

RESEARCH PAPER

Inhibiting endocannabinoid biosynthesis: a novel approach to the treatment of constipation

Correspondence

Keith A. Sharkey, Department of Physiology and Pharmacology, University of Calgary, Hospital Drive N.W., Calgary, AB 3330, Canada. E-mail: ksharkey@ucalgary.ca

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M Bashashati¹, Y Nasser², C M Keenan¹, W Ho¹, F Piscitelli³, M Nalli⁴, K Mackie⁵, M A Storr^{2,6}, V Di Marzo³ and K A Sharkey¹

¹Hotchkiss Brain Institute and Snyder Institute for Chronic Diseases, Department of Physiology and Pharmacology, University of Calgary, Calgary, AB, Canada, ²Division of Gastroenterology, Department of Medicine, University of Calgary, Calgary, AB, Canada, ³Endocannabinoid Research Group, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Pozzuoli, Italy, ⁴Dipartimento di Chimica e Tecnologie del Farmaco, Sapienza Università di Roma, Rome, Italy, ⁵Department of Psychological and Brain Sciences, Gill Center for Biomolecular Science, Indiana University, Bloomington, IN, USA, and ⁶II Medical Department, Klinikum Groshadern, Ludwig Maximilians University of Munich, Munich, Germany

BACKGROUND AND PURPOSE

Endocannabinoids are a family of lipid mediators involved in the regulation of gastrointestinal (GI) motility. The expression, localization and function of their biosynthetic enzymes in the GI tract are not well understood. Here, we examined the expression, localization and function of the enzyme diacylglycerol lipase- α (DAGL α), which is involved in biosynthesis of the endocannabinoid 2-arachidonoylglycerol (2-AG).

EXPERIMENTAL APPROACH

Cannabinoid CB₁ receptor-deficient, wild-type control and C3H/HeJ mice, a genetically constipated strain, were used. The distribution of DAGL α in the enteric nervous system was examined by immunohistochemistry. Effects of the DAGL inhibitors, orlistat and OMDM-188 on pharmacologically induced GI hypomotility were assessed by measuring intestinal contractility *in vitro* and whole gut transit or faecal output *in vivo*. Endocannabinoid levels were measured by mass spectrometry.

KEY RESULTS

DAGL α was expressed throughout the GI tract. In the intestine, unlike DAGL β , DAGL α immunoreactivity was prominently expressed in the enteric nervous system. In the myenteric plexus, it was colocalized with the vesicular acetylcholine transporter in cholinergic nerves. In normal mice, inhibiting DAGL reversed both pharmacologically reduced intestinal contractility and pharmacologically prolonged whole gut transit. Moreover, inhibiting DAGL normalized faecal output in constipated C3H/HeJ mice. In colons incubated with scopolamine, 2-AG was elevated while inhibiting DAGL normalized 2-AG levels.

CONCLUSIONS AND IMPLICATIONS

DAGL α was expressed in the enteric nervous system of mice and its inhibition reversed slowed GI motility, intestinal contractility and constipation through 2-AG and CB₁ receptor-mediated mechanisms. Our data suggest that DAGL α inhibitors may be promising candidates for the treatment of constipation.

Abbreviations

2-AG, 2-arachidonoylglycerol; DAG, diacylglycerol; DAGL, diacylglycerol lipase; EFS, electrical field stimulation; FAAH, fatty acid amide hydrolase; GI, gastrointestinal; MAGL, monoacylglycerol lipase; RQ, relative quantification; vAChT, vesicular acetylcholine transporter

Tables of Links

TARGETS
Enzymes^a
DAGL α , diacylglycerol lipase α (DGL α)
DAGL β , diacylglycerol lipase β (DGL β)
FAAH, fatty acid amide hydrolase
MAGL, monoacylglycerol lipase
NOS
GPCRs^b
CB ₁ receptors
CB ₂ receptors
Ion channels^c
TRPV1 channels
Transporters^d
VACHT, vesicular acetylcholine transporter (SLC18A3)

LIGANDS
2-AG, 2-arachidonoylglycerol
Anandamide, N-arachidonylethanolamide
AM251
Bethanechol
JZL184
Loperamide
Orlistat
Scopolamine

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (^{a,b,c,d}Alexander *et al.*, 2013a,b,c,d).

Introduction

Endocannabinoids are a family of lipid mediators which are increasingly being recognized for their important intercellular and intracellular signalling functions (Piomelli, 2003; Di Marzo and De Petrocellis, 2012; Galve-Roperh *et al.*, 2013). Classically, endocannabinoids act at the cannabinoid CB₁ and CB₂ receptors, the receptors for the psychotropic constituent of cannabis, Δ^9 -tetrahydrocannabinol (Howlett, 2002; Pertwee *et al.*, 2010). The two best-described endocannabinoids are N-arachidonylethanolamide (anandamide) and 2-arachidonoylglycerol (2-AG). They are synthesized from membrane lipids as they are required. The endocannabinoid system and the enzymes involved in their biosynthesis and degradation have been fully described in recent review articles (Dinh *et al.*, 2002; Izzo and Sharkey, 2010; Ueda *et al.*, 2010; Di Marzo, 2011; Di Marzo and De Petrocellis, 2012; Blankman and Cravatt, 2013).

Here, we have studied 2-AG and its biosynthesis. 2-AG is the most abundant endocannabinoid and its synthesis occurs following activation of phosphoinositide-specific phospholipase C which hydrolyzes inositol phospholipids producing diacylglycerol (DAG). The hydrolysis of DAG by the *sn*-1-selective DAG lipases α (DAGL α) and DAGL β leads to the formation of 2-AG (Bisogno *et al.*, 2003; Ueda *et al.*, 2011; Blankman and Cravatt, 2013). DAGL α and DAGL β are proteins of 1042 and 672 amino acids, respectively, and have extensive homology in their membrane and substrate binding domains. They have similar activities and their encoding genes are highly conserved between mouse and human. DAGL α cleaves equally well most fatty acids from the *sn*-1 position of DAGs, while DAGL β has a slight preference for linoleic, oleic, arachidonic and stearic acid. In the adult mouse brain, DAGL α is much more abundant than DAGL β and both are localized to the postsynaptic dendrite. This

localization correlates with the postsynaptic synthesis of 2-AG, as a retrograde regulator of excitatory neurotransmission (Bisogno *et al.*, 2003). In the small and large intestine of rats, mRNA for both DAGL α and DAGL β is present, although the former was more abundant than the latter (Iannotti *et al.*, 2013).

The distribution of endocannabinoids, their CB receptors, and their degradative enzymes, have been well characterized in the gastrointestinal (GI) tract (Izzo and Camilleri, 2008; Izzo and Sharkey, 2010; Abalo *et al.*, 2012; Alhouayek and Muccioli, 2012). Under physiological conditions, endocannabinoids are produced, giving rise to a 'tone' that serves as a mechanism to limit basal GI motility, principally by regulating the release of ACh from enteric nerves (Pertwee, 2001; Izzo and Camilleri, 2008; Izzo and Sharkey, 2010). To date, however, there is no well-defined role for 2-AG in the regulation of GI motility and only little is known about the expression and localization of DAGLs in the enteric nervous system as modulators of GI motility. Because cannabinoid receptors reduce GI contractility and propulsive motility, we considered that by inhibiting the biosynthesis of their endogenous ligands, we might reduce the level of endocannabinoid tone and hence its braking action, thereby enhancing motility. In this study, we focused on the distribution and function of the biosynthetic enzyme DAGL because in contrast to N-acyl phosphatidylethanolamine PLD, inhibitors of DAGL are available and inhibiting DAGL α gives us the opportunity to study 2-AG tone as a potential regulator of GI motility.

Methods

Full details of specific methods are found in the Supporting Information Appendix S1. In general, all assays were con-

ducted in a blinded fashion and the data were generated by an investigator unaware of the treatments administered.

Animals

All animal care and experimental procedures complied with the guidelines of the Canadian Council of Animal Care, and were approved by the University of Calgary Animal Care Committee. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 151 mice were used in the experiments described here.

Male C57BL/6N wild-type (WT) mice were supplied by Charles River (Montreal, QC, Canada) and CB₁ receptor-deficient mice (CB₁^{-/-}) were bred at the University of Calgary mouse breeding facility as described previously (Storr *et al.*, 2010). C3H/HeJ mice, which have a spontaneous mutation in TLR4 gene (*Tlr4^{lps-d}*) and are chronically constipated (Anitha *et al.*, 2012), and their control background mice, C3H/HeOuj, (Jackson Laboratory, Bar Harbor, ME, USA), all at 5–12 weeks of age, were used in these studies.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Duncan *et al.*, 2008; Bashashati *et al.*, 2012). Full details are found in the Supporting Information Appendix S1. Briefly, whole-mount preparations were incubated with the primary rabbit antibody against DAGL α or DAGL β (1:50–1:500, optimal dilution 1:100) raised by Ken Mackie, Indiana University, Indianapolis, IN, USA (Katona *et al.*, 2006; Jain *et al.*, 2013) alone or in double-labelling experiments with the vesicular acetylcholine transporter (vAChT; Phoenix Pharmaceuticals Inc., Burlingame, CA, USA), substance P (Medicorp Inc., Montreal, QC, Canada) or neuronal NOS (Sigma-Aldrich Ltd., Oakville, ON, Canada). Positive DAGL α labelling was completely abolished by pre-absorption with 100 $\mu\text{g}\cdot\text{mL}^{-1}$ of the immunizing peptide.

Real-time PCR

Real-time PCR was performed as described previously (Bashashati *et al.*, 2012). Full details are found in the Supporting Information Appendix S1. Briefly, TaqMan gene expression assay kit for the DAGL α gene (Mm00813830-m1; Applied Biosystems, Frederick, MD, USA) was used for the real-time PCR. Relative quantification (RQ) value was defined as fold changes in mRNA expression compared with a calibrator sample from the stomach and presented as mean \pm SEM of RQ values in each group.

Assessment of intestinal contractility in vitro

Assessment of intestinal contractility was performed as previously described (Storr *et al.*, 2010; Bashashati *et al.*, 2012). Briefly, longitudinal segments of the ileum and distal colon of mice were studied. Electrical field stimulation (EFS) was applied and the effects of cumulative concentrations of the DAGL inhibitors (orlistat or OMDM-188; 1–5 μM ; 15 min apart) on contractile responses to EFS were investigated. In additional experiments, the tension generated by bethanechol (10 μM) in the absence of and at a maximum concentration of the DAGL inhibitors used for the EFS experiments were compared.

In separate sets of experiments, cumulative concentrations of the muscarinic antagonist scopolamine (1–100 nM) or the μ -opioid receptor agonist loperamide (10–100 nM) were added to the organ bath. Scopolamine and loperamide concentration-dependently decreased EFS-induced contractility. To see if inhibiting DAGL or CB₁ receptor antagonism could reverse the effect of scopolamine or loperamide on EFS contractility, orlistat (5 μM), OMDM-188 (1 μM) or AM251 (100 nM) were added to the organ bath 15 min before the first concentration of scopolamine or loperamide.

Before the addition of drugs, the mean of three successive EFS contractions were used as the internal control (100%). Also, vehicle controls were performed for all experiments. The amplitude of contractions in the presence of the compounds was reported as the percentage of the internal control.

GI transit studies in vivo

Whole gut transit was performed as described (Storr *et al.*, 2010). Briefly, mice were acclimated for 60 min in individual transparent plastic cages without bedding. Orlistat (1 mg·kg⁻¹) or OMDM-188 (1 mg·kg⁻¹) were injected i.p. Twenty minutes later, 200 μL of an Evans blue (5% Evans blue; Sigma-Aldrich, St. Louis, MO, USA; 5% gum arabic; Sigma-Aldrich, USA) marker was gavaged into the stomach. The time taken to detect Evans blue in the faeces (in min) was recorded and presented as the whole gut transit time.

The direct effect of 2-AG on GI motility has not been well investigated. In a previous study, we showed that inhibiting monoacylglycerol lipase (MAGL) attenuates whole gut transit in mice (Duncan *et al.*, 2008). Therefore, in the current study, we tested the effect of 2-AG (10 mg·kg⁻¹, i.p.) on whole gut transit in the presence and absence of the MAGL inhibitor JZL184 (18 mg·kg⁻¹, i.p.) in WT and in CB₁ gene-deficient mice.

In preliminary experiments, we established that scopolamine (0.5 mg·kg⁻¹, i.p.) or loperamide (0.5 mg·kg⁻¹, i.p.) significantly delayed whole gut transit in the mouse. To see if the inhibition of DAGL could ameliorate the slowed GI transit in these models, mice were pretreated with scopolamine or loperamide followed 20 min later by the gavage of Evans blue. Twenty minutes before or 2 h after the gavage of marker, orlistat (1 mg·kg⁻¹) or OMDM-188 (1 mg·kg⁻¹) were injected i.p. and subsequently mice were monitored for coloured stools. To see if the potential effects of DAGL inhibitors are mediated through the CB₁ receptors, CB₁^{-/-} mice were used as control for each set of experiments.

Treatment with the DAGL inhibitors 2 h after the gavage gave us a dynamic range for demonstrating the effects of these compounds because vehicle 2 h after the gavage did not speed up the presence of coloured stool (whole gut transit time: 203.6 \pm 14.0 min) compared with animals treated with vehicle before gavage (whole gut transit time: 185 \pm 9.4 min) (*t*-test; *P* value > 0.05) and loperamide or scopolamine consistently increased the transit time to more than 200 min.

Faecal output studies

C3H/HeJ mice and their background mice, C3H/HeOuj were injected with orlistat or OMDM-188 (1 mg·kg⁻¹, i.p.) or their vehicle. After 20 min, the animals were transferred to separate cages without any bedding and covered with lids. Faecal

pellets were collected every 15 min and weighed immediately for a total duration of 1 h for each mouse. Total stool weight was calculated and the result was presented as the percentage of stool weight in vehicle-treated background mice.

Identification and quantification of endocannabinoids

Ileal or colonic tissues (distal colon) from WT mice were incubated with orlistat (5 μM), OMDM-188 (1 μM) or vehicle for 15 min. In some experiments, the tissues were collected at this point. In another set of experiments, cumulative concentrations of scopolamine (1, 10 and 100 nM; 15 min intervals between consecutive concentrations) were added to the bath and the tissues were collected at 60 min. This protocol was designed to mimic the organ bath contractility studies described earlier.

Additionally, to see if the DAGL inhibitors alter the levels of endocannabinoids *in vivo*, we collected and analysed intestinal tissues (as described above) from *C3H/HeJ* and *C3H/HeOuj* mice 45 min after treatment with orlistat, OMDM-188 (1 mg·kg⁻¹, i.p.) or vehicle.

Collected tissues (60 mg of ileum; 50 mg of colon) were homogenized in 0.6 mL of chloroform/methanol/Tris HCl 50 mM (2:1:1) containing 10 pmol of d8-anandamide and 50 pmol of d5-2-AG per sample and extracted with 0.6 ml of chloroform four times. The lipid-containing organic phase was dried down under nitrogen, weighed, and pre-purified by open-bed chromatography on silica gel (pore size 60 Å, 70–230 mesh, Sigma Aldrich) mini-columns. Fractions were obtained by eluting the column with 99:1, 90:10 and 50:50 (v/v) chloroform/methanol. The 90:10 fraction was used for AEA and 2-AG quantification by LC/MS using an HPLC Shimadzu (LC10ADVP; Kyoto, Japan) coupled to a single quadrupole mass spectrometer (LCMS2020) equipped with an APCI interface, as previously described and using selected ion monitoring (SIM) at M + 1 values for the two compounds and their deuterated homologues, as described by Di Marzo *et al.*, (2001). Briefly, AEA and 2-AG were separated using a Discovery C18 column (15 cm × 4.6 mm; I.D. 5 μm ; Supelco) and eluted with an isocratic flow of methanol:water (85:15, 0.1% acetic acid). LC-10ADVP HPLC pumps were used to deliver solvent at a flow rate of 1 ml/min. Samples were injected in 10 μl using an autosampler (SIL-LOADvp). Ions were generated using a capillary voltage of 4.5 kV and APCI temperature was set at 400°C. The levels of the compounds were expressed as pmol g⁻¹ (anandamide) or nmol g⁻¹ (2-AG) of wet tissue weight.

Data analysis

Data are presented as mean \pm SEM of experiments. One or two-way ANOVA followed by appropriate *post hoc* tests were used for comparison of more than two means.

Materials

For the organ bath intestinal contractility studies and the endocannabinoid measurements, stock solutions (0.01 M) of the DAGL inhibitors OMDM-188 and orlistat (Cayman Chemical, Ann Arbor, MI, USA) and the CB₁ receptor antagonist AM251 (Tocris, Ellisville, MO, USA) were prepared in

DMSO and the muscarinic antagonist scopolamine hydrobromide, loperamide and bethanechol (all from Sigma-Aldrich) were dissolved in water.

For the *in vivo* experiments, the animals were treated with 2-AG (Tocris), the MAGL inhibitor JZL184 (Tocris), OMDM-188, orlistat, scopolamine or loperamide dissolved in normal saline containing 2.5% DMSO and 2.5% Tween 80.

The IC₅₀ values of OMDM-188 and orlistat (tetrahydrolipstatin) for inhibiting DAGL α are 0.016 and 1 μM respectively. The concentrations of OMDM-188 (1 μM) and orlistat (5 μM) for inhibiting DAGL in the organ bath, and of JZL184 (16 mg·kg⁻¹) for the *in vivo* experiments, were selected based published data (Ortar *et al.*, 2008; Min *et al.*, 2010; Zhang *et al.*, 2011).

Results

DAGL α is present in the enteric nervous system of mice

DAGL α immunoreactivity was detected as pericellular labelling surrounding unlabelled enteric neurons in the myenteric plexus of mouse ileum and colon (Figure 1). This pericellular labelling was present around many, but not all, myenteric neurons. Punctate labelling was also observed on muscle cells throughout the circular muscle. No immunoreactivity to DAGL β was observed in either the ileum or the colon. Double-labelling revealed that DAGL α colocalizes to a large extent with the vAChT (Figure 1A), but only to a minimal extent with either substance P or NOS immunoreactivity in both the ileum (Figure 1A) and colon (Figure 1B).

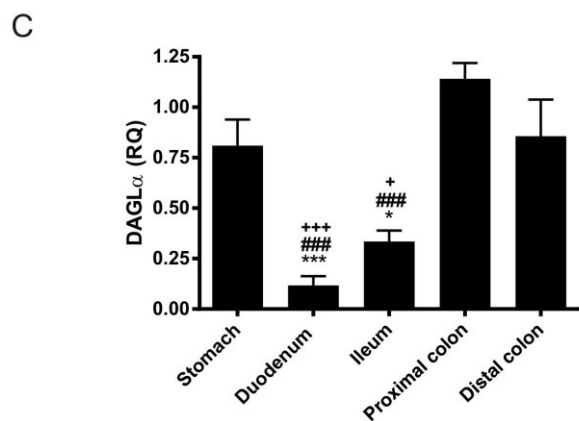
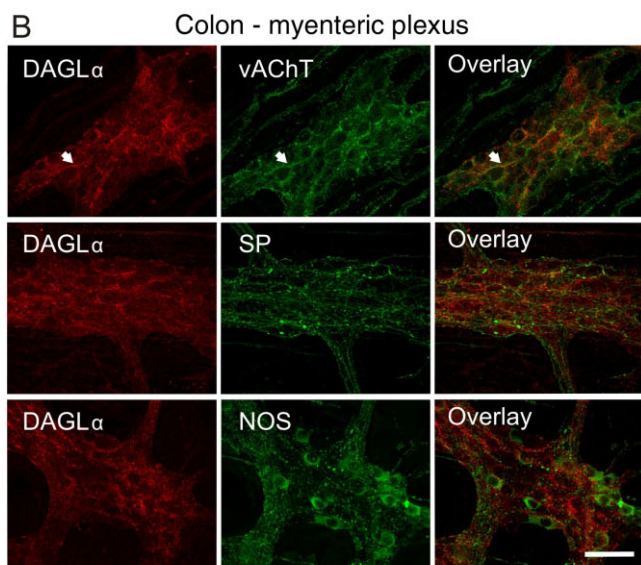
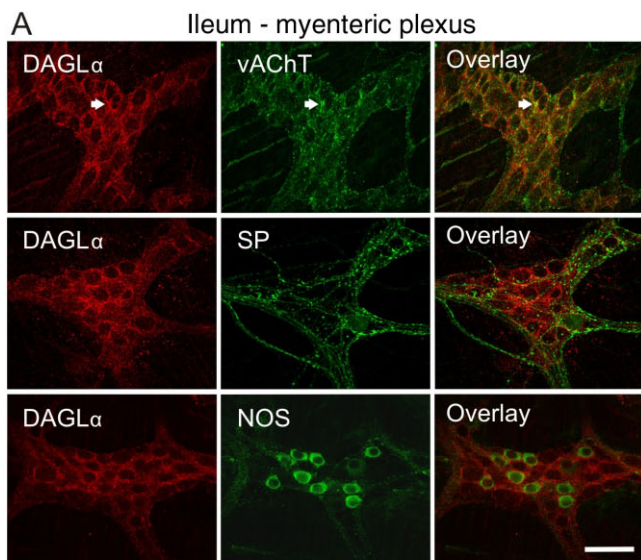
To confirm that the DAGL α gene was expressed in the GI tract, we performed real-time PCR. Real-time PCR showed that DAGL α mRNA was expressed throughout the length of the mouse GI tract, with the highest levels of expression in the stomach and colon (Figure 1C).

2-AG regulates GI motility *in vivo*

To examine if 2-AG has any effect on GI transit, we tested the effects of exogenous 2-AG *in vivo*. 2-AG had no effects on whole gut transit when administered alone, but in the presence of JZL184, which inhibits its metabolic inactivation, transit was dramatically slowed (Figure 2). The effects of JZL184 either alone or together with 2-AG were absent in CB₁^{-/-} mice, suggesting that they are mediated by CB₁ receptors.

DAGL inhibitors do not change baseline intestinal contractility or motility

We next examined whether inhibiting DAGL activity would alter ileal and colonic contractility *in vitro* or whole gut transit *in vivo* under basal conditions. Incubation of the ileal and colonic preparations with either orlistat or OMDM-188 did not have any effect on EFS-induced contractility (EFS: 100.1 \pm 0.6% and 95.3 \pm 2.1% of initial contraction in the ileum and colon with orlistat and 97.9 \pm 2.6% and 96.2 \pm 8.5% of initial contraction in the ileum and colon with OMDM-188 up to 5 μM respectively). Similarly, these drugs had no effect on bethanechol-induced contractility (99.2 \pm 3.4% and 106.1 \pm 8.3% of initial bethanechol contraction in the ileum and



colon with orlistat and $103.6 \pm 6.9\%$ and $97.7 \pm 17.9\%$ of initial bethanechol contraction in the ileum and colon with OMDM-188 up to $5 \mu\text{M}$ respectively). Consistent with these data, i.p. injection of orlistat ($1 \text{ mg}\cdot\text{kg}^{-1}$) or OMDM-188 ($1 \text{ mg}\cdot\text{kg}^{-1}$) did not alter whole gut transit in WT mice (188.0

Figure 1

DAGL α immunoreactivity and mRNA expression in the mouse GI tract. DAGL α immunoreactivity is present in punctate terminals and nerve processes surrounding enteric neurons in the myenteric plexus of the ileum (A) and colon (B). DAGL α colocalizes with the vAChT in the myenteric plexus (arrowheads) but only to a minimal extent with either substance P (SP) or NOS immunoreactivity in both ileum and colon. Scale bars: $50 \mu\text{M}$. DAGL α mRNA is expressed throughout the GI tract (C). The relative expression is significantly lower in the duodenum and ileum compared with the colon and stomach. $n = 3\text{--}5$ mice per group. F (degrees of freedom): $F(4,15) = 20.34$, $P < 0.001$; one-way ANOVA; Bonferroni *post hoc* test. * $P < 0.05$, *** $P < 0.001$, compared with stomach; ### $P < 0.001$, compared with proximal colon; + $P < 0.05$, +++ $P < 0.001$, compared with distal colon.

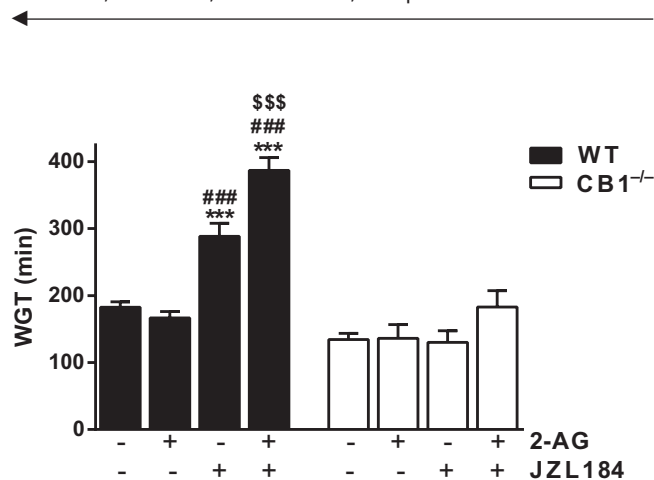


Figure 2

Effects of 2-AG and the MAGL inhibitor JZL184 on whole gut transit (WGT) in mice. 2-AG ($10 \text{ mg}\cdot\text{kg}^{-1}$, i.p.) did not change WGT in WT or CB1^{-/-} mice. However, JZL184 ($18 \text{ mg}\cdot\text{kg}^{-1}$, i.p.) prolonged WGT and enhanced the effect of 2-AG in the WT but not the CB1^{-/-} mice. $n = 4\text{--}14$ mice per group; F (degrees of freedom) for interaction: $F(3,56) = 13.62$, $P < 0.001$; two-way ANOVA; Bonferroni *post hoc* test. *** $P < 0.001$, compared with WT vehicle; ### $P < 0.001$, compared with WT 2-AG alone; \$\$\$ $P < 0.001$, compared with WT JZL184 alone.

± 32.5 min after orlistat and 191.7 ± 15.9 min after OMDM-188 treatment vs. 203.6 ± 14.0 min in controls, $P > 0.05$).

Inhibiting DAGL partly reverses the effects of scopolamine and loperamide on ileal and colonic contractility in vitro

It has become clear that the endocannabinoid system plays an important role in conditions where motility is disturbed (Izzo and Camilleri, 2008; Izzo and Sharkey, 2010). We therefore wondered whether blocking the synthesis of 2-AG when motility was reduced would reveal an action of this ligand. We first reduced contractility in the organ bath using scopolamine ($1\text{--}100 \text{ nM}$) or loperamide ($10\text{--}100 \text{ nM}$), both of which inhibited EFS-induced contractility in the ileum and colon of WT mice. The DAGL inhibitors (OMDM-188 $1 \mu\text{M}$ and orlistat $5 \mu\text{M}$) significantly reversed the effects of both scopolamine ($10\text{--}100 \text{ nM}$) and loperamide (10 nM) on the ileal and

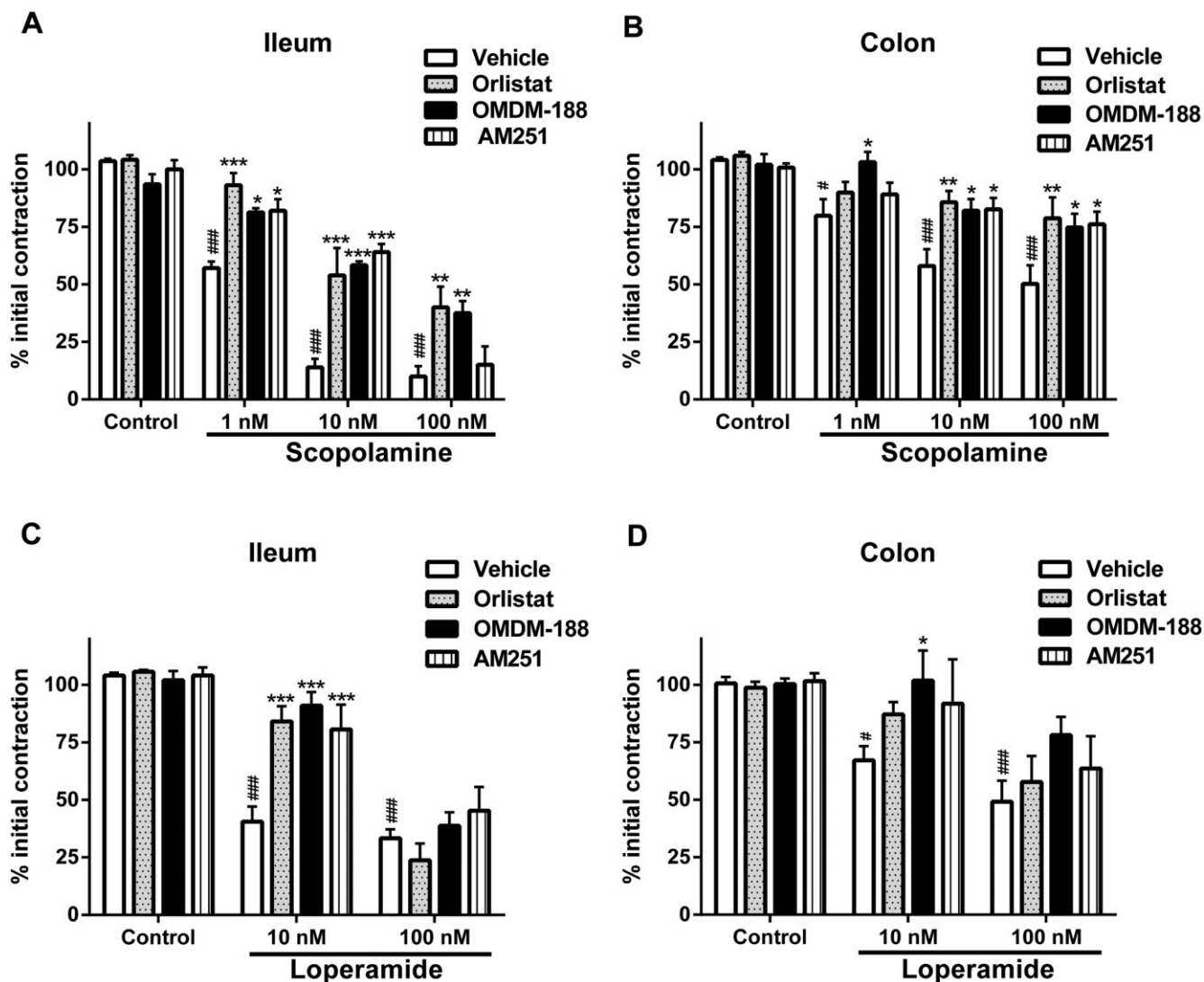


Figure 3

Effects of DAGL inhibitors on pharmacologically inhibited EFS contractility of the mouse ileum (A and C) and colon (B and D). Scopolamine (A and B) or loperamide (C and D) were used at the doses indicated to reduce contractility. Inhibiting DAGL with either orlistat (5 μ M) or OMDM-188 (1 μ M) reversed the effects of scopolamine or loperamide on the EFS contractility. The CB₁ inverse agonist, AM251 (100 nM), also reversed the inhibitory effect of scopolamine and loperamide on the EFS contractility. $n = 3-8$ mice per group; # and ### $P < 0.05$ and $P < 0.001$, respectively, control vehicle compared with scopolamine vehicle or loperamide vehicle; F (degrees of freedom) for interaction of panel A: F (9,40) = 4.63, $P < 0.001$; panel B: F (9,48) = 1.61, $P > 0.05$ [scopolamine treatment: F (3,48) = 25.16, $P < 0.001$, DAGL inhibitors F(3,48) = 7.81, $P < 0.001$]; panel C: F (6,33) = 3.88, $P < 0.01$ and panel D: F(6,30) = 0.74, $P > 0.05$ [loperamide treatment: F (2,30) = 17.32, $P < 0.001$, DAGL inhibitors: F (3,30) = 2.70, $P = 0.06$]. two-way ANOVA; Bonferroni *post hoc* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with respective vehicle at each concentration.

colonic EFS-induced contractility (Figure 3). Furthermore, incubating tissues with the CB₁ receptor antagonist AM251 (100 nM) reversed the effects of both scopolamine and loperamide to about the same extent as the DAGL inhibitors (Figure 3), suggesting that an endocannabinoid ligand acting at CB₁ receptors was acting to limit contractility. Importantly, AM251, similar to the two DAGL inhibitors, was inactive in unstimulated preparations (Figure 3) despite it being an inverse agonist, suggesting that only after stimulation by scopolamine or loperamide, are the endogenous 2-AG levels sufficiently increased to inhibit contractility so that the inhibition of its synthesis/action can result in an effect.

Inhibiting DAGL normalizes drug-induced GI transit in vivo

These intriguing data led us to conduct an *in vivo* transit study. Scopolamine (0.5 mg·kg⁻¹, i.p.) and loperamide (0.5 mg·kg⁻¹, i.p.) substantially slowed whole gut transit in WT and CB₁^{-/-} mice. Basal whole gut transit was slightly, but not statistically significantly, faster in the CB₁^{-/-} mice compared with WT controls in agreement with the *in vitro* AM251 data obtained above. Orlistat (1 mg·kg⁻¹) and OMDM-188 (1 mg·kg⁻¹) given 2 h after Evans blue reversed the effects of both scopolamine and loperamide on whole gut transit in

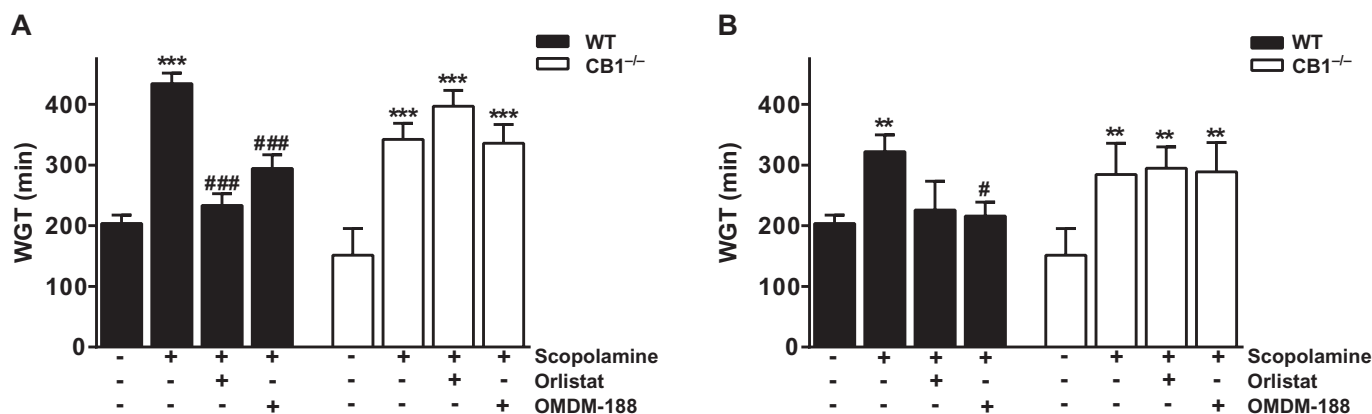


Figure 4

Effects of DAGL inhibitors on whole gut transit (WGT). Mice were treated with scopolamine (A, 0.5 mg·kg⁻¹, i.p.) or loperamide (B, 0.5 mg·kg⁻¹, i.p.) that significantly reduced WGT (increasing the transit time) in the WT or the CB1^{-/-} mice. Orlistat (1 mg·kg⁻¹, i.p.) or OMDM-188 (1 mg·kg⁻¹, i.p.) reversed the effect of scopolamine and loperamide on WGT in the WT but not the CB1^{-/-} mice. Note that neither orlistat nor OMDM-188 had any effect on transit when given alone in untreated animals. $n = 4-12$ mice per group; F (degrees of freedom) for interaction of panel A: $F(3,55) = 11.15$, $P < 0.001$ and panel B: $F(3,36) = 4.32$, $P < 0.05$; two-way ANOVA; Bonferroni *post hoc* test. ** $P < 0.01$, *** $P < 0.001$ compared with vehicle in WT or CB1^{-/-} mice; # $P < 0.05$, ### $P < 0.001$, compared with scopolamine or loperamide in WT mice.

WT mice, but had no effects in CB1^{-/-} mice (Figure 4). When the DAGL inhibitors were given 20 min before loperamide or scopolamine, they were minimally effective (data not shown).

Inhibiting DAGL increases faecal output in C3H/HeJ mice

As described earlier (Anitha *et al.*, 2012), C3H/HeJ mice had significantly lower faecal output compared with the background strain C3H/HeOuJ mice. Inhibiting DAGL, particularly with OMDM-188, increased faecal output in the C3H/HeJ mice, but had no significant effects in the C3H/HeOuJ mice (Figure 5).

Inhibiting DAGL differentially reduces 2-AG levels in the mouse ileum and colon

We measured the levels of 2-AG and anandamide in the ileum and colon to assess if we could detect changes caused by DAGL inhibitors *in vitro*. Orlistat (5 μ M) and OMDM-188 (1 μ M) given alone did not change either 2-AG or anandamide levels in the ileum or colon under basal conditions, in agreement with their lack of effect on EFS contractility under these conditions (Figure 6). Scopolamine tended to increase 2-AG levels in the colon, but not the ileum (Figure 6). In the scopolamine-treated colon, orlistat tended to decrease, and OMDM-188 clearly reduced, 2-AG levels (Figure 6). Neither compound altered 2-AG levels in the scopolamine-treated ileum. Finally, orlistat produced an unexpected rise in anandamide levels in scopolamine-treated ileum, but not in the colon (Figure 6). However, the anandamide : 2-AG ratio (scopolamine alone: 4.47 ± 0.32) was significantly increased in the colon after both orlistat (8.78 ± 0.31) and OMDM-188 (13.58 ± 1.01) treatments (ANOVA, $F(3,7) = 17.08$, $P < 0.01$).

Based on the *in vivo* experiments, constipated C3H/HeJ mice tended to have a higher colonic levels of 2-AG compared with the background C3H/HeOuJ mice, and orlistat

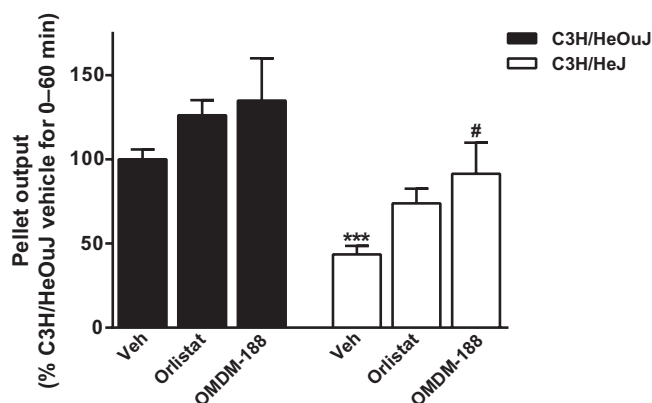


Figure 5

Effects of DAGL inhibitors on faecal output of C3H/HeJ and the background C3H/HeOuJ mice. Monitoring the faecal output for 1 h revealed that C3H/HeJ mice had significantly less output compared with the background strain mice. *** $P < 0.001$; two-way ANOVA; Bonferroni *post hoc* test. OMDM-188 (1 mg·kg⁻¹, i.p.) but not orlistat (1 mg·kg⁻¹, i.p.) significantly enhanced faecal output of the constipated C3H/HeJ mice; $n = 8-19$ mice per group. # $P < 0.05$, compared with faecal output of vehicle-treated C3H/HeJ mice; two-way ANOVA; Bonferroni *post hoc* test; (F (degrees of freedom) for interaction: $F(2,86) = 0.19$, $P > 0.05$ [genotype: $F(1,86) = 33.79$, $P < 0.001$, treatment: $F(2,86) = 7.83$, $P < 0.001$]).

(1 mg·kg⁻¹) significantly decreased 2-AG levels in the former group, suggesting that the enhanced faecal output in these mice after DAGL inhibition was mediated by endocannabinoid down-regulation (Figure 7).

Discussion and conclusions

Complex and sophisticated neural mechanisms are involved in the regulatory control of GI motility and our understand-

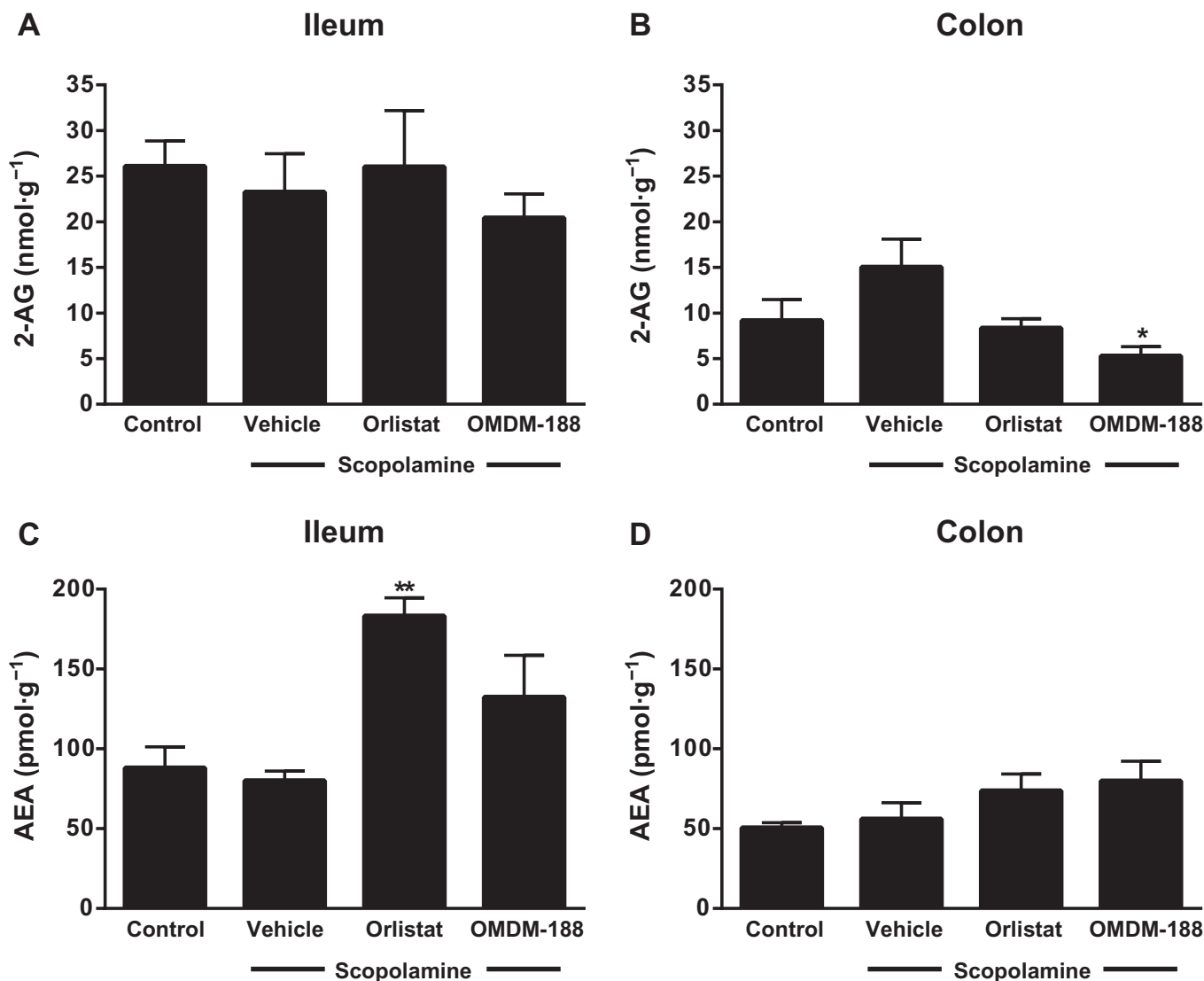


Figure 6

Effects of DAGL inhibitors on intestinal endocannabinoid levels in WT mice. Pre-incubation of the colon with scopolamine (1–100 nM) induced a tendency towards an increase in 2-AG levels and the DAGL inhibitors orlistat (5 μ M) and OMDM-188 (1 μ M) reversed this effect. Inhibiting DAGL increased anandamide (AEA) levels in the ileum. $n = 3$ –5 mice per group; F (degrees of freedom) for panel A: $F(3,13) = 0.43$, $P > 0.05$; panel B: $F(3,9) = 4.23$, $P < 0.05$; panel C: $F(3,11) = 9.73$, $P < 0.01$ and panel D: $F(3,9) = 1.36$, $P > 0.05$; one-way ANOVA; Bonferroni *post hoc* test. * $P < 0.05$. ** $P < 0.01$, compared with vehicle.

ing of these neural mechanisms has advanced considerably over the last 20 years with the discoveries of lipid mediators, including the endocannabinoids (Izzo and Camilleri, 2008; Kasperek *et al.*, 2008; Izzo and Sharkey, 2010). A role for endocannabinoids in the regulation of GI motility has become more relevant with the discovery of (i) single nucleotide polymorphisms such as a functional variant in the fatty acid amide hydrolase (FAAH) gene (C385A) which is associated with irritable bowel syndrome-diarrhoea symptoms and acceleration of colonic transit (Camilleri *et al.*, 2008) and (ii) pharmacogenomic studies such as one indicating that mutation of the CB₁ receptor gene (rs 806378) modifies the effect of the non-selective cannabinoid agonist dronabinol on colonic phasic pressure activity in humans (Wong *et al.*,

2011). Despite these findings and while there is considerable evidence for the expression and function of CB receptors in the GI tract, and the enzymic pathways of endocannabinoid degradation (Izzo and Sharkey, 2010), the sites of endocannabinoid biosynthesis remain uncertain. In the present study, we have shown that DAGL α , the principal enzyme for 2-AG biosynthesis, unlike DAGL β , is localized in the enteric nervous system. We further demonstrated that 2-AG can regulate motility via CB₁ receptors and in a manner controlled by MAGL. Finally, by inhibiting DAGL activity with a clinically available DAGL inhibitor, orlistat, and a specific DAGL inhibitor, OMDM-188, and thus potentially reducing 2-AG biosynthesis in the GI tract, we have identified a novel mechanism to enhance contractility in the small and large

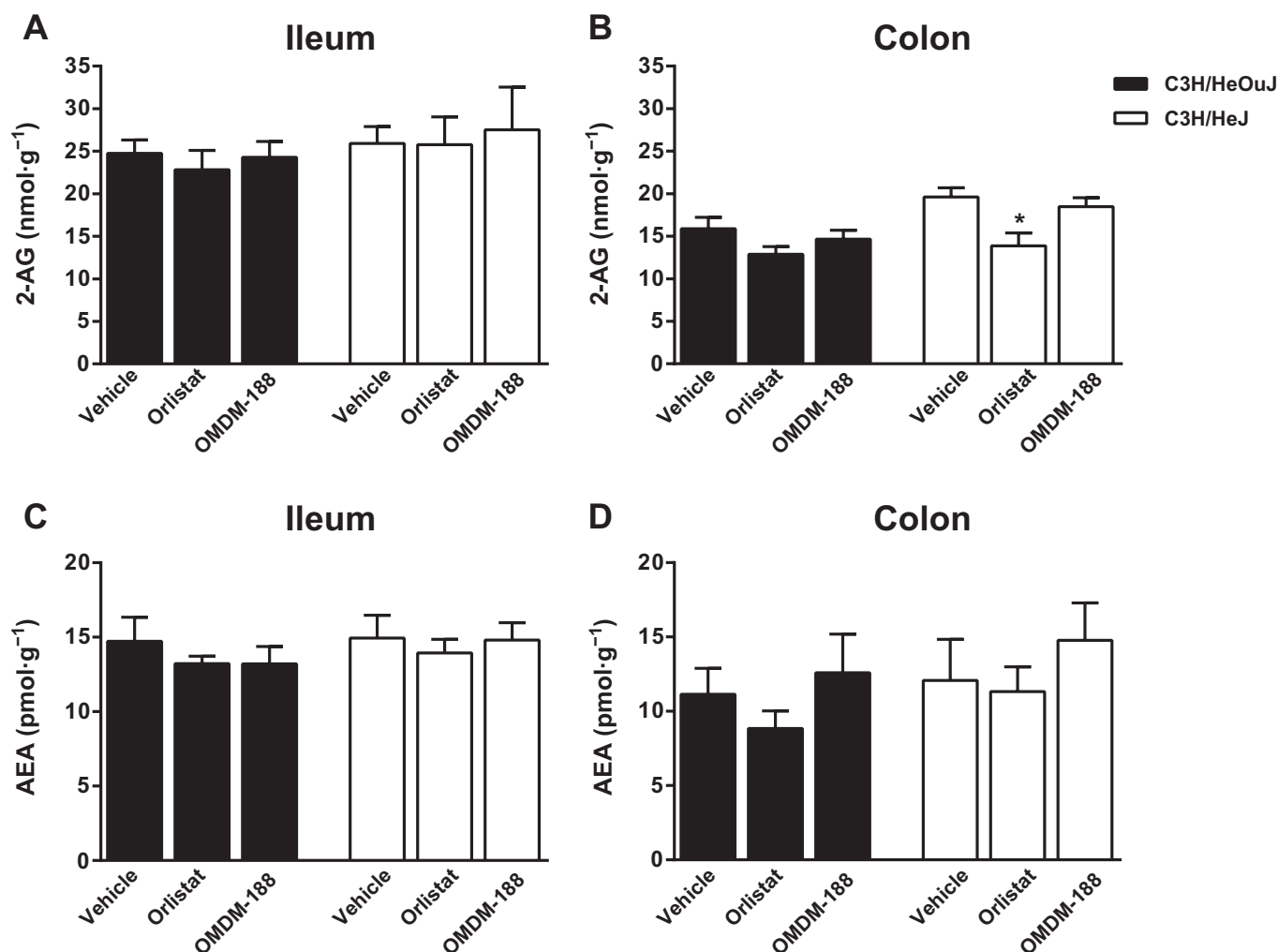


Figure 7

Effects of DAGL inhibitors on the intestinal endocannabinoid levels in C3H/HeOuJ and C3H/HeJ mice. 2-AG tended to be higher in the colon of C3H/HeJ compared with C3H/HeOuJ mice. $P = 0.06$. Orlistat significantly decreased 2-AG levels in the colon of C3H/HeJ mice. * $P < 0.05$ compared with vehicle in the C3H/HeJ group. The dose of orlistat and OMDM-188 was $1 \text{ mg}\cdot\text{kg}^{-1}$ i.p. $n = 6\text{--}7$ mice per group. F (degrees of freedom) for treatment (panel B): $F(2,34) = 7.55$, $P < 0.01$; two-way ANOVA; Bonferroni *post hoc* test.

intestine and propulsive motility in three models of reduced GI transit.

Mechoulam *et al.* first isolated 2-AG from dog intestine and showed that this endogenous lipid mediator had cannabinimimetic effects (Mechoulam *et al.*, 1995). However, the role of 2-AG in the GI tract remains somewhat elusive because of its rapid metabolism. In the current study, when 2-AG hydrolysis was reduced by MAGL inhibition, whole gut transit time was prolonged. Adding exogenous 2-AG with the MAGL inhibitor further increased whole gut transit time through a CB₁ receptor-mediated mechanism. These data extend previous reports that showed that MAGL was present in the enteric nervous system and that by inhibiting MAGL, whole gut transit in mice was slowed (Duncan *et al.*, 2008). Using a mouse model of non-steroidal anti-inflammatory drug-induced gastric ulceration, Kinsey *et al.* demonstrated that blocking MAGL significantly reduced gastric damage and inflammatory cytokines, although exogenous 2-AG alone

had no anti-inflammatory effects, very similar to the results we obtained with motility (Kinsey *et al.*, 2013). In this study too, the effects of MAGL inhibition were mediated by CB₁ receptors. Along similar lines, JZL184 administration protected mice against the development of acute colitis induced with trinitrobenzene sulphonic acid (Alhouayek *et al.*, 2011). In this model, the actions of JZL184 were mediated by both CB₁ and CB₂ receptors, reflecting either regional differences or differences that are due to the pathophysiology of the gastric versus colonic inflammation.

In 2003, Bisogno *et al.* showed that DAGL α mRNA was expressed in the human and mouse GI tract (Bisogno *et al.*, 2003). This has been extended recently by Iannotti *et al.* who demonstrated that DAGL α and DAGL β mRNA was expressed in the rat GI tract (Iannotti *et al.*, 2013). Using antibodies to these enzymes, the first evidence for the distribution of DAGLs was shown by Marquez *et al.* (2009). They showed DAGL α immunoreactivity on the apical surface of epithelial

cells, on some plasma cells in the lamina propria and densely distributed in the muscularis mucosae and muscularis externa of the human colon. Numerous DAGL α positive nerve fibres were observed around unlabelled ganglion cells in the myenteric plexus. In this respect, the pattern of labelling was similar to our observations in the mouse myenteric plexus. In contrast to our findings in the mouse, they also demonstrated DAGL β in the human colonic myenteric plexus, where it was localized in neurons and nerve fibres. The reason for these differences remains to be determined. We have extended these observations and showed that DAGL α is localized on cholinergic nerves and to a much lesser extent on those expressing NO synthase or substance P. In the case of the NOS, this is perhaps not too surprising, as these nerves do not seem to express any components of the endocannabinoid system (Coutts *et al.*, 2002; MacNaughton *et al.*, 2004). In contrast, because substance P is colocalized in excitatory cholinergic nerves in the enteric nervous system (Kunze and Furness, 1999) then we might have expected to see a greater degree of overlap in the immunoreactivity. This apparent disparity may reflect the fact that the majority of substance P immunoreactivity is in a different cellular domain to the membrane bound nature of the DAGL α and so overlap is not obvious. Further studies are needed to examine the subcellular localization of DAGL α in the enteric nervous system to clarify this matter.

The pericellular localization of DAGL α around enteric neurons is consistent with a postsynaptic source of the endocannabinoid 2-AG in the enteric nervous system. Recently, a detailed electrophysiological analysis was performed on neurons of the mouse myenteric plexus (Hons *et al.*, 2012). This study revealed endocannabinoid-mediated retrograde transmission in the enteric nervous system, indicating that CB $_1$ receptors inhibit transmitter release at enteric synapses and depress synaptic strength basally and in an activity-dependent manner. Although the specific molecular identity of the endocannabinoid was not identified in this study, by analogy with the CNS, the most likely candidate is 2-AG which is released and acts at presynaptic CB $_1$ receptors (Di Marzo, 2011). Our data would support a similar role of 2-AG in the enteric nervous system.

Previous studies have raised the possibility that anandamide tonically controls GI motility *in vivo* and this was found to be the case in our model system (Izzo *et al.*, 2001; Pinto *et al.*, 2002; Bashashati *et al.*, 2012). Here, we have hypothesized that 2-AG also exerted an endogenous tone controlling intestinal contractility *in vitro* and GI transit *in vivo*. An important and novel finding described in the present study is that DAGL inhibitors did not alter evoked contractility or 2-AG levels under basal conditions in either the ileum or colon *in vitro* and whole gut transit or faecal output *in vivo* in healthy mice. This observation suggests that basal endocannabinoid tone to inhibit gut contractility is instead largely under the control of anandamide, as previously suggested by Pinto *et al.* (2002) and Capasso *et al.* (2005). In fact, unlike anandamide, 2-AG is a product of, and intermediate in, triglyceride digestion in the intestine, which is mediated by lipases other than DAGL (Wood *et al.*, 2010; Cascio, 2013). If such dietary and digestive pathways were spatially and temporally organized in a way to activate CB $_1$ receptors, thereby reducing GI contractility, this might cause constipation after

any fatty meal, which is obviously not the case. It is, instead, only the 2-AG that is derived from DAGL α and produced upon stimulation that can activate CB $_1$ receptors on myenteric neurons and inhibit contractility. Another explanation for our findings, however, is that there are alternative pathways for the biosynthesis of the endocannabinoid 2-AG in the gut that maintain 2-AG levels in the face of DAGL inhibition. For example, 2-AG can be synthesized by lysophosphatidylinositol-selective PLC (Muccioli, 2010; Ueda *et al.*, 2011; Murataeva *et al.*, 2014). However, evaluation of the role of alternative pathways needs molecular data and specific inhibitors for this enzyme, which are not available at this time. Our measurements of 2-AG levels in the ileum and colon are not inconsistent with this second possibility. Conversely, it is predictable that inhibiting MAGL, which blocks the degradation of the entire GI 2-AG pool including exogenous 2-AG, represents an artificial (i.e. 'pharmacological' rather than 'physiological') condition sufficient to strongly and indirectly activate CB $_1$ receptors and hence significantly affect basal motility. Further studies are required to examine the sources and metabolic pathways of 2-AG in the intestine.

Indeed, although we have not detected DAGL β , lack of evidence should not necessarily be taken as evidence of lack of a role of this isoform in controlling 2-AG biosynthesis in states of reduced motility. In fact, orlistat inhibits with similar efficacy both DAGL α and DAGL β (Bisogno *et al.*, 2003), whereas OMDM-188 has so far only been tested on DAGL α (Ortar *et al.*, 2008). However, given the similarity between the catalytic triads of the two enzymes, their very similar primary sequence near the binding site, serine lipase, lipase 3 motif (Bisogno *et al.*, 2003; Ortar *et al.*, 2008) and because OMDM-188 was developed from a chemical modification of orlistat, it is expected that this compound is also not preferentially selective for either of the two isoforms of the enzyme. Selective DAGL α and β inhibitors have only just been developed (Baggelaar *et al.*, 2013; Hsu *et al.*, 2013) and hence any final conclusion on the role of DAGL β will have to await the commercial availability of such compounds.

Finding that these compounds had no effect under physiological conditions prompted us to examine whether they may have effects when motility was reduced pharmacologically, a common clinical situation. Two drugs that are known to decrease evoked contractility in the intestine are scopolamine and loperamide (Kachur *et al.*, 1986; Krueger *et al.*, 2013). Interestingly, when we reduced evoked contractility with scopolamine, we observed that OMDM-188 and orlistat could partly or fully reverse the effects in both the ileum and colon depending on the dose of scopolamine employed. Moreover, the two DAGL inhibitors, which had a relatively short effective half-life *in vivo* also reversed the scopolamine-induced prolongation of whole gut transit, an action of the muscarinic antagonist that mimics slow transit constipation. These data suggest that, following scopolamine administration, 2-AG formation contributes, via CB $_1$ receptor activation, to the reduction of GI motility and that this effect can be attenuated by the DAGL inhibitors. We confirmed that CB $_1$ receptors were responsible for this effect using antagonists and CB $_1$ ^{-/-} mice. Using a second model that mimics the common problem of opioid-induced constipation, we observed very similar results and that treatment with DAGL inhibitors could partly reverse evoked hypocontractility and

restore motility *in vivo*. These exciting findings suggest that either at the neuromuscular junction and/or in the myenteric plexus, 2-AG biosynthesis can be up-regulated over basal levels via GPCRs (including cholinergic muscarinic and μ -opioid receptors) and contribute to constipation via inhibition of cholinergic transmission following CB₁ receptor activation. When 2-AG biosynthesis is inhibited, these mechanisms are also inhibited and motility is increased. It is not known how such up-regulation of 2-AG levels (which we investigated here only *in vitro* with scopolamine) occurs. It is important to emphasize that the DAGL inhibitors, orlistat and OMDM-188, reduced stimulated, and not the basal or pre-existing, levels of 2-AG also in isolated neurons, brain slices, hypothalamus and liver (Bisogno *et al.*, 2009; 2013; Alger and Kim, 2011).

As mentioned earlier, in the presence of scopolamine, 2-AG levels in the colon tended to increase and were significantly reduced by the DAGL inhibitor OMDM-188, in agreement with our functional data. Interestingly, and unexpectedly, orlistat increased anandamide levels in the ileum after incubation with scopolamine. Increased anandamide levels obtained by blocking FAAH have been previously shown to reduce 2-AG levels in the brain (Maccarrone *et al.*, 2008). This negative cross-talk between anandamide and 2-AG has been suggested to be mediated by TRPV1 channels, as postsynaptic activation of TRPV1 channels by anandamide subsequently inhibited DAGL α and decreased 2-AG levels (Di Marzo and Cristino, 2008). It is possible that, in turn, 2-AG exerts a negative effect on anandamide levels and that inhibition of 2-AG biosynthesis results in increased anandamide levels. However, DAGL $\alpha^{-/-}$ mice exhibit lower, rather than higher, anandamide levels in some brain regions (Yoshino *et al.*, 2011). To our knowledge, our findings are the first evidence of an effect of blocking DAGL α on anandamide levels. Understanding the mechanism underlying this phenomenon therefore needs further investigation. Unfortunately, inhibitors of anandamide biosynthesis are not currently available to see if reciprocal interactions between these endocannabinoids occur in the GI tract. At any rate, neither of the two DAGL inhibitors tested here affected anandamide levels in the colon, although there was a trend based on the increased anandamide : 2-AG ratio in this organ after treatment with DAGL inhibitors in the presence of scopolamine. The differences between colon and ileum endocannabinoid levels may be attributed to possible differences in the expression of DAGL α in these regions as well as differences in the cholinergic components of these GI organs. Indeed, in a previous publication, we have shown that despite being blocked in the ileum, EFS contractility in the colon is not completely blocked by atropine (Bashashati *et al.*, 2012) and in the present study, we have shown that inhibition of EFS contractility by scopolamine is weaker in the colon than in the ileum, although whether cholinergic-endocannabinoid cross-talk can explain the differential response to DAGL inhibition in the ileum and colon needs further investigation.

When we administered DAGL inhibitors to C3H/HeJ mice which have constipation because of a genetic defect that leads to the loss of a population of NOS immunoreactive neurons (Anitha *et al.*, 2012), we could restore physiological GI motility. Quantification of the endocannabinoids revealed that C3H/HeJ mice tended to have higher colonic 2-AG levels

compared with the background mice and orlistat significantly decreased 2-AG levels in the colon of these mice. Therefore, besides the loss of NOS neurons, endocannabinoid imbalance may also contribute to the phenomenon of constipation in these mice. It is possible that the loss of NOS neurons in the myenteric plexus enhances neuronal activity in this region and this increases the local levels of 2-AG such that inhibiting DAGL reveals the role of this mediator in synaptic inhibition. Further detailed electrophysiological studies are required to assess if this is the case. Importantly, although OMDM-188 was more efficacious than orlistat at restoring GI motility in C3H/HeJ mice, it did not significantly reduce 2-AG levels in either the ileum or colon of these animals. This suggests that there might be more than inhibition of 2-AG biosynthesis in the mechanism of the anti-constipation action of OMDM-188 or that its inhibition of DAGL may occur at sites different from the ileum and colon, but still involved in the control of GI motility.

In conclusion, here, we have shown that DAGL α , the principal enzyme for 2-AG biosynthesis, is localized in the enteric nervous system. We demonstrated that 2-AG regulates motility via CB₁ receptors but that under basal conditions this mechanism is absent. However, in states of reduced motility, we discovered that inhibiting DAGL is a novel mechanism to enhance contractility in the small and large intestine and to propel motility in three models of slow transit. As constipation is a major clinical problem and new approaches to combat this condition are required, our data suggest that DAGL inhibitors may be promising candidate molecules for such conditions. Therefore, besides MAGL inhibitors, such as JZL184, which are potential treatments for diarrhoea (Ramesh *et al.*, 2011), targeting DAGL is a promising treatment strategy for constipation. These findings may provide a new therapeutic approach for patients with chronic, slow transit or drug-induced constipation.

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Author contributions

M. B., C. M. K., M. A. S., V. D. and K. A. S. designed the studies. M. B., Y. N., C. M. K., W. H. and F. P. conducted the experiments. M. B., Y. N., C. M. K., W. H., F. P., V. D. and K. A. S. analysed data. M. N. and K. M. provided the unique reagents. K. M., M. A. S., V. D. and K. A. S. obtained grant support. All the authors contributed to the writing of the paper, revising the manuscript and approving the final version for submission.

Conflict of interest

The authors have no competing interests.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

<http://dx.doi.org/10.1111/bph.13114>

Appendix S1 Supplemental Methods.