 2002; Fegley *et al.*, 2005). Inhibiting FAAH produces behavioural effects consistent with activation of the CB_1 receptor, for example, anti-anxiety, analgesia and anti-inflammatory actions (Chang *et al.*, 2006; Scherma *et al.*, 2008; Schlosburg *et al.*, 2009). However, typical adverse behavioural effects of CB agonists (e.g. catalepsy) are not observed in animals treated with FAAH inhibitors or seen in mice deficient in the FAAH gene (Cravatt *et al.*, 2001; Kathuria *et al.*, 2003). These features make FAAH inhibition an attractive therapeutic option for circumstances where CB receptor activation may be beneficial (Cravatt and Lichtman, 2003; Petrosino and Di Marzo, 2010).

AEA, PEA and OEA are able to reduce intestinal motility in mice (Capasso *et al.*, 2001; Pinto *et al.*, 2002; Cluny *et al.*, 2009). While there is compelling evidence in support of the EC system regulating intestinal motility, the role of FAAH remains somewhat enigmatic. FAAH is expressed throughout the wall of the gut; in the ENS, epithelial cells and immune cells (Katayama *et al.*, 1997; Capasso *et al.*, 2005; De Filippis *et al.*, 2008a; Marquez *et al.*, 2009). It is up-regulated in sepsis and colitis, but not in colon cancer (Ligresti *et al.*, 2003; De Filippis *et al.*, 2008a; Marquez *et al.*, 2009). However, FAAH-deficient mice do not display reduced GI motility and may (Capasso *et al.*, 2005) or may not have elevated intestinal levels of AEA despite the increased intestinal PEA and OEA levels (Fegley *et al.*, 2005).

Treatment with the FAAH inhibitor URB597 blocks FAAH activity, but does not elevate levels of fatty acid ethanolamides (FAE) in the small intestine, in contrast to the CNS (Fegley *et al.*, 2005); however, another FAAH inhibitor, AA-5-HT, decreases intestinal motility and increases intestinal AEA and PEA (Capasso *et al.*, 2005). The differences in findings of the studies on FAAH inhibitors and FAAH-deficient mice require further investigation.

Despite the discrepancies that have been seen on the levels of FAEs after treatment with different FAAH inhibitors and in FAAH-deficient mice, recent studies showed a significant association between FAAH genotype and different phenotypes of functional GI disorders (Camilleri *et al.*, 2008). Moreover, patients with Crohn's disease and ulcerative colitis with a FAAH gene polymorphism are more prone to a severe disease phenotype and an earlier disease onset respectively (Storr *et al.*, 2009a).

Given the therapeutic potential of FAAH to regulate intestinal motility, we have investigated a recently developed FAAH inhibitor, AM3506 (Godlewski *et al.*, 2010) in a model of enhanced GI transit induced by LPS. It has been shown that a low dose of LPS increases GI motility without causing inflammation. This model of GI hypermotility mimics some features of functional GI motor disorders (Ceregrzyn *et al.*, 2001; Mathison *et al.*, 2004; Duncan *et al.*, 2008).

We hypothesized that the newly developed potent FAAH inhibitor AM3506 (Godlewski *et al.*, 2010) would normalize intestinal dysmotility, through activation of the EC system. Our goal was to evaluate whether FAAH inhibitors might have therapeutic potential in regulating abnormal GI motility, an approach that could be translated to humans.

Methods

Animals

Male Swiss albino mice (CD1, Charles River, St. Constant, QC, Canada), male CB_1-deficient mice ($CB_1^{-/-}$) on a predominant C57BL/6N background, male FAAH-deficient mice on C57BL/6N background and their wild-type pairs at 5–8 weeks of age were used and genotyped as previously described (Cravatt *et al.*, 2001; Marsicano *et al.*, 2002). Mice were housed with free access to food and water, and were allowed 1 week of acclimatization at a constant temperature (22°C) under a 12:12 h light–dark cycle before the study was com-

menced. All animal care and experimental procedures complied with the Guidelines of the Canadian Council on Animal Care and were approved by the University of Calgary Animal Care Committee.

LPS treatment for in vitro *experiments*

For the *in vitro* experiments, LPS (100 μg·kg^{-1}; *Escherichia coli* 026:B6; Sigma-Aldrich, St. Louis, MO, USA) or saline was injected i.p., and mice were killed 90 min later by cervical dislocation (Mathison *et al.*, 2004; Duncan *et al.*, 2008). The *in vivo* experiments are discussed separately below.

Determination of tissue myeloperoxidase (MPO) activity

MPO activity was assessed as a marker of neutrophil infiltration (Krawisz *et al.*, 1984; Storr *et al.*, 2009b). Samples of ileum or distal colon of LPS or saline-treated CD1 mice were homogenized in hexadecyltrimethyl ammonium bromide (HTAB) buffer (0.5% HTAB; Sigma-Aldrich). The homogenate was centrifuged, and supernatant was added to potassium phosphate buffer, containing O-dianisidine hydrochloride and H_2O_2. Absorbance was measured at 460 nm (Thermo Fischer Labsystems Multiskan, Thermo Scientific, Ottawa, ON, Canada), and MPO activity was expressed in U g^{-1} of wet tissue weight and was calculated from a standard curve performed on purified peroxidase enzyme (Sigma-Aldrich).

Histology

As detailed before (Storr *et al.*, 2009b), segments of distal colon or ileum of LPS or saline-treated CD1 mice were fixed overnight in Zamboni's fixative (2% paraformaldehyde, 15% picric acid; pH 7.4) at 4°C. Tissues were then rinsed in PBS and cross and sagittal sections of the specimens were cryoprotected in PBS containing 20% sucrose for 24 h. Specimens were embedded in optimum cutting temperature (Tissue-Tek, Sakura Finetechnical, Tokyo, Japan), cryostat-sectioned (12 μm) and mounted onto poly-D-lysine-coated slides. Sections were stained with haematoxylin and eosin and examined using a Zeiss Axioplan microscope (Carl Zeiss, Toronto, ON, Canada).

RNA extraction, cDNA generation and quantitative PCR

Distal colon and ileum of LPS- or saline-treated CD1 mice stored at −20°C in RNAlater (Qiagen, Mississauga, ON, Canada) were used for RNA extraction. Total RNA was extracted using the QIAGEN RNeasy Plus Mini Kit (Qiagen, Mississauga, ON, Canada). cDNA was generated from 1 μg of total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). In some experiments, reverse transcriptase was withheld from the reaction mixture.

TaqMan Gene Expression assay kits for the CB_1 (Mm00432621_s1), CB_2 (Mm0438286_m1), FAAH (Mm00515684_m1) and TNF (Mm00443258_m1) genes were purchased from Applied Biosystems (Frederick, MD, USA). The rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (VIC) from Applied Biosystems was used as the internal control. GAPDH expression was unchanged after treatment of the animals with LPS. Triplicate samples of each cDNA were amplified by real-time PCR in the ABI Prism 7000 Sequence Detection System (Applied Biosystems; 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min). Results were analysed using the ABI Prism 7000 SDS software (Applied Biosystems). Relative quantification (RQ) values, defined as fold changes in mRNA expressions, were calculated using the 'ΔΔ Ct' method. Briefly, a sample of ileum from a saline-treated animal was selected as the calibrator. The Ct values of other samples were calculated and presented as fold decrease or increase relative to the calibrator and presented as mean ± SEM of RQ values in each group.

Immunohistochemistry

Ileum and distal colon of LPS- or saline-treated CD1 mice were removed and immersed in ice-cold PBS (10 min) containing 1 μM nicardipine (Sigma-Aldrich). Tissues were opened along the mesenteric border, and were stretched and pinned mucosal side up in Sylgard (Dow Corning, Midland, MI, USA)-coated Petri dishes. Tissue samples were fixed by overnight immersion in 4% paraformaldehyde. Full-wall thickness cryostat sections or whole mount preparations were washed in PBS (3 × 10 min) containing 0.1% Triton X-100 (Sigma), incubated with a rabbit anti-FAAH primary antibody (1:500, provided by KM), which was raised against the last 103 amino acids of rat FAAH (Helliwell *et al.*, 2004) for 48 h at 4°C. After 48 h, tissues were washed again in PBS (3 × 10 min) and incubated with the donkey anti-rabbit CY3 (Jackson ImmunoResearch, West Grove, PA, USA; 1:100) as a secondary antibody for 90 min at room temperature, mounted in bicarbonate-buffered glycerol, examined using a Zeiss Axioplan fluorescence microscope and photographed with a Sensys digital camera (Photometrics, Tucson, AZ, USA) using V for Windows software (version 3.5; Digital Optics Ltd; Auckland, New Zealand). Intestinal tissues from FAAH-deficient mice were used as a negative control (Supporting Information Figure S1).

Assessment of FAAH activity

Ileal and distal colonic samples of LPS- or saline-treated CD1 mice were incubated at 37°C in Krebs solution with or without AM3506 (100 nM) for 15 min. Tissues were washed three times with Krebs solution and immediately frozen in liquid nitrogen. Frozen tissues were then homogenized in ice-cold 10 mM Tris-HCl buffer pH 7.6 containing 1 mM EDTA and centrifuged to remove cell debris. Supernatants were transferred to other tubes, and protein amounts were adjusted to 20 μg for each reaction. Homogenates were incubated with a mix containing 400 pmol of cold AEA and 500 fmol of [^{3}H]-AEA (specific activity – 60 Ci·mmol^{-1}; American Radiolabeled Chemicals, St. Louis, MO, USA) for 10 min at 37°C. FAAH activity was quantified by the amount of the [^{3}H]-ethanolamine released from [^{3}H]-AEA labelled on the ethanolamine moiety, as described previously (Fowler *et al.*, 2000; Liu *et al.*, 2003). Samples were done in triplicates and the results are presented as pmol AEA hydrolyzed min^{-1} mg^{-1} protein.

Identification and quantification of ECs

Quantification of EC levels was performed as described previously (Williams *et al.*, 2006; 2007; Wood *et al.*, 2008). Frozen samples of ileum and distal colon of LPS- or saline-treated CD1 mice pretreated with AM3506 (0.5 mg·kg^{-1}) or its

vehicle were weighed before homogenization in ice-cold acetone:PBS (pH 7.4; 3:1) and centrifuged. After the acetone had been evaporated under nitrogen, 100 µL PBS, one volume of methanol and two volumes of chloroform were added to the remaining supernatant,. The chloroform layer was dried under nitrogen stream and reconstituted in methanol before LC/MS/MS analysis. Chromatographic separation was achieved using an Agilent Zorbax SB-CN column (2.1 × 50 mm, 5 mm) on a Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Electron, San Jose, CA, USA) with an Agilent 1100 HPLC on the front end (Agilent Technologies, Wilmington, DE, USA). Eluted peaks were ionized via atmospheric pressure chemical ionization in positive mode and detected by multiple reaction monitoring. Deuterated internal standards were used for standard curves, and the levels of AEA, PEA, OEA or 2-AG g^{-1} tissue were determined.

Assessment of intestinal contractility

Segments of ileum and distal colon of LPS- or saline-treated mice were removed and immersed in ice-cold oxygenated Krebs solution. Intestinal segments (1 cm) were tied with a silk thread and suspended in an organ bath filled with oxygenated Krebs solution (95% O_2 and 5% CO_2) at 37°C. The preparations were attached to an isometric force transducer, were positioned between platinum electrodes and allowed to equilibrate under 500 mg tension (Harvard Apparatus, model 50–7905, Kent, UK). Mechanical activity of the muscle was enhanced by a transducer amplifier (Harvard Apparatus, model 50-7970), relayed to a bioelectric amplifier (Hewlett-Packard, model 8811A, Mississauga, ON, Canada). The data were converted using a CED 1401 PLUS analogue-to-digital converter (Cambridge Electronic Design, Cambridge, UK.) running into Spike 3 software (Cambridge Electronic Design).

We assessed the effect of LPS on tension generated by cumulative addition of bethanechol (1–30 µM) and on electrically-evoked contractions and relaxations of the ileum and colon. Each experiment was done on fresh ileal or colonic tissues, and different tissues were used for each experiment. Electrical field stimulation (EFS) was applied by a Grass Stimulator (S88 stimulator, Grass, Quincy, MA, USA) at 1, 2, 4 and 8 Hz (10 s stimulation duration, 0.5 ms pulse duration, 60 V). EFS relaxations were recorded in the presence of atropine (1 µM) and guanethidine (5 µM). After these studies, the tissues were stimulated at 4 Hz (0.5 ms pulse duration; 60 V) continuously, and the effects of AM3506 or CB receptors agonists and antagonists were investigated. In separate experiments, we assessed if AM3506 altered tension generated by bethanechol (300 nM–10 µM).

AM3506 or CB agonists were added to the organ bath, and 15 min incubation time was allowed for each drug. Before the addition of drugs, the mean of three successive EFS contractions or relaxations were used as an internal control (100%). The amplitude of contractions and relaxations were measured and in the presence of drugs, were reported as the percentage of the internal control. In every set of experiments, vehicle controls were performed.

GI transit and faecal output studies

Mice were transferred from their home cage into individual transparent plastic cages without bedding. After 60 min acclimatization, any faecal pellets were removed from the cage, and mice were administered LPS (100 µg·kg^{-1}, i.p.) or physiological saline. The individual cages were inspected for the presence of faecal pellets for the next 90 min, and the total weight of faecal output was measured. After this, 200 µL of a charcoal (10% charcoal, 5% gum arabic) marker was gavaged into the stomach to investigate the upper GI transit, which is a gross measure of gastric emptying plus small intestinal transit. The mice were killed 15 min later, and the small intestine was removed. The distance travelled by the marker was measured and expressed as a percentage of the total length of the small intestine (pylorus to caecum). To determine whether FAAH inhibition affects intestinal motility, AM3506 (0.5 mg·kg^{-1}, i.p.) or vehicle was injected 20 min before the LPS or saline injection. In separate experiments, vehicle, AM251 (0.5 mg·kg^{-1}, i.p.) or AM630 (1 or 5 mg·kg^{-1}, i.p.) were administered 20 min before AM3506 injection to determine the CB receptor responsible for the effects.

Whole-gut transit was assessed in FAAH-deficient mice and their aged-matched wild-types. Whole-gut transit was performed as described previously (Cluny *et al.*, 2009). Briefly, mice were transferred to individual cages without bedding and were left to acclimatize for 1 h. Then the animals were gavaged with 200 µL of an Evans' blue (5% Evans' blue, 5% gum arabic) marker. The latency to the detection of Evans' blue in the faeces (in min) was recorded and presented as whole-gut transit time.

Assessment of locomotor activity

Ambulatory locomotor activity was measured 90 min after LPS or saline injection using an infrared beam activity monitor (Columbus Instruments, Columbus, OH, USA). AM3506 (0.5 mg·kg^{-1}, i.p.) or vehicle was injected 20 min before the mice were administered an injection of LPS (100 µg·kg^{-1}, i.p.) or physiological saline. Mice were pre-exposed to the recording equipment in the morning and the experiments were done in the afternoon of the same day. Each individual mouse was placed in the apparatus, and the ambulatory count was recorded over a 10 min period. Movement of the mice was recorded as the ambulatory activity count when the infrared beams were sequentially broken. The effect of WIN55,212-2 (5 mg·kg^{-1}) on locomotor activity was used as a positive control.

Drugs

AM3506 was dissolved in ethanol for *in vitro* experiments. AM3506 is a later generation sulphonyl fluoride analogue with enhanced affinity and selectivity for FAAH (Figure 1A) (Deutsch *et al.*, 1997; Godlewski *et al.*, 2010). Experiments on human recombinant FAAH showed that AM3506 has IC_{50} of 48 nM for inhibition of FAAH activity, and it is three times more potent compared with its structural analogue phenylmethylsulphonyl fluoride. AM3506 has low affinity for CB_1 (5.77 µmol·l^{-1}) and moderate affinity for CB_2 receptors (192 nmol·l^{-1}). AM3506 is twice as potent in inhibiting FAAH compared with URB597 (Godlewski *et al.*, 2010). The dose and concentration of AM3506 were selected based on our previous study that showed specific FAAH blockage at 100 nm *in vitro* and 0.5 mg·kg^{-1} *in vivo* (Godlewski *et al.*, 2010). The CB_1/CB_2 receptor agonist WIN55,212-2; CB_2 recep-

tor agonist JWH-133; CB_1 receptor antagonist AM251; and CB_2 receptor antagonist AM630 were purchased from Tocris (Ellisville, MI, USA) and dissolved in 100% ethanol. AM630 was dissolved in 100% dimethyl sulphoxide (DMSO) for *in vitro* experiments. The maximum concentration of ethanol and DMSO used in the organ bath had no effect on contractility. For the *in vivo* experiments AM3506, AM251 and AM630 were dissolved in 2% DMSO, 1% Tween 80 in physiological saline (vehicle). Bethanechol, atropine, guanethidine and tetrodotoxin (TTX) were purchased from Sigma-Aldrich and dissolved in water. The concentration of stock solutions for AM3506, WIN55,212-2, JWH133, AM630, AM251, guanethidine and atropine were 0.01 M and for bethanechol 1 M, these were diluted immediately before the experiments.

Statistics

Data are presented as mean ± SEM of *n* experiments, which indicates the number of individual mice. Student's *t*-test was used to compare a single treatment mean with control mean, while one-way or two-way ANOVA followed by Bonferroni *post hoc* testing were used for the analysis of multiple measurements. A value of $P < 0.05$ was considered statistically significant.

Results

The effects of LPS treatment on inflammation, FAAH, CB_1 and CB_2 expression

LPS treatment caused no histological evidence of inflammation (not shown) or granulocyte infiltration, as determined by MPO activity (Figure 1B). Nevertheless, LPS gave rise to immune activation as shown by enhanced TNF mRNA expression (Figure 1C). FAAH mRNA levels were significantly higher in the ileum than the colon. LPS treatment had no

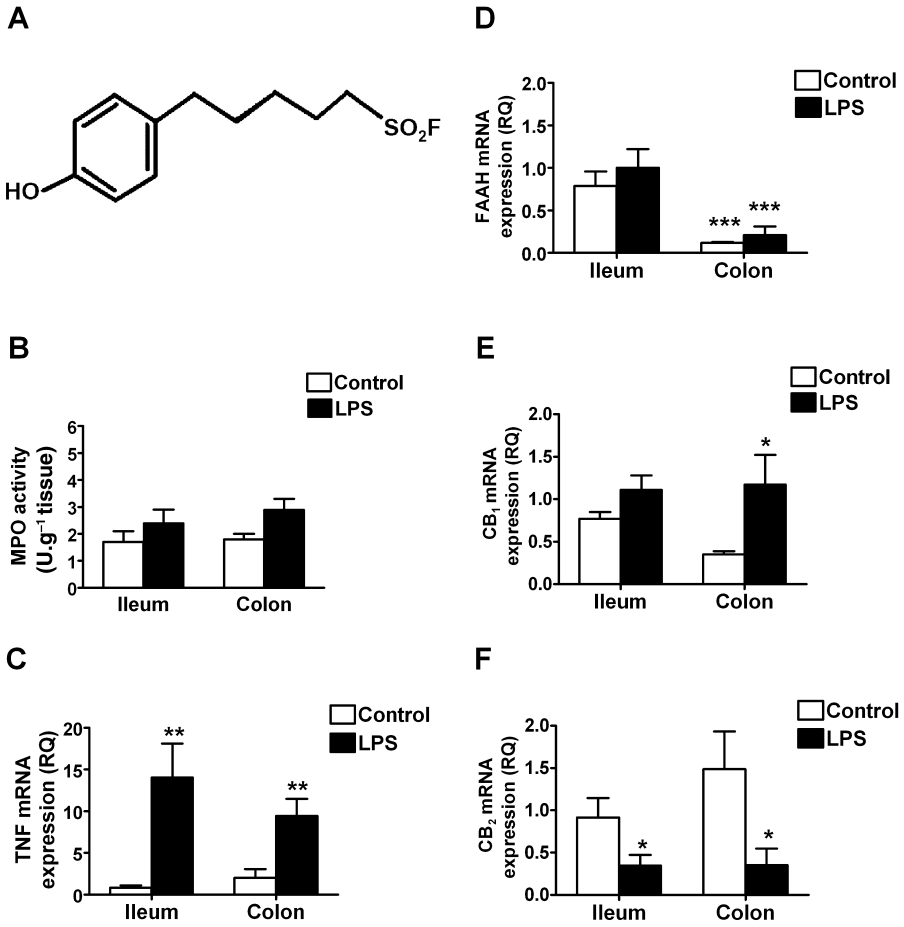

Figure 1

(A) Chemical structure of AM3506. AM3506 is a structural analogue of phenylmethylsulphonyl fluoride. (B) MPO activity; (C) TNF; (D) FAAH mRNA; (E) CB_1 receptor; and (F) CB_2 receptor expression in the ileum and distal colon of CD1 mice treated with saline (control) or LPS (100 $\mu g \cdot kg^{-1}$, i.p.). There were no significant changes in MPO activity after LPS treatment, although TNF mRNA was significantly increased in both ileum and distal colon. LPS treatment had no effect on FAAH mRNA, which was significantly higher in the ileum than the colon. CB_1 receptor mRNA was increased in the distal colon, and CB_2 receptor mRNA was reduced in both regions of the gut by LPS treatment. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control in (E, C and F) and compared with ileum in (D); $n = 4–6$ per group.

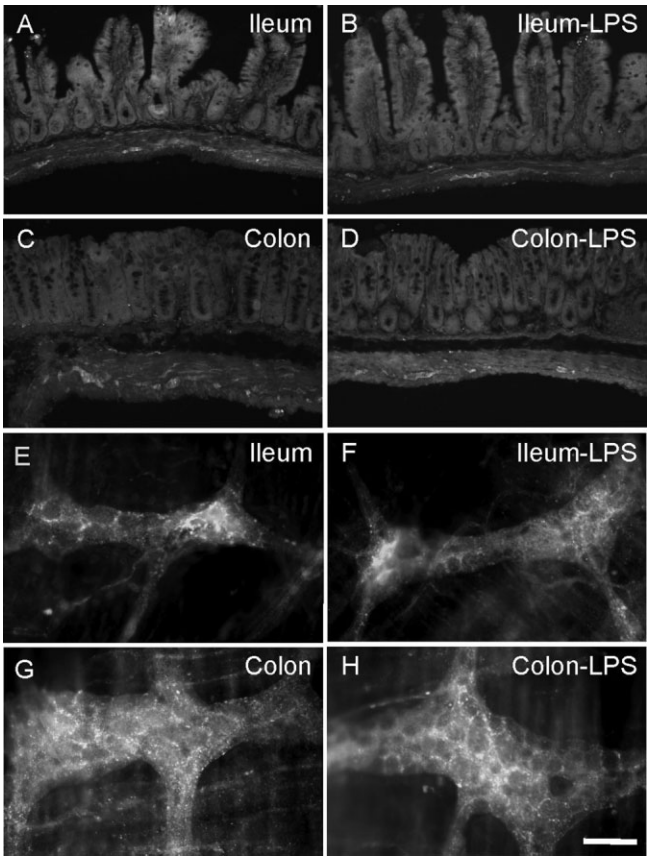

Figure 2

FAAH immunoreactivity in full-wall thickness sections (A–D) and whole mount preparations of the myenteric plexus (E–H) of the ileum and distal colon from animals treated with saline and LPS. LPS (100 μg·kg^{-1}, i.p.) treatment did not alter the distribution of FAAH (B, D, F and H). Scale bars: 100 μm (A–D) and 50 μm (E–H).

effect on the levels of FAAH expression in either region of the GI tract (Figure 1D). CB_1 receptor mRNA levels were increased in the colon, while CB_2 receptor mRNA levels were significantly reduced in both ileum and colon by LPS treatment (Figure 1E, F). Despite the regional variation in FAAH mRNA expression, FAAH immunoreactivity was present in neurons and nerve fibres and/or terminals in the ileal and colonic myenteric plexus (Figure 2), in smooth muscle, and epithelial cells. In the ileum, some neurons displayed higher levels of expression than others, the significance of which is not known. The pattern of FAAH immunoreactivity remained unchanged after LPS treatment (Figure 2). No immunoreactivity was observed in FAAH-deficient mice.

Effect of LPS treatment on FAAH activity and EC levels

The activity of FAAH regulates the levels of ethanolamides in the gut. LPS treatment did not change FAAH activity in the ileum or the colon of mice (Figure 3A, B). After incubation with AM3506 (100 nM), we observed a significant reduction in FAAH activity in the ileum of LPS-treated mice (Figure 3A). LPS treatment had no effect on the levels of AEA, 2-AG, PEA and OEA (Figure 4). In LPS-treated mice, which were pretreated with AM3506 (0.5 mg·kg^{-1}), there was a significant increase in AEA levels the ileum and the tended to be increased in the colon (Figure 4A, B). As previously reported (Pinto *et al.*, 2002), levels of 2-AG were over a thousand times higher than AEA in both regions of the gut. 2-AG tended to be decreased in the ileum and colon of LPS-treated mice that were pretreated with AM3506, although this did not reach significance with a multiple group comparison.

The effects of LPS treatment on GI contractility

Electrical stimulation of the mouse ileum produced a largely monophasic contraction, which was converted to a

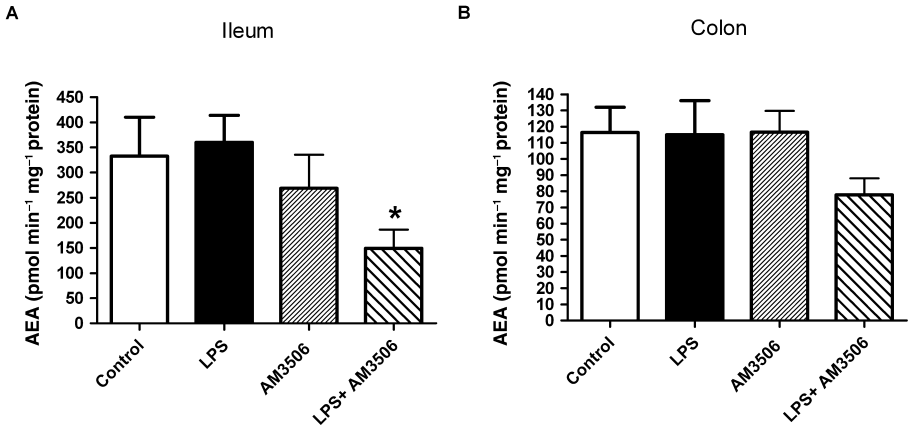

Figure 3

FAAH activity in the ileum (A) and the distal colon (B) of CD1 mice. Note that FAAH activity in the colon of control group was significantly lower than in the ileum ($P < 0.05$). LPS (100 μg·kg^{-1}, i.p.) treatment or incubation with AM3506 (100 nM) did not inhibit FAAH activity in the ileum and the distal colon. After incubation with AM3506 (100 nM), a significant reduction in the FAAH activity of the ileum of LPS-treated mice was observed (A). *$P < 0.01$ compared with control; n = 6–10 per group.

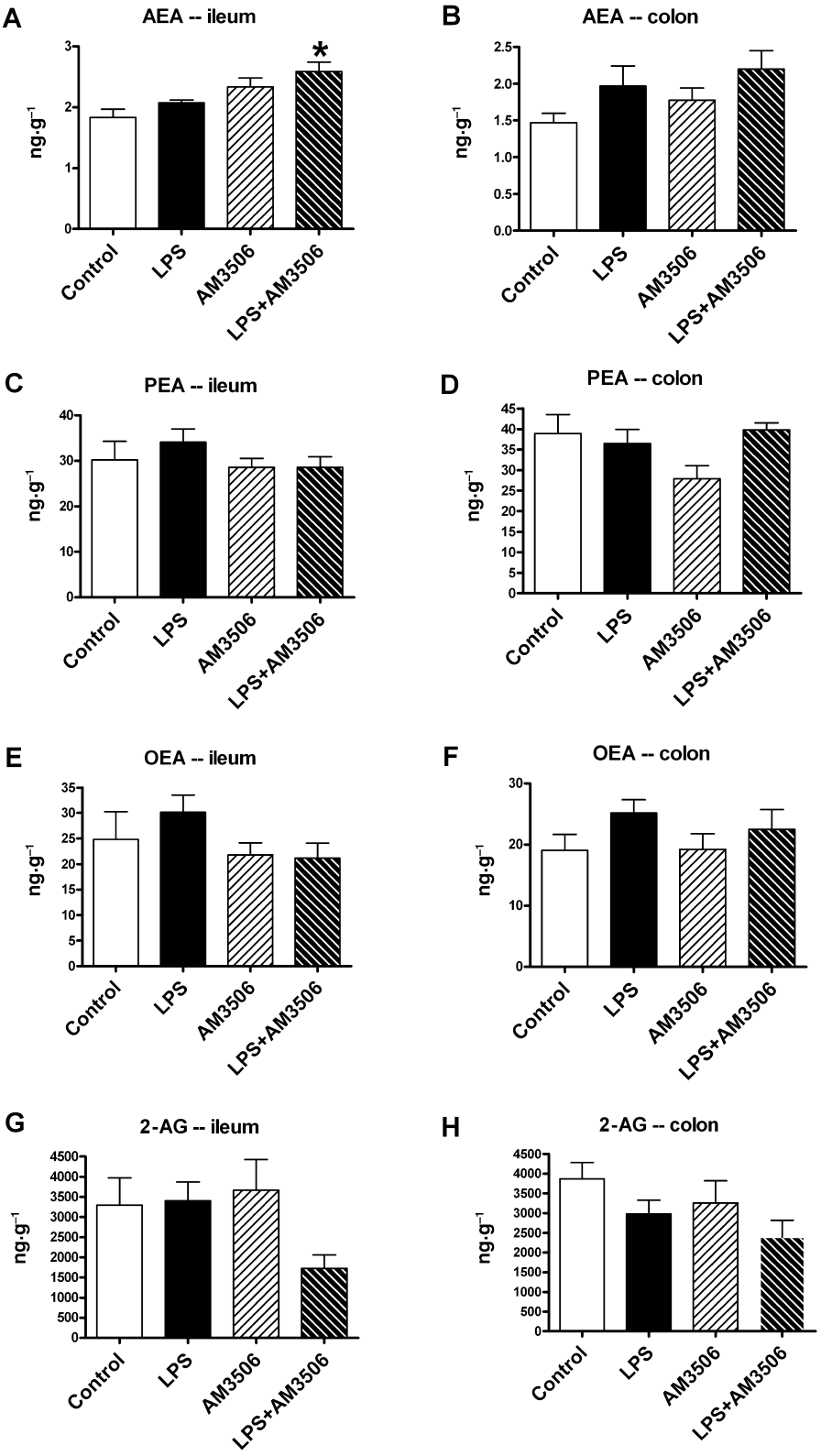

Figure 4

FAE and 2-AG levels in the ileum and distal colon of saline-treated (control) or LPS (100 μg·kg^{-1}, i.p.)-treated mice pretreated with AM3506 (0.5 mg·kg^{-1}, i.p.) or its vehicle. AM3506 treatment enhanced AEA levels in the ileum (A), but not the colon (B) of LPS-treated mice. There were no significant differences in the levels of PEA (C and D), OEA (E and F) or 2-AG (G and H) in the ileum (C, E and G) or colon (D, F and H). *$P < 0.05$ compared with control; n = 3–10 per group.

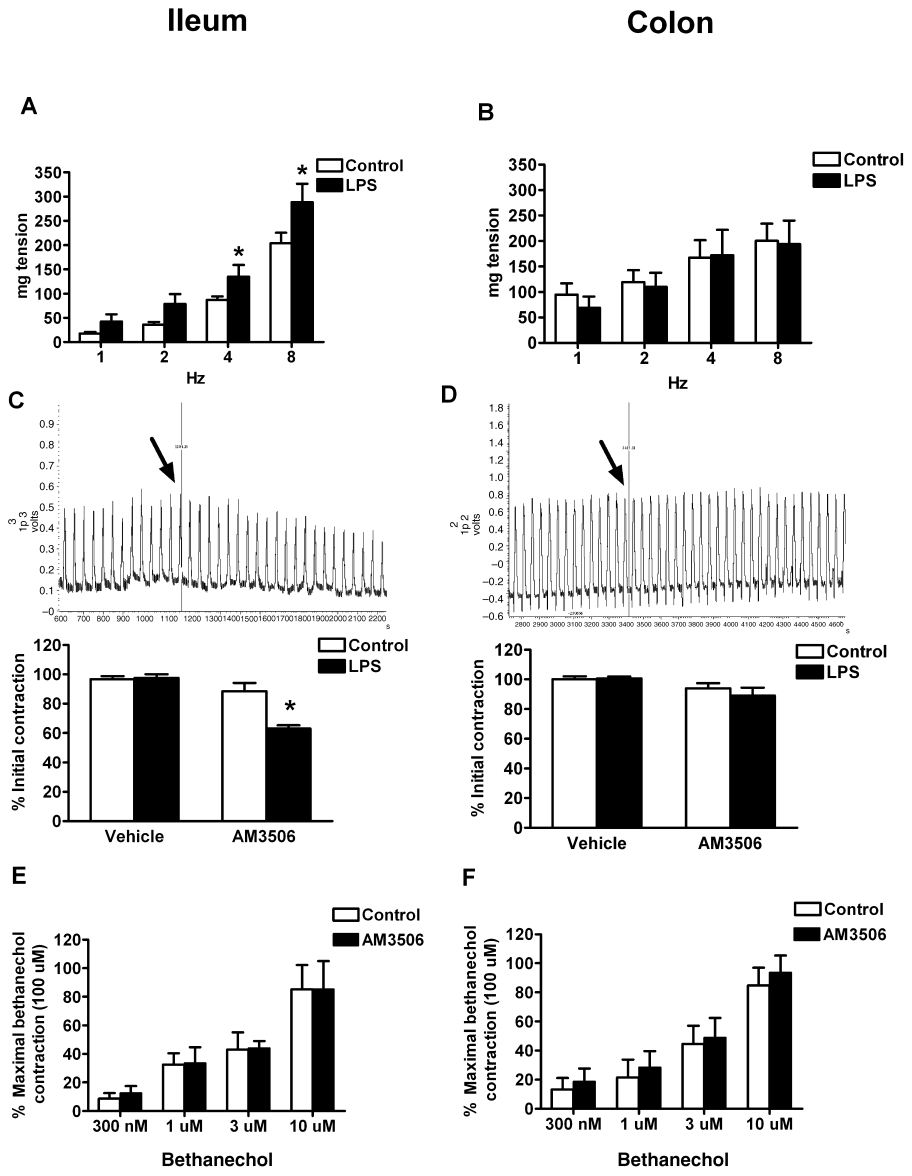

Figure 5

Effect of LPS (100 μg·kg^{-1}, i.p.) and AM3506 (100 nM) on EFS-evoked contractions (4 Hz in C and D) of mouse ileum (A and C) and distal colon (B and D) and bethanechol(300 nM–10 μM)-induced contractions of mouse ileum and distal colon (E and F). Note that LPS enhanced contractility in the ileum (A) but not the distal colon (B) of the CD1 mice at all frequencies. (C) AM3506 reduced ileal contractions in LPS-treated CD1 mice but not in control mice. The trace represents the effect of AM3506 on ileal EFS contractility in a LPS-treated mouse. The arrow shows the time that tissue was exposed to AM3506 (100 nM). (D) AM3506 had no effect on colonic electrical contractions in control and LPS-treated animals. The trace represents the effect of AM3506 on colonic EFS contractility in a LPS-treated mouse. The arrow shows the time that tissue was exposed to AM3506 (100 nM). (E and F) Contractilities induced by bethanechol were not changed after LPS treatment. *$P < 0.05$ compared with control; $n = 4$–8 per group in all cases.

relaxation under NANC conditions after adrenergic and cholinergic blockade with guanethidine and atropine. The mouse colon typically gave a relaxation followed by a contraction after electrical stimulation, and this was converted to a larger relaxation and a smaller contraction in NANC conditions. We assessed the effect of LPS on ileal contractility in the mouse ileum and colon (Figure 5A and B). We observed a frequency-dependent contraction of the ileum, which was significantly enhanced at 4 and 8 Hz stimulations in tissues taken from LPS-treated mice (Figure 5A). Similarly, we observed a frequency-dependent contraction in the mouse colon, but by contrast, this was not enhanced in animals treated with LPS (Figure 5B). In control or LPS-treated mice, the contractions evoked by EFS in the ileum were completely abolished by atropine (1 μM) and TTX (100 nM) (data not shown). In contrast, atropine reduced colonic contractions by only 31.7 ± 5.5% ($P < 0.01$, $n = 4$) and TTX abolished them, albeit at a higher concentration (2 μM). LPS did not change the

bethanechol-induced contractions (1–30 μM) or EFS-induced relaxations in either the ileum or colon (data not shown). The effects of bethanechol (10 μM) were not altered by TTX (100 nM), but they were completely blocked by atropine (1 μM).

Effect of FAAH inhibition on intestinal contractility

Addition of AM3506 (100 nM) to the organ bath caused a reduction of electrically evoked contractions in the ileum of LPS-treated mice, but not that of normal animals (Figure 5C). The magnitude of the reduction (37 ± 6%) seen in LPS-treated mice was similar to the extent of the enhancement seen after LPS treatment (48 ± 8%), suggesting that FAAH inhibition normalizes contractility. In the same manner that LPS had no effect on contractility of the colon, AM3506 also had no effect on either normal or LPS-treated colonic contractility (Figure 5D). AM3506 did not alter bethanechol contractility in the ileum and colon (Figure 5E and F).

The effect of AM3506 on the ileum of LPS-treated mice was completely abolished in $FAAH^{-/-}$ mice (Figure 6A). To test whether the effects of FAAH inhibition by AM3506 on GI contractility are mediated via CB_1 and/or CB_2 receptors, we used CB_1 and CB_2 receptor antagonists, as well as $CB_1^{-/-}$ mice. The CB_1 receptor antagonist AM251 (100 nM) completely abolished the effect of AM3506 (100 nM) in the ileum of LPS-treated mice, as did the CB_2 receptor antagonist AM630 (300 nM) (Figure 6B). A combination of both antagonists also completely abolished the effects of AM3506. The actions of AM3506 were absent in the ileum of $CB_1^{-/-}$ mice (Figure 6C). AM251 and AM630 at the concentrations used in this study did not change EFS- or bethanechol-induced contractilities.

Effect of CB_1 and CB_2 receptor agonists on EFS-induced contractions after LPS treatment

Because LPS treatment could alter the expression and/or function of CB receptors, we next examined the effects of CB agonists in LPS-treated animals.

WIN55,212-2 (10–100 nM), a prototypical CB agonist, reduced ileal contractility evoked by EFS in a concentration-dependent manner (Figure 7A), but did not significantly reduce colonic contractility at these concentrations (Figure 7B). After LPS treatment, the effects of WIN55,212-2 were virtually identical in the ileum; however, it significantly decreased electrical contractility in the colon (Figure 7B), consistent with the observed increase in CB_1 receptor mRNA expression. The effect of WIN55,212-2 in the ileum of saline or LPS-treated mice was abolished by the CB_1 receptor antagonist AM251 (100 nM) but not by the CB_2 receptor antagonist AM630 (300 nM) (Figure 7A). After LPS treatment, the effects of WIN55,212-2 (100 nM) in the colon was partially reversed by 100 nM AM251 (Figure 7B). AM630 (300 nM) did not change the response to WIN55,212-2 in the colon (Figure 7B).

To assess if there is a role for CB_2 receptors in GI contractility in the mouse, we tested the selective CB_2 receptor agonist JWH-133. JWH-133 reduced electrically-evoked contractions at the highest concentration (10 μM) in the ileum, but was without effect in the colon (Figure 7C and D). The effect of JWH-133 in the ileum was completely blocked by the

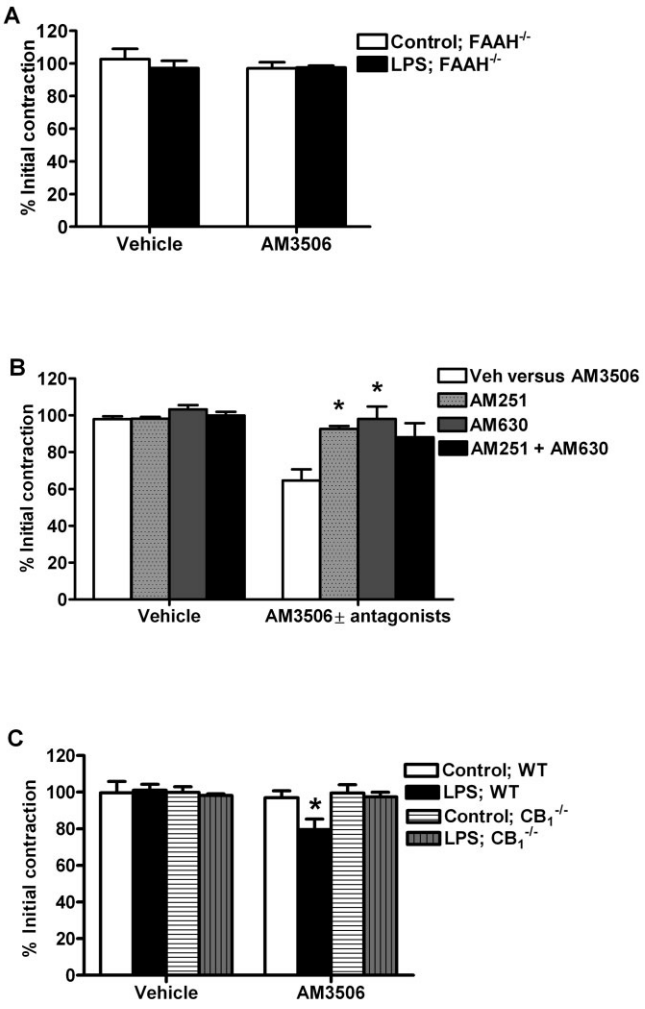

Figure 6

Effect of AM3506 (100 nM) on ileal contractions in gene-deficient mice and in the presence of CB receptor antagonists. (A) AM3506 did not change EFS contractility in control or LPS(100 μg·kg^{-1}, i.p.)-treated $FAAH^{-/-}$ mice. (B) AM251 (100 nM) and AM630 (300 nM) blocked the effect of AM3506 on ileal contractility in LPS treated CD1 mice. (C) AM3506 had no effect on ileal contractions of vehicle or LPS-treated $CB_1^{-/-}$ mice; however, it decreased EFS contractility in C57BL/6N wild-type mice treated with LPS (100 μg·kg^{-1}, i.p.). *$P < 0.05$ compared with AM3506 in (B) and compared with vehicle in (C); n = 4–8 per group in all cases.

CB_2 receptor antagonist AM630. After LPS treatment, JWH-133 was slightly more potent in the ileum, completely reversed by AM630 and ineffective in the colon (Figure 7C and D).

Effect of LPS treatment and FAAH inhibition on intestinal transit

Upper GI transit was increased in LPS-treated mice (Figure 8A), as it is in rats (Mathison *et al.*, 2004). AM3506 (0.5 mg·kg^{-1}) had no effect on transit in vehicle-treated mice, but slowed the LPS-enhanced upper GI transit back to control levels (Figure 8A). The effect of AM3506 was abolished by

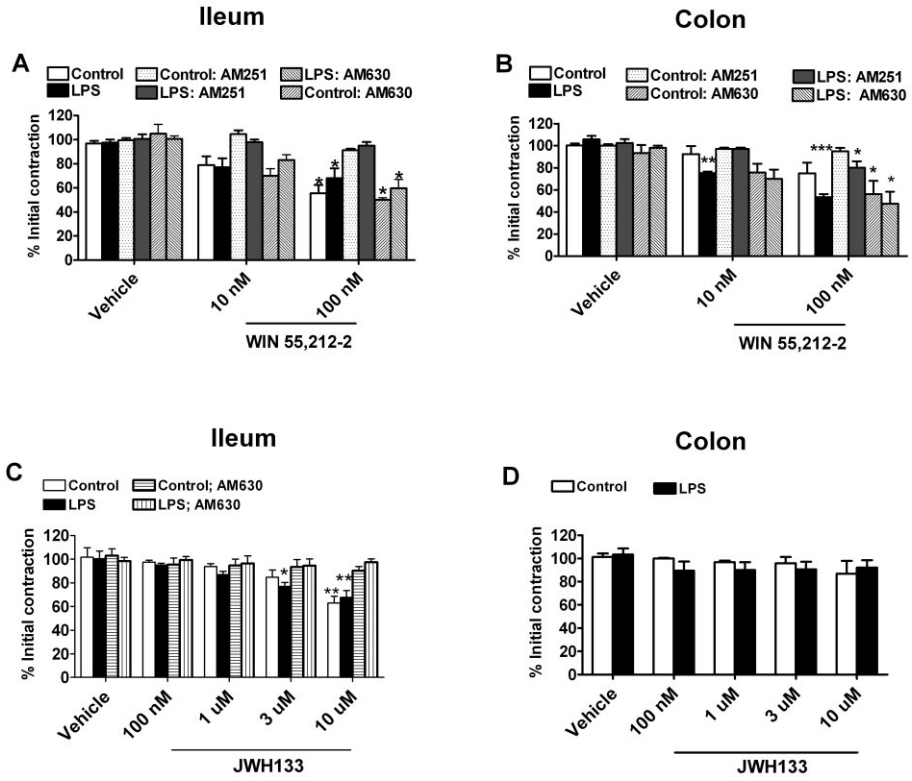

Figure 7

The effect of WIN55,212-2 (10–100 nM) and JWH133 (100 nM–10 μM) in the ileum and distal colon of CD1 mice after treatment with saline (control) or LPS (100 μg·kg^{-1}, i.p.). (A) WIN55,212-2 significantly reduced contractility of the ileum in control and LPS-treated mice to about the same extent and this effect was fully reversed by the CB_1 receptor antagonist AM251 (100 nM) but not the CB_2 receptor antagonist AM630 (300 nM). (B) WIN55,212-2 reduced contractility of the colon in control mice, but this was only significant after LPS treatment. AM251 did not fully reverse the effects of WIN55,212-2 (100 nM) after LPS treatment in the distal colon. (C) JWH133 concentration-dependently reduced contractions evoked by EFS in the ileum. This effect was abolished by AM630 (300 nM). (D) JWH133 had no effect on evoked contractions in the distal colon. *P < 0.05, **P < 0.01, ***P < 0.001 compared with vehicle; n = 3–6 per group for all panels.

AM251 (0.5 mg·kg^{-1}) and the highest dose of AM630 (5 mg·kg^{-1}) (Figure 8A). AM251 and AM630 at these doses did not alter upper GI transit when administered alone in both control and LPS-treated animals (data not shown).

Effect of LPS treatment and FAAH inhibition on faecal output

In order to assess the effects of LPS and FAAH inhibition on faecal output, we measured the mass of faecal output over 90 min. The total weight of faecal output significantly increased in LPS-treated mice (Figure 8B). This enhanced faecal output was completely reversed by AM3506 (0.5 mg·kg^{-1}), which alone had no significant effect on control stool output (Figure 8B). AM251 abolished the effect of AM3506 on total stool output; indicating that the effect of AM3506 is mediated by CB_1 receptors. AM630 had no effect on AM3506 at either dose used (Figure 8B). AM251 and AM630 alone at the doses used did not change total faecal output in either control or LPS-treated animals (data not shown).

Effect of AM3506 on locomotor activity

Finally, in order to examine whether AM3506 produces any cataleptic effects, we examined the motor activity of mice treated with AM3506. AM3506 (0.5 mg·kg^{-1} i.p.), compared with WIN55,212-2, did not change the ambulatory score in saline-treated mice; however, LPS-treated animals that were/were not pretreated with AM3506 (0.5 mg·kg^{-1}) showed a similar decrease in ambulatory score (Figure 8C).

GI motility in FAAH-deficient mice

Whole gut and upper GI transit were identical in FAAH-deficient mice compared with the wild-type mice (Figure 9A and B). LPS increased upper GI transit in the wild-type but not in the FAAH-deficient mice (Figure 9B).

Discussion and conclusions

FAAH is an important enzyme whose activity is involved in the regulation of the levels of ECs and their related FAEs (Puffenbarger, 2005; Ahn *et al.*, 2008; Fezza *et al.*, 2008). FAAH-deficient mice display a behavioural and functional phenotype; they have heightened pain thresholds, reduced anxiety and, among other things, are protected from the development of experimental colitis (Cravatt *et al.*, 2001; Massa *et al.*, 2004; Moreira *et al.*, 2008; Wise *et al.*, 2008).

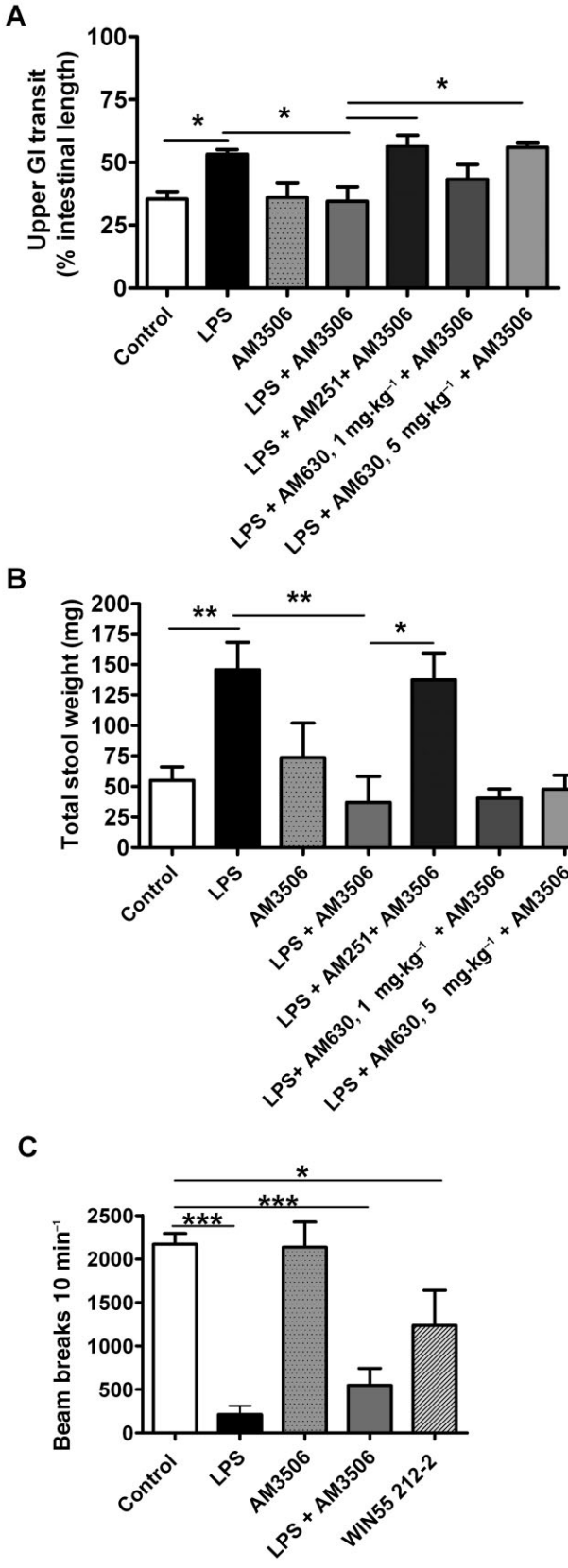

Figure 8

Effect of AM3506 (0.5 mg·kg^{-1}, i.p.) on upper GI transit of a charcoal marker (A) and total stool weight (B) of control and LPS (100 μg·kg^{-1}, i.p.)-treated mice. (A) AM3506 reversed the enhanced transit produced by LPS while having no effect alone. The effect of AM3506 was reversed by AM251 and the higher dose of AM630. (B) AM3506 reversed the enhanced stool output produced by LPS while having no effect alone. The effect of AM3506 was reversed by AM251, but not by AM630. (C) Effect of LPS (100 μg·kg^{-1}, i.p.) and AM3506 (0.5 mg·kg^{-1}, i.p.) on ambulatory motor activity of mice; AM3506 did not decrease ambulatory motor activity. LPS decreased ambulatory activity to the same extent in AM3506- or vehicle-pretreated mice. WIN55,212-2 (5 mg·kg^{-1}) significantly decreased ambulatory activity. *P < 0.05, **P < 0.01, ***P < 0.001, bar indicates significant differences between the groups. n = 4–14 per group.

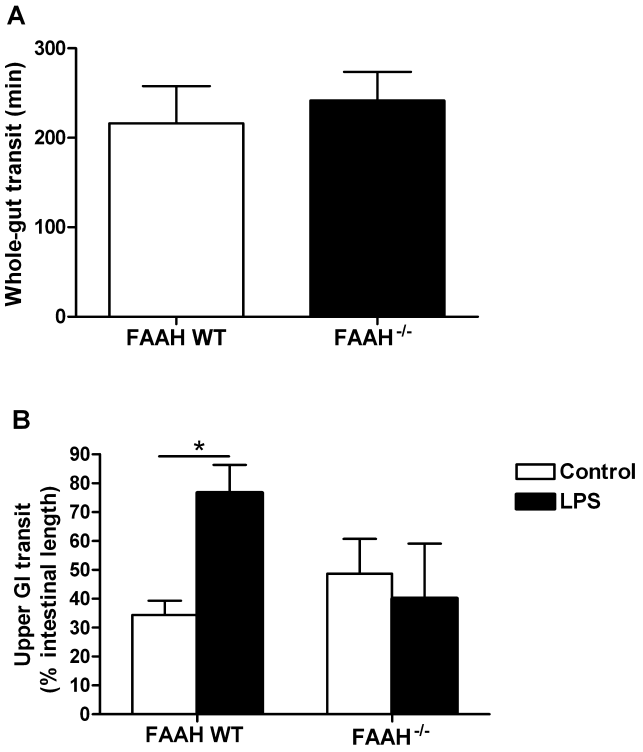

Figure 9

GI transit in FAAH-deficient (FAAH$^{-/-}$) and wild-type (WT) mice. (A) Whole-gut transit was identical in both groups. (B) Upper GI transit was identical in both groups under control conditions; however, LPS (100 μg·kg^{-1}, i.p.) increased transit in wild-type but not the FAAH$^{-/-}$ mice. *P < 0.05; n = 4–8 per group.

FAAH inhibitors produce a very similar spectrum of actions to that of genetic ablation of FAAH (Gaetani *et al.*, 2003; Schlosburg *et al.*, 2009). In the GI tract, FAAH inhibitors protect animals from the development of colitis and against the development of aberrant crypt foci, considered to be precancerous lesions of the colon (Izzo *et al.*, 2008; Storr *et al.*, 2008). These compounds offer significant promise as therapeutic agents because they do not appear to cause the unwanted central actions of CB agonists, even though their

effects can largely be blocked by CB_1 receptor antagonists and are not observed in CB_1 receptor gene-deficient mice (Petrosino and Di Marzo, 2010). We have investigated a novel FAAH inhibitor AM3506 (Godlewski *et al.*, 2010) in a model of intestinal dysmotility and shown that this compound normalizes LPS-enhanced small intestinal motility *in vitro* and *in vivo*, without obvious behavioural side effects or actions in normal animals.

Previously, we demonstrated in rats that the EC system is involved in enhanced motility in the LPS model, with the notable finding that the enhanced intestinal contractility and motility were regulated by CB_2 receptors (Mathison *et al.*, 2004; Duncan *et al.*, 2008). Here we have extended these findings to a mouse model and have focused on the role of FAAH. Examination of the gut from LPS-treated mice revealed no obvious structural changes or evidence of leucocyte infiltration. Nevertheless, immune activation was demonstrated by the increased TNF mRNA levels in the ileum and colon of LPS-treated mice, and this mechanism probably underlies the functional alterations observed (Ceregrzyn *et al.*, 2001). Ileus, histological changes and increased macrophage activation in the mouse intestine have been reported in mouse models of sepsis induced with much higher doses of LPS than we used (de Winter *et al.*, 2005; De Filippis *et al.*, 2008b). Both increased and decreased GI motility can be seen after LPS, depending on the dose, timing between injection and assessments of GI motility, the region of the GI system that is investigated and the type of LPS.

In this study we used a model of intestinal dysmotility in the absence of overt intestinal inflammation. Our model has features of acute gastroenteritis and irritable bowel syndrome (IBS). The enteric nervous system has been implicated in dysmotility in IBS (Gershon, 2005). In this regard it is worth noting that the ENS is activated in the LPS model (Mathison *et al.*, 2004; Duncan *et al.*, 2008). A second feature of this LPS model is its association with increased excitability of colonic nociceptive dorsal root ganglion neurons (Ochoa-Cortes *et al.*, 2010). This is consistent with the enhanced visceral sensitivity in IBS patients (Ohman and Simren, 2010).

Interestingly, in the colon, LPS did not have any effect on EFS-evoked contractility, although faecal output *in vivo* was increased. This is possibly due to increased secretomotor activity, central actions or the enhanced transit of small intestinal contents into the large intestine. The latter is less likely because of the lack of sensitivity to CB_2 receptor antagonists. In the ileum, contractility is completely cholinergic whereas in the colon, it is partially cholinergic (Mule *et al.*, 2007a,b) as confirmed in this study. The differences in the motor responses in the ileum versus the colon may be because of the balance of cholinergic versus non-cholinergic excitation. LPS did not appear to have a direct effect on intestinal smooth muscle motor activity or enteric inhibitory motor neurons, as the response did not change for bethanechol- or EFS-induced relaxation. Thus, it appears that our model is largely one of enhanced ACh release in the ENS. Since CB_1 and CB_2 receptors are localized to enteric cholinergic nerves and CB receptors regulate the release of ACh, our focus on modulation of the EC system offers the possibility to target these nerves with a degree of specificity.

The EC system is activated in the LPS model as previously demonstrated (Mathison *et al.*, 2004; Duncan *et al.*, 2008; Li *et al.*, 2010). This state of activation does not seem to depend on changes to CB receptor expression or on enhanced EC levels. CB_1 receptor mRNA in the ileum was not affected by LPS treatment to any great extent, although LPS significantly elevated CB_1 receptor mRNA levels in the colon. This finding helps to explain the slightly greater potency of the CB agonist WIN55,212-2 after LPS treatment in the colon. In contrast, CB_2 receptor mRNA expression was reduced by LPS treatment in the ileum and colon. A similar reduction in CB_2 receptor expression in response to LPS has previously been reported in immune cells (Lee *et al.*, 2001). However, it is apparent from our findings, and previous reports, that despite a reduced expression, CB_2 receptor function in the small intestine is increased by LPS treatment (Mathison *et al.*, 2004; Duncan *et al.*, 2008). In fact, we observed what appears to be a fully functional CB_2 receptor in our model, since AM3506 inhibited both small intestinal contractility *in vitro* and upper GI motility *in vivo* in a manner that was reversed by the CB_2 receptor antagonist AM630 and, as well, the CB_2 receptor agonist JWH-133 reduced electrically evoked contractions *in vitro*. While CB_2 receptor mRNA is present in both the ileum and colon, no functional CB_2 receptor actions were found in the colon. The reasons for these regional differences is not clear, but these findings suggest that targeting the CB_2 receptor in small bowel pathophysiology with endogenous ligands represents an attractive therapeutic option because it has no psychotropic central actions and is apparently inactive in the healthy gut.

The main goal of the study was to examine whether inhibition of FAAH represents a mechanism to reverse dysmotility in animals treated with LPS. Here we showed that AM3506 was able to inhibit enhanced contractility evoked by EFS *in vitro* in tissues from animals treated with LPS, but not under control conditions. Our immunohistochemical localization of FAAH in the enteric nervous system suggests that AM3506 is acting there, which is also consistent with the functional activity of AM3506 *in vitro*. Perhaps more importantly, AM3506 was effective *in vivo* at reducing the enhanced intestinal motility and faecal output in LPS-treated mice.

AM3506 had virtually no effect on the GI motility of healthy animals and GI transit was identical in FAAH-deficient and wild-type mice. Moreover, we have shown that AM3506 does not inhibit FAAH activity in the ileum of the healthy animals and does not alter intestinal AEA, 2-AG, PEA and OEA levels. Capasso *et al.* (2005) showed that FAAH-deficient mice have similar intestinal transit compared with the wild-type mice; however, in their hands, the FAAH inhibitor AA-5-HT reduced intestinal transit, partially through a CB_1 receptor-mediated mechanism of action. The reason for the differences between Capasso's findings and our results may relate to the different specificities between the FAAH inhibitors or other factors. AA-5-HT antagonizes transient receptor potential vanilloid 1 with an IC_{50} of 36.8–39.9 nM, which is far lower compared than its IC_{50} for FAAH (1–12 μM). Moreover, AA-5-HT is known to increase not only the intestinal levels of AEA, but also those of 2-AG and PEA, which may reduce intestinal motility. On the other hand, AM3506 has affinity for CB_1 and CB_2 at 5.77 μM and 192 nM, respectively; however, the K_i of AA-5-HT for CB_1 and CB_2 receptors is more than 50 and 10 μM, respectively (Maione *et al.*, 2007; Godlewski *et al.*, 2010). In agreement with our

findings, in a recent study that examined three different FAAH inhibitors, that is, AA-5-HT, PMSF and URB597, it was shown that these inhibitors caused no changes in electrically-evoked contractility of the small intestine in rats and guinea-pigs (Makwana *et al*., 2010). These data support the idea that under physiological conditions, inhibition of FAAH produces little effect.

In the presence of LPS, AM3506 significantly decreased FAAH activity in the ileum. Moreover, AM3506 increased AEA levels in the ileum in LPS-treated mice. In another study, Capasso *et al*. (2008) showed that cannabidiol, which is a cannabis-derived non-psychotropic compound, normalizes croton oil-induced hypermotility in the small intestine of mouse without affecting motility in the control mice, and AA-5-HT abolished the effect of cannabidiol. These findings, as well as the recent publication by De Petrocellis *et al*. (2011) about the role of cannabidiol in the inhibition of FAAH activity, emphasize the role of FAAH in the regulation of GI dysmotility in pathological conditions.

Another interesting finding of the current study was the lack of enhancement of the upper GI transit in LPS-treated FAAH-deficient mice. This suggests a role of FAAH in LPS-enhanced motility in the GI tract and supports our pharmacological experiments, which showed that inhibiting FAAH normalizes upper GI motility in LPS-treated wild-type mice. Whether this is due to an anti-inflammatory role of FAEs or other mechanisms needs further investigation.

We did not observe any change in the colonic contractility after LPS and/or AM3506 treatments. Moreover, we observed a far lower expression of FAAH mRNA and FAAH activity in the colon, which is in agreement with the lack of functional effects of AM3506 in this region. Given the similar baseline levels of ECs in the ileum and colon, there might be at least two enzymes capable of hydrolyzing AEA in different regions of the GI tract. One is FAAH, the other is like FAAH in some respects but not in terms of its sensitivity to AM3506 or LPS, explaining how AM3506 could inhibit FAAH and also reverse the actions of LPS *in vivo*. A similar conclusion regarding AEA hydrolase activity was drawn by Fegley *et al*. (2005) who examined another FAAH inhibitor, URB597, in the GI tract. Although URB597 clearly inhibited AEA hydrolysis in the duodenum and was inactive in FAAH gene-deficient mice, the levels of AEA, OEA and PEA were unaffected by this compound (Fegley *et al*., 2005). One potential candidate is the acid amidase enzyme discovered by Ueda *et al*. (2001), found in the gut, albeit at higher levels in the small intestine. Other possibilities include the ability of colonic bacteria to metabolize ECs or differences in EC biosynthesis between the regions of the gut. These alternative possibilities require further investigation.

Interestingly, *in vivo*, we observed that the actions of AM3506 on stool output from the colon were entirely CB_1 receptor-sensitive, whereas in the ileum, it appears that there is a CB_1 and CB_2 receptor component to the enhanced motility. These results contrast with those from an earlier study in rats that found a purely CB_2-mediated response (Mathison *et al*., 2004; Duncan *et al*., 2008). AM3506 did not reduce locomotor behaviours in mice suggesting that it was not acting as an exogenous CB agonist, which would be expected to reduce locomotion. These findings support the notion that FAAH inhibitors represent a new class of potential therapeutic agents in the treatment of disorders of GI motility and inflammation (Capasso *et al*., 2005; Storr *et al*., 2008).

The expression of FAAH may be regulated up or down by LPS according to the conditions. In a report by De Filippis *et al*. (2008a) FAAH protein expression in the small intestine during sepsis was increased, but in isolated lymphocytes, LPS reduced FAAH gene and protein expression (Maccarrone *et al*., 2001). Therefore, it seems that the regulation of ECs and fatty acid amide levels are under complex control, and their levels vary in the gut dependent upon several conditions (Gomez *et al*., 2002; Darmani *et al*., 2005). This is likely to be a reflection of both synthesis and degradation through a variety of enzymatic pathways. This idea is supported by the findings of Pinto *et al*. (2002), who reported 100-fold greater activity of an AEA hydrolase in the colon than in the ileum. Further characterization of the enzymes of EC synthesis and degradation in the GI tract are required.

In conclusion, we have shown that inhibition of FAAH normalizes enhanced motility *in vitro* and *in vivo* in the mouse GI tract through the EC system. Inhibition of FAAH with compounds, which appear to be devoid of the side effects of exogenous CBs, may represent a novel treatment for abnormal GI motility in conditions such as IBS.

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Conflict of interests

GK and AM are co-inventors on patent application 200100261674 published 14 October 2010 covering AM3506. The other authors declare they have no competing interests.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 FAAH immunoreactivity in whole mount preparations of the myenteric plexus of the distal colon from a wild-type (A, WT) and FAAH-deficient (KO) mouse (B). Note that in FAAH $^{-/-}$ mice, there was no immunoreactivity observed. Scale bar: 50 μm.

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