

# The identification of peroxisome proliferator-activated receptor alpha-independent effects of oleoylethanolamide on intestinal transit in mice

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**Abstract** Oleoylethanolamide (OEA) is an endogenous lipid produced in the intestine that mediates satiety by activation of peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ). OEA inhibits gastric emptying and intestinal motility, but the mechanism of action remains to be determined. We investigated whether OEA inhibits intestinal motility by activation of PPAR $\alpha$ . PPAR $\alpha$  immunoreactivity was examined in whole mount preparations of mouse gastrointestinal (GI) tract. The effect of OEA on motility was assessed in wildtype, PPAR $\alpha$ , cannabinoid CB<sub>1</sub> receptor and CB<sub>2</sub> receptor gene-deficient mice and in a model of accelerated GI transit. In addition, the effect of OEA on motility was assessed in mice injected with the PPAR $\alpha$  antagonist GW6471, transient receptor potential vanilloid 1 antagonist SB366791 or the glucagon-like peptide 1 antagonist exendin-3(9-39) amide. PPAR $\alpha$  immunoreactivity was present in neurons in the myenteric and submucosal plexuses throughout the GI tract. OEA inhibited upper GI transit in a dose-dependent manner, but was devoid of an effect on whole gut transit or colonic propulsion. OEA-induced inhibition of motility was still present in PPAR $\alpha$ , CB<sub>1</sub> and CB<sub>2</sub> receptor gene-deficient mice and in the presence of GW6471, SB366791 and exendin-3(9-39) amide, suggesting neither PPAR $\alpha$  nor the cannabinoids and other likely

receptors are involved in mediating the effects of OEA. OEA blocked stress-induced accelerated upper GI transit at a dose that had no effect on physiological transit. We show that PPAR $\alpha$  is found in the enteric nervous system, but our results suggest that PPAR $\alpha$  is not involved in the suppression of motility by OEA.

**Keywords** enteric nervous system, intestinal motility, oleoylethanolamide, peroxisome proliferator-activated receptor alpha, stress enhanced motility.

## INTRODUCTION

Peroxisome proliferator-activated receptors (PPAR) are nuclear receptors that heterodimerize with the retinoid-X receptor and, upon ligand-induced activation, lead to the transcription of target genes. Three isoforms of these receptors have been identified: PPAR $\alpha$ , PPAR $\beta/\delta$  and PPAR $\gamma$ . Activation of PPAR $\alpha$  modulates the expression of genes involved in the regulation of lipid metabolism, including those encoding for apolipoproteins and enzymes involved in  $\beta$ -oxidation.<sup>1</sup> As such, PPAR $\alpha$  expression is high in regions involved in lipolysis: enterocytes, hepatocytes, cardiomyocytes and the proximal tubules of the kidney.<sup>2</sup> PPAR $\alpha$  is located throughout the gastrointestinal (GI) tract with the highest levels in the duodenum and relatively low levels of expression in the colon.<sup>2</sup> This receptor is localized to the mucosa of the GI tract where its activation upregulates the expression of genes encoding for proteins involved in lipid metabolism and fatty acid oxidation.<sup>3,4</sup>

Endogenous ligands for PPAR $\alpha$  include a variety of fatty acids<sup>5</sup> and fatty acid amides, such as the pluripotent signalling molecules oleoylethanolamide (OEA)<sup>6</sup> and palmitoylethanolamide.<sup>7</sup> OEA is synthesized in

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Received: 18 August 2008

Accepted for publication: 14 October 2008

the GI tract on demand in response to increases in intracellular calcium from cells expressing its biosynthetic enzymes. *N*-acyltransferase catalyzes the synthesis of *N*-oleoyl-phosphatidylethanolamide (NOPE) from the precursors oleic acid and phosphatidylcholine. NOPE is then cleaved by phospholipase-D producing OEA.<sup>8</sup> Like other fatty acid ethanolamides, OEA is degraded to oleic acid and ethanolamide by the enzymatic action of fatty acid amide hydrolase (FAAH), which is also expressed in the GI tract.<sup>9</sup> Another enzyme, palmitoylethanolamide-preferring acid amidase, present in the small intestine, also contributes to the hydrolysis of OEA.<sup>10</sup>

Oleylethanolamide exerts its role in energy homeostasis via activation of PPAR $\alpha$ , resulting in the stimulation of fatty acid oxidation and lipolysis<sup>11</sup> and the attenuation of food intake.<sup>6,12</sup> PPAR $\alpha$  and OEA levels in the mouse intestine are under circadian regulation, with levels of both the receptor mRNA and agonist content increasing during the light phase.<sup>6</sup> OEA biosynthesis in the rat small intestine is suppressed during fasting periods and increased upon re-feeding.<sup>12</sup> Furthermore, OEA levels are increased in the stomach, but not in the small intestine, in mice fed a high-fat diet for 14 weeks.<sup>13</sup>

Oleylethanolamide has been shown to inhibit gastric emptying<sup>13</sup> and transit of markers in the upper GI tract in mice.<sup>14</sup> The mechanism of action of OEA in inhibiting motility has not been determined. Due to the close correlation in the localization of PPAR $\alpha$  and regions of OEA production, we hypothesized that the inhibitory action of OEA may be mediated via PPAR $\alpha$  in the enteric nervous system. As the motility of the GI tract is regulated by neurons of the myenteric plexus, we determined the localization of PPAR $\alpha$  in the enteric nervous system and sought to determine if the actions of OEA were mediated by PPAR $\alpha$ . We also investigated the actions of OEA in a model of accelerated GI transit to explore its potential role in GI pathophysiology.

## METHODS

### Animals

Male albino guinea pigs (180–250 g; Charles River, Montreal, QC, Canada), male Sprague–Dawley rats (175–250 g; Charles River) and male and female C57BL/6 mice (wildtype; 19–26 g; Charles River) were housed in plastic sawdust floor cages and allowed free access to tap water and standard laboratory chow, unless otherwise stated.

Male PPAR $\alpha$ <sup>-/-</sup> (B6.129S4-*Ppara*<sup>tm1Gonz</sup> N12) mice, previously backcrossed to C57BL/6 mice for 12 generations by the supplier were purchased from Taconic (Germantown, NY, USA). Two breeding pairs of heterozygous CB<sub>1</sub><sup>+/-C57BL/6N</sup> mice were obtained from Dr B. Lutz (Johannes Gutenberg University, Mainz, Germany) and two breeding pairs of heterozygous CB<sub>2</sub><sup>+/-C57BL/6N</sup> mice were obtained from Dr N. Buckley (California State Polytechnical University, Pomona, CA, USA) and bred in our facility to obtain CB<sub>1</sub><sup>-/-C57BL/6N</sup> and CB<sub>2</sub><sup>-/-C57BL/6N</sup> mice respectively. Animals used in these studies were backcrossed from both heterozygous and homozygous breeding pairs to C57BL/6N for six generations and were used at the same age (female, CB<sub>1</sub><sup>-/-</sup>: 7–8 weeks and CB<sub>2</sub><sup>-/-</sup>: 9–11 weeks) and maintained under the same conditions as the wildtype mice. All CB<sub>1</sub><sup>-/-15</sup> and CB<sub>2</sub><sup>-/-16</sup> mice were genotyped using established protocols and were confirmed as homozygous gene-deficient animals (CB<sub>1</sub><sup>-/-C57BL/6N</sup>, CB<sub>2</sub><sup>-/-C57BL/6N</sup>) prior to inclusion in the study.

All experimental procedures were approved by the University of Calgary Animal Care Committee and were carried out in accordance with the guidelines of the Canadian Council on Animal Care.

### Immunohistochemistry

Animals were killed by an overdose of halothane (guinea pigs), sodium pentobarbitol (rats) or via cervical dislocation (mice). The GI tract from the stomach to the distal colon was removed and placed in phosphate-buffered saline (PBS) containing 10  $\mu$ mol L<sup>-1</sup> sodium nifedipine. Tissue from the stomach, duodenum, jejunum, ileum and distal colon was pinned and fixed in Zamboni's fixative overnight at 4 °C. Whole mount preparations of longitudinal muscle-myenteric plexus and submucosal plexus were dissected from the fixed tissue. The mucosal layer was removed from the tissue and the submucosal plexus below was carefully dissected away from the underlying circular muscle. The circular muscle was removed leaving the longitudinal muscle-myenteric plexus. The samples were washed three times at 5-min intervals, in PBS containing 0.1% Triton X-100 and incubated in the primary antibody rabbit anti-PPAR $\alpha$  (1 : 1000; #228-114, Fitzgerald Industries International Inc., Concord, MA, USA) for 48 h at 4 °C. Tissues were then washed three times in PBS containing 0.1% Triton X-100 and incubated for 1 h at room temperature in donkey anti-rabbit CY3 (1 : 100; #711-165-152, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Tissues were washed and mounted in bicarbonate-buffered glycerol and then viewed using a Zeiss

Axioplan fluorescence microscope (Carl Zeiss, Jena, Germany) with images captured using a Sensys digital camera (Photometrics, Tucson, AZ, USA). PPAR $\alpha$  staining of the rat liver was used as a positive control. Negative controls were carried out excluding either the primary or secondary antibodies. Specificity of the PPAR $\alpha$  antibody was demonstrated using a blocking peptide (1 and 10  $\mu\text{mol L}^{-1}$ ; #229-102, Fitzgerald Industries International Inc.). In further experiments, tissues were double labelled to determine the cellular localization of the PPAR $\alpha$  immunoreactivity. Mouse ileal myenteric plexus preparations were dissected as described above and were incubated in the primary antibody mouse antiprotein gene product 9.5 (PGP 9.5) (1 : 1000; #13C4, Ultraclone Ltd., Isle of Wight, England) for 48 h at 4 °C. Tissues were then washed three times in PBS containing 0.1% Triton X-100 and incubated for 1 h at room temperature in goat anti-mouse FITC (1 : 50; #115-096-062, Jackson ImmunoResearch Laboratories Inc.). These tissues were then washed and incubated in the primary antibody rabbit anti-PPAR $\alpha$  for 48 h at 4 °C. Tissues were then washed three times in PBS containing 0.1% Triton X-100 and incubated for 1 h at room temperature in donkey anti-rabbit CY3.

### ***In vivo* transit studies**

**Whole gut transit studies** Three days prior to the experiment mice were individually housed. On the day of the experiment, mice were transferred to individual plastic cages without bedding and were left to acclimatize to the cage for 1 h. Mice were administered an i.p. injection of OEA (10, 20 or 40 mg kg $^{-1}$ ) or vehicle (4% DMSO, 2% Tween 80 in physiological saline) 30 min before being gavaged (using a 3-cm, 20-G gavaging needle) with 200  $\mu\text{L}$  of an Evans' blue (5% Evans' blue, 5% gum arabic) marker. Mice were returned to their individual cages (*ad libitum* access to food and water) and the latency to the detection of Evans' blue in the droppings was recorded. As a positive control the  $\mu$ -opioid receptor agonist loperamide (1.25 mg kg $^{-1}$ ) was administered to mice using the same protocol. This dose has been shown to slow transit in our preliminary experiments.

**Upper GI transit studies** Mice were fasted for 10–14 h prior to the start of the experiment with *ad libitum* access to water. Mice were administered an i.p. injection of OEA (10, 20 or 40 mg kg $^{-1}$ ), the specific PPAR $\alpha$  agonist GW7647 (10, 20 or 40 mg kg $^{-1}$ ) or vehicle 30 min before being gavaged (using a 3-cm, 20-G gavaging needle) with 200  $\mu\text{L}$  of a charcoal (10% charcoal, 5% gum arabic) marker. Fifteen minutes later

mice were killed via cervical dislocation and the intestine from the region of the pyloric sphincter to the ileo-caecal junction was removed. Without stretching the tissue the length of the intestine and distance travelled by the marker was recorded.

In further experiments, mice received an i.p. injection of vehicle or the PPAR $\alpha$  antagonist GW6471 (5, 10 or 20 mg kg $^{-1}$ ), the transient receptor potential vanilloid 1 (TRPV1) antagonist SB366791 (0.5 or 2 mg kg $^{-1}$ ) or the glucagon-like peptide 1 (GLP-1) antagonist exendin-3(9-39) amide (0.1 mg kg $^{-1}$ ) 30 min before being administered OEA (40 mg kg $^{-1}$  i.p.) or vehicle. The doses of SB366791 were based on previously published *in vivo* studies.<sup>17</sup> For GW6471, we chose a wide range of doses as this compound did not appear to have been used by i.p. administration *in vivo* in mice previously. For exendin-3(9-39) we chose a dose that alone would not have actions on the GI tract. Higher doses reduce gastric emptying when given alone.<sup>18</sup> Transit of the upper GI tract was then measured as outlined above. In other experiments, C57BL/6, PPAR $\alpha$  $^{-/-}$ , CB1 $^{-/-}$  or CB2 $^{-/-}$  mice were administered an i.p. injection of OEA (40 mg kg $^{-1}$ ) or vehicle and transit was measured as outlined above.

**Colonic propulsion** Mice were lightly anaesthetized with isoflurane before a 2.5-mm spherical glass bead was inserted 2 cm intrarectally. The latency to the expulsion of the bead was recorded. This was repeated two more times, at 1 h 30-min intervals and the overall mean of the three trials per mouse was calculated. Mice were injected i.p. with OEA (10, 20 or 40 mg kg $^{-1}$ ), loperamide (1.25 mg kg $^{-1}$ ) or vehicle 30 min before the first bead trial only. Colonic propulsion between bead trials 1, 2 and 3 was comparable.

**Accelerated upper GI transit** Preliminary experiments showed that moving group housed mice from their home cage individually into a transparent plastic cage without a sawdust floor or bedding, termed 'novel environment', for 45 min, accelerated upper GI transit time. Mice were fasted 10–14 h prior to the start of the experiment with *ad libitum* access to water. Mice were injected with an OEA (10, 20 or 40 mg kg $^{-1}$ , i.p.) or vehicle injection and placed individually into the novel environment described above. Other mice received an i.p. vehicle injection and were returned to their home cage, with their cage mates, and served as controls for baseline upper GI transit. Thirty minutes later mice were gavaged with a charcoal marker and upper GI transit was measured, as detailed above, 15-min postgavage.

## Drugs

Oleylethanolamine, 2-[[4-[2-[[[Cyclohexylamino]carbonyl][4-cyclohexylbutyl]amino]ethyl]phenyl]thio]-2-methylpropanoic acid (GW7647), [(2S)-2-[[[1Z]-1-Methyl-3-oxo-3-[4-(trifluoromethyl)phenyl]-1-propenyl]amino]-3-[4-[2-(5-methyl-2-phenyl-4-oxa zolyl)ethoxy]phenyl]propyl]-carbamic acid ethyl ester (GW6471), 4'-Chloro-3-methoxycinnamanilide (SB366791) and exendin-3(9-39) amide were purchased from Tocris (Ellisville, MI, USA). Loperamide hydrochloride was purchased from Sigma-Aldrich (St Louis, MO, USA). All drugs were dissolved in a vehicle of 4% DMSO, 2% Tween 80 in physiological saline. Injections were administered at 10  $\mu$ L g<sup>-1</sup> body weight.

## Statistical analysis

Data are expressed as the mean  $\pm$  SEM and analyzed using either a one way analysis of variance, two way analysis of variance or Kruskal–Wallis test followed by Bonferroni's or Dunn's *post hoc* test as appropriate.  $P < 0.05$  was considered significant.

## RESULTS

### Immunohistochemistry

We determined the presence of PPAR $\alpha$  in the myenteric (MP) and submucosal (SMP) plexuses of the GI tract using immunohistochemistry. PPAR $\alpha$  immunoreactivity was found in the MP of the stomach and in both the MP and SMP of the duodenum, jejunum, ileum and distal colon in mouse tissues (Fig. 1). A similar pattern of PPAR $\alpha$  immunoreactivity was observed in rat and guinea-pig tissues (Fig. S1). Double labelling of the mouse ileal MP revealed that the nuclei of neuronal cell bodies were brightly stained while weaker staining was observed in the cytoplasm (Fig. S2). Preabsorption of the PPAR $\alpha$  antibody completely abolished PPAR $\alpha$  staining. Furthermore, staining with this PPAR $\alpha$  antibody was observed in the positive control tissue, rat liver (data not shown).

### In vivo transit studies

The positive control, loperamide, increased whole gut transit time from a mean  $\pm$  SEM of 188.8  $\pm$  30.9 s in vehicle-treated mice to 323.0  $\pm$  49.6 s ( $P < 0.05$ ) and slowed colonic propulsion (vehicle: 285.7  $\pm$  54.2 s; loperamide: 576.8  $\pm$  149.8 s,  $P < 0.05$ ). OEA administered at doses of 10, 20 and 40 mg kg<sup>-1</sup> did not modify whole gut transit (Fig. 2A) or have an effect on colonic

propulsion (Fig. 2C). However, OEA produced a dose-dependent inhibition of upper GI transit (Fig. 2B).

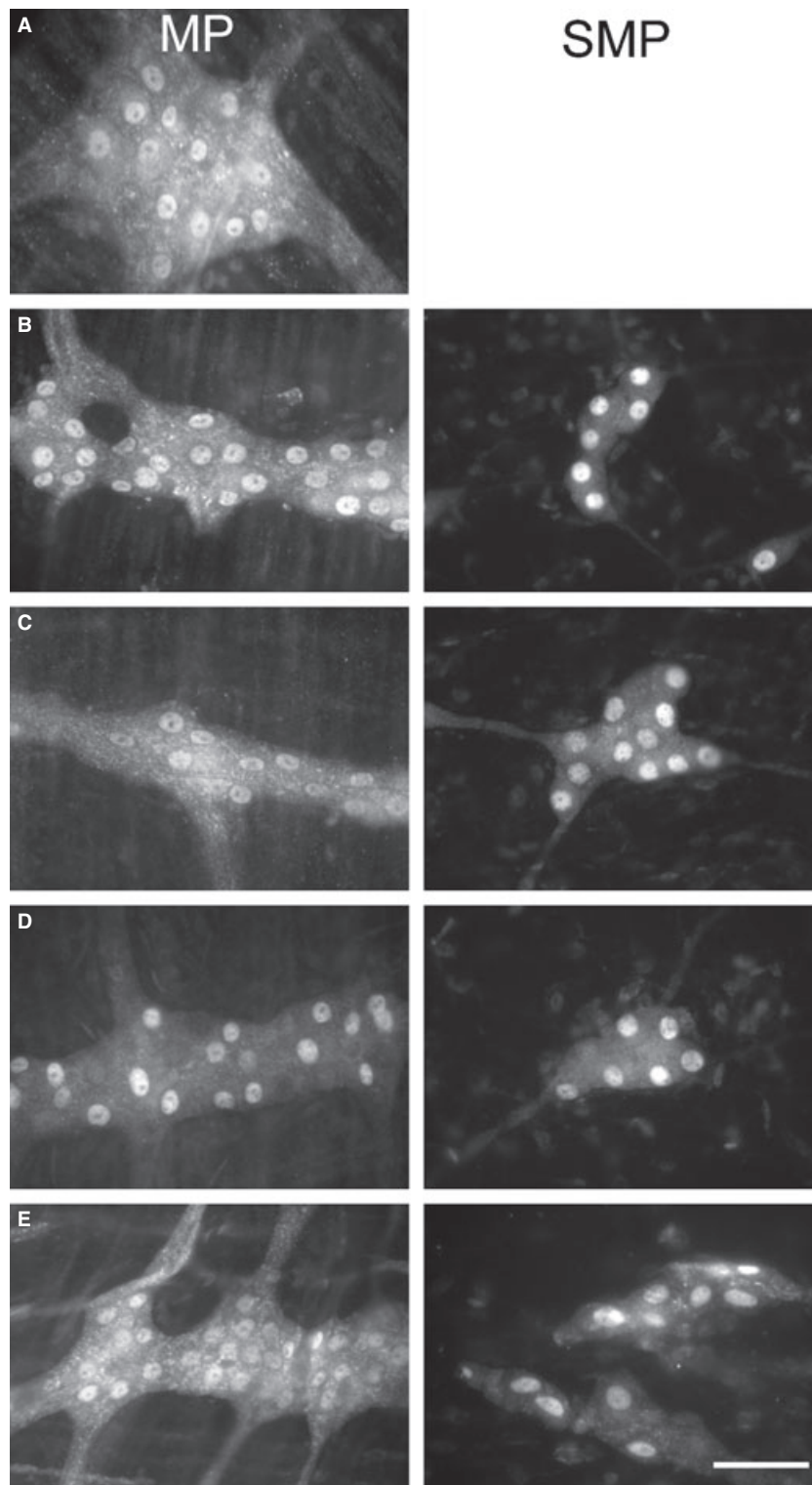
We then investigated whether the inhibitory action of OEA was mediated by PPAR $\alpha$ . The PPAR $\alpha$  antagonist GW6471 failed to block OEA-induced inhibition of upper GI transit, with transit in mice treated with 5, 10 or 20 mg kg<sup>-1</sup> GW6471 and OEA (40 mg kg<sup>-1</sup>) comparable ( $P > 0.05$ ) to that in mice treated with OEA alone (Fig. 3A). GW6471 (20 mg kg<sup>-1</sup>) had no effect on transit when administered alone (Fig. 3A). To further investigate the involvement of PPAR $\alpha$  in OEA-induced inhibition of transit, PPAR $\alpha$  gene-deficient mice were used. A significant reduction in transit of the charcoal marker by OEA was observed in PPAR $\alpha$ <sup>-/-</sup> mice (Fig. 3B). Transit along the upper GI tract in the PPAR $\alpha$ <sup>-/-</sup> mice treated with vehicle was comparable ( $P > 0.05$ ) to that in the wildtype mice treated with vehicle. Furthermore, the specific PPAR $\alpha$  agonist GW7647 did not modify upper GI transit at doses of up to 40 mg kg<sup>-1</sup> (Fig. 3C).

Gastrointestinal transit in CB<sub>1</sub><sup>-/-</sup> and CB<sub>2</sub><sup>-/-</sup> animals tended to be enhanced over that observed in control mice (Fig. 4A), but OEA (40 mg kg<sup>-1</sup>) had very similar effects in these animals (Fig. 4A). Neither the TRPV1 antagonist SB366791, at doses of 0.5 and 2 mg kg<sup>-1</sup> (Fig. 4B), nor the GLP-1 antagonist exendin-3(9-39) amide, at a dose of 0.1 mg kg<sup>-1</sup> (Fig. 4C), blocked the reduction in GI transit induced by OEA (40 mg kg<sup>-1</sup>). SB366791 (2 mg kg<sup>-1</sup>) or exendin-3(9-39) amide (0.1 mg kg<sup>-1</sup>) alone did not modify transit.

A 45-min exposure to novel environment stress accelerated transit of the upper GI from 57.5  $\pm$  2.6% intestine length (mean  $\pm$  SEM) in vehicle-treated non-stressed mice to 71  $\pm$  1.6% in vehicle-treated stressed mice ( $P < 0.001$ ). OEA at all doses tested (10, 20 and 40 mg kg<sup>-1</sup>) blocked enhanced upper GI transit (Fig. 5A). The lower dose of 10 mg kg<sup>-1</sup> OEA had no effect on normal non-stressed transit (Fig. 2B) yet reversed stress-induced acceleration of transit to non-stressed levels (Fig. 5).

## DISCUSSION

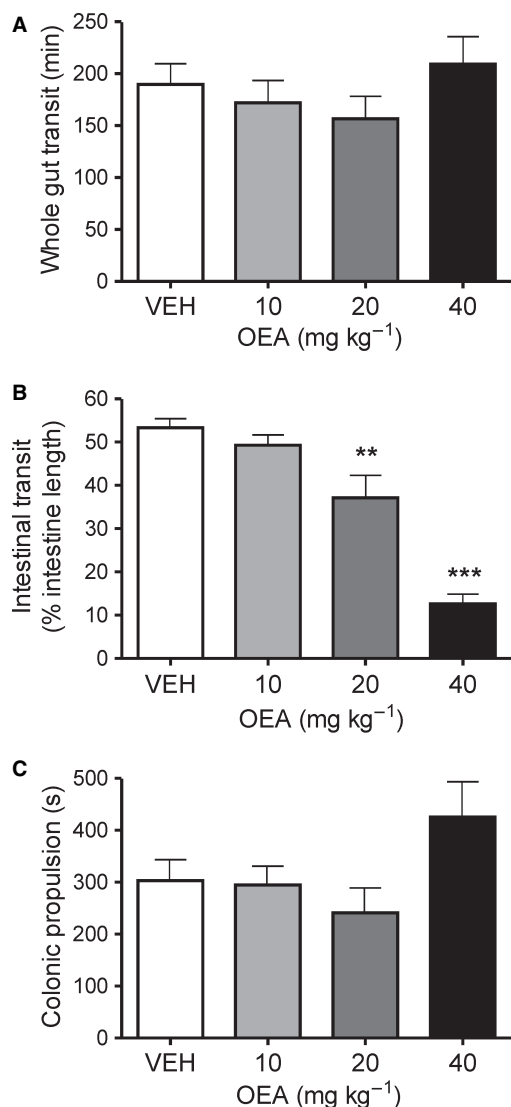
We report the presence of PPAR $\alpha$  in neurons of the enteric nervous system. However, it appears unlikely that this receptor mediates the inhibitory action of OEA on upper GI transit. Furthermore, receptors that are activated by OEA both directly, such as the TRPV1 receptor, and indirectly, cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptors and GLP-1 receptors appear not to mediate this action either. OEA blocked stress-induced accelerated upper GI transit at a dose that has no effect on physiological transit.



**Figure 1** Peroxisome proliferator-activated receptor alpha immunoreactivity in the myenteric plexus (MP) of the stomach (A), and the MP and submucosal plexus (SMP) of the duodenum (B), jejunum (C), ileum (D) and distal colon (E) of the mouse. Scale bar: 50  $\mu$ m.

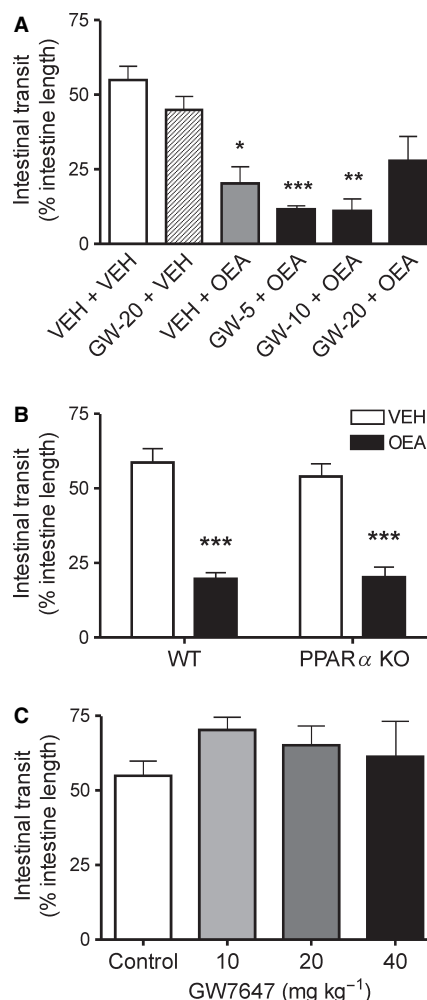
This study has revealed the presence of PPAR $\alpha$  on neurons of the enteric nervous system. Neurons of both the submucosal and the myenteric plexus expressed PPAR $\alpha$  immunoreactivity, which was

primarily located in the nuclei, though the nucleoli were unlabelled. This pattern of localization has also been shown in rat cortical neurons<sup>19</sup> and is expected of a nuclear receptor. PPAR $\alpha$  activation has been reported



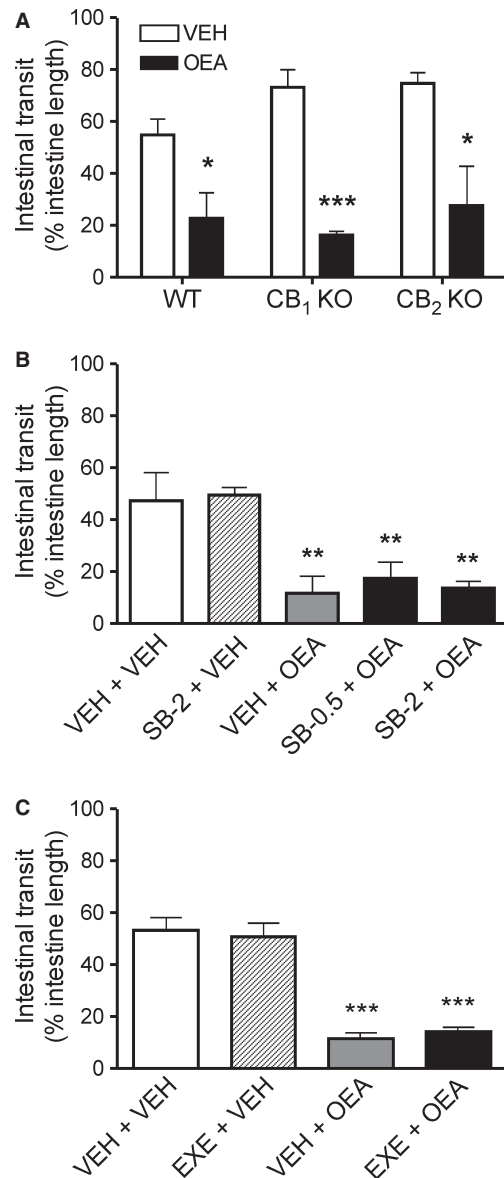
**Figure 2** The effect of vehicle (4% DMSO, 2% Tween 80 in physiological saline; i.p.) or oleoylethanolamide (OEA) (10, 20 or 40 mg kg<sup>-1</sup>; i.p.) on whole gut transit (A), upper GI transit (B) and colonic propulsion (C) in mice. Bars represent the mean  $\pm$  SEM,  $n = 6$ –16 per group. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  denote a significant difference to vehicle control.

to repress cellular processes involved in smooth muscle contraction in the mouse intestine.<sup>4</sup> Also, PPAR $\alpha$  upregulates the expression of the serotonin transporter SERT during fasting in mice,<sup>3</sup> resulting in termination of the excitatory action of serotonin on contractility of the intestine. The myenteric plexus contains the motor neurons responsible for the regulation and coordination of motility of the GI tract. As such, the localization of PPAR $\alpha$  in the myenteric plexus led us to investigate whether the activation of these receptors could modify motility. OEA is a high



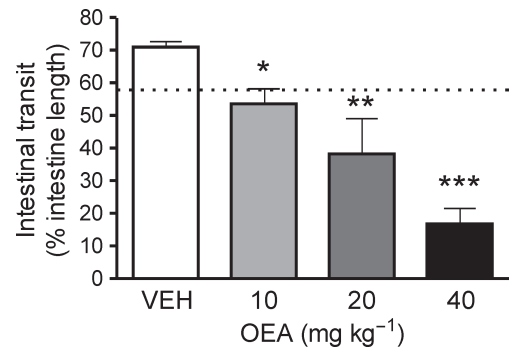
**Figure 3** (A) The effect of vehicle (4% DMSO, 2% Tween 80 in physiological saline; i.p.) or the peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ) antagonist GW6471 (5, 10 or 20 mg kg<sup>-1</sup>; i.p.) administered 30 min prior to vehicle or oleoylethanolamide (OEA) (40 mg kg<sup>-1</sup>; i.p.) on upper gastrointestinal (GI) transit. (B) The effect of vehicle or OEA (40 mg kg<sup>-1</sup>; i.p.) on upper GI transit in wildtype (WT) and PPAR $\alpha$  knockout (KO) mice. (C) The effect of vehicle or GW7647 (10, 20 or 40 mg kg<sup>-1</sup>; i.p.) on upper GI transit in mice. Bars represent the mean  $\pm$  SEM,  $n = 3$ –9. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  denote a significant difference to the corresponding vehicle control.

affinity agonist of PPAR $\alpha$  (EC<sub>50</sub> = 120 nmol L<sup>-1</sup>).<sup>6</sup> It is via activation of this nuclear receptor that OEA induces a state of satiety, increasing latency to eating while having no effect on meal size in free-feeding rats.<sup>20</sup> OEA has been shown to inhibit gastric emptying in mice through an, as yet, unidentified mechanism<sup>13</sup> and so we investigated the possible PPAR $\alpha$ -mediated actions of OEA in GI transit. We confirmed previous findings that OEA dose-dependently inhibits upper intestinal transit<sup>14</sup> and we show for the first time that



**Figure 4** (A) The effect of vehicle (4% DMSO, 2% Tween 80 in physiological saline; i.p.) or oleoylethanolamide (OEA) ( $40 \text{ mg kg}^{-1}$ ; i.p.) on upper gastrointestinal (GI) transit in wildtype (WT), CB<sub>1</sub> and CB<sub>2</sub> knockout (KO) mice. There was a trend for increased transit in both the CB<sub>1</sub> and CB<sub>2</sub> KO mice, but this did not reach statistical significance ( $P = 0.09$ ). The effect of vehicle, the transient receptor potential vanilloid 1 antagonist SB366791 ( $0.5$  or  $2 \text{ mg kg}^{-1}$ ; i.p.), B) or the glucagon-like peptide 1 antagonist exendin-3(9-39) amide [EXE(9-39),  $0.1 \text{ mg kg}^{-1}$ ; i.p., C] administered 30 min prior to vehicle (4% DMSO, 2% Tween 80 in physiological saline; i.p.) or OEA ( $40 \text{ mg kg}^{-1}$ ; i.p.) on upper GI transit. Bars represent the mean  $\pm$  SEM,  $n = 3-7$ . \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  denote a significant difference to the corresponding vehicle control.

colonic transit in mice is not modified by OEA. To determine whether the inhibitory action was mediated by PPAR $\alpha$ , we took two approaches: a pharmacological



**Figure 5** The effect of vehicle (4% DMSO, 2% Tween 80 in physiological saline; i.p.) or oleoylethanolamide (OEA) ( $10$ ,  $20$  or  $40 \text{ mg kg}^{-1}$ ; i.p.) on upper gastrointestinal (GI) transit in novel-environment stressed mice. Bars represent the mean  $\pm$  SEM,  $n = 6-18$ . \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  denote a significant difference to the vehicle control. The dotted line represents mean normal upper GI transit in non-stressed mice ( $n = 16$ ,  $57.5 \pm 2.6\%$ ). Note that at  $10 \text{ mg kg}^{-1}$  OEA had no significant effect in non-stressed mice (see Fig. 2B).

approach and a gene knockout approach. Although a high dose of the PPAR $\alpha$  antagonist GW6471 partially reversed OEA-induced inhibition of upper intestinal transit, attenuation of transit induced by OEA was still observed in PPAR $\alpha$  deficient mice, suggesting that this receptor does not mediate the effect of OEA on GI motility. Furthermore, neither the PPAR $\alpha$  antagonist GW6471 nor the PPAR $\alpha$  agonist GW7647 induced a change in motility, and basal transit in PPAR $\alpha$  deficient mice was comparable to that in wildtype mice, implying that PPAR $\alpha$  is not involved in the regulation of motility in the mouse upper GI tract. These results complement the finding that a PPAR $\alpha$  antagonist failed to inhibit OEA-induced gastric emptying in mice.<sup>13</sup>

Both OEA and the endocannabinoid anandamide are fatty acid ethanolamides that share similar biosynthetic and catabolic pathways; both are synthesized on demand from precursors located in the plasma membrane and are degraded by FAAH. Unlike anandamide, OEA has very little affinity for the CB<sub>1</sub> and CB<sub>2</sub> receptors ( $\text{EC}_{50} = > 30\,000 \text{ nmol L}^{-1}$ ).<sup>21,22</sup> However, OEA has been shown to inhibit the breakdown of anandamide.<sup>23</sup> Indeed, exogenous OEA is reported to increase endogenous intestinal levels of this endocannabinoid,<sup>14</sup> which therefore has the potential to induce an effect via indirect activation of CB<sub>1</sub> receptors. Anandamide inhibits transit in the small intestine<sup>24</sup> and so we investigated whether OEA could mediate inhibition of transit through an indirect action on cannabinoid receptors by enhancing endocannabinoid levels. Aviello *et al.*<sup>13</sup> have shown that neither SR141716 nor the CB<sub>2</sub> receptor antagonist SR144528

could reverse OEA-induced inhibition of gastric emptying. However, another group<sup>14</sup> has described an incomplete blockade of OEA-induced inhibition of upper intestinal transit by the CB<sub>1</sub> receptor antagonist SR141716, suggesting a partial involvement of the CB<sub>1</sub> receptors. Our findings using both cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptor gene-deficient mice suggest that OEA-induced inhibition of intestinal transit does not involve the activation of the cannabinoid receptors. Another fatty acid ethanolamide, palmitoylethanolamide, which is structurally similar to OEA, has also been shown to inhibit upper GI transit in mice in a non-cannabinoid receptor-mediated fashion.<sup>25</sup>

Although OEA has been shown to activate TRPV1 with an EC<sub>50</sub> in the micromolar range (2  $\mu\text{mol L}^{-1}$ )<sup>26</sup> it appears unlikely that this receptor mediates the inhibitory action of OEA on intestinal transit. This study has demonstrated an inability of the high-affinity TRPV1 antagonist SB366791; at similar doses to those which reversed capsaicin-induced hypothermia and vasodilatation in the knee joint,<sup>17</sup> to reverse the effect of OEA. Furthermore, this antagonist induced no change in intestinal transit alone.

Oleoylethanolamide is also an agonist of the G-protein receptors GPR119 and GPR55 with an EC<sub>50</sub> of 2.9  $\mu\text{mol L}^{-1}$ <sup>27</sup> and 440 nmol L<sup>-122</sup> respectively. GPR119 is a G-protein coupled receptor with high levels of mRNA expression in the stomach and small intestine as well as in the pancreas.<sup>28</sup> The activation of GPR119 stimulates the release of GLP-1; a peptide that inhibits intestinal transit through the GLP-1 receptor.<sup>29</sup> Due to the distribution of the receptor and the stimulated release of an inhibitory mediator of both gastric and intestinal motility, we hypothesized that this receptor could mediate the actions of OEA on transit. As GPR119 knockout mice and pharmacological tools are not commercially available, we examined the effect of the GLP-1 receptor antagonist, exendin-3(9-39) amide on the action of OEA. We discovered that antagonism of the GLP-1 receptor did not reverse OEA-induced inhibition of transit. While this does not rule out GPR119 as the mediator of this action of OEA, it does indicate that it is not mediated by downstream stimulation of GLP-1 release.

GPR55 is suggested to be a novel cannabinoid receptor that has distinct signalling pathways to CB<sub>1</sub> and CB<sub>2</sub> receptors.<sup>30</sup> In the periphery, GPR55 is predominantly localized in the small intestine and the adrenal tissues<sup>22</sup> however, there is no evidence, as yet, of the functional role of intestinally located GPR55. As pharmacological tools and gene knockout animals are not commercially available, we were unable to investigate the involvement of this receptor

in the mediation of OEA-induced inhibition of transit. However, it is intriguing to think that OEA may be an endogenous ligand for this receptor in the intestine.

Small intestinal levels of OEA fluctuate on a circadian cycle, increasing during the day when animals are resting, and do not eat, and decreasing at night when animals are feeding.<sup>6</sup> Also, feeding status regulates the biosynthesis of OEA in the small intestine of rats, with OEA levels in this region being depressed in a starved state and returning to baseline levels upon refeeding.<sup>12</sup> This only occurs in the small intestine and is not seen in the stomach.<sup>12,31</sup> Hence, OEA levels in the small intestine are increased following eating and the increased endogenous OEA levels observed during this time could be contributing to the jejunal brake, slowing upper GI transit for maximal nutrient absorption. Indeed OEA increases fatty acid uptake in intestinal cells<sup>32</sup> and induces lipolysis by activating PPAR $\alpha$ , through non-genomic signalling.<sup>11</sup> Furthermore, the presence of a lipid emulsion (but not protein or carbohydrate) in the duodenum of rats induces a rapid increase in jejunal OEA levels.<sup>33</sup> As the jejunal brake is initiated by the presence of fat in the small intestine,<sup>34</sup> these data suggest that endogenous OEA is likely to be a mediator of this phenomenon.

Enhanced upper GI transit is reported in diarrhoea-predominant irritable bowel syndrome (D-IBS) patients<sup>35</sup> and research suggests that GI symptoms of IBS are brought about and/or exacerbated by stress.<sup>36</sup> The reported effects of psychological stress on the modification of GI transit in animal models are conflicting, due in part to the diverse stressors used. For example, the presence of a hungry cat was shown to inhibit upper GI transit in mice,<sup>37</sup> while in gerbils a 2-h exposure to a novel environment had no effect on upper GI transit.<sup>38</sup> However, consistent with motility changes observed in D-IBS patients, small bowel transit was accelerated in humans experiencing an electric shock avoidance stressor,<sup>39</sup> and also in rats that were subjected to a flooded cage floor.<sup>40</sup> This study has shown that a 45-min exposure to a novel environment accelerates upper GI transit in mice. We have demonstrated that OEA is capable of blocking stress-induced acceleration of transit, without slowing transit below control baseline levels, and at a dose which has no inhibitory effect on normal transit. These findings may reveal a novel site of action of OEA that is not present under physiological conditions or via one that is upregulated under stress conditions. This has implications for OEA to be developed as a treatment for pathophysiological states of enhanced motility of the small bowel, either through administration of exogenous OEA or by raising endogenous OEA levels.

In summary, we have revealed the localization of the nuclear receptor PPAR $\alpha$  in the enteric nervous system. The functional data from this study suggest that the fatty acid amide OEA does not mediate inhibition of upper GI transit via activation of these receptors. Furthermore, the attenuating actions of OEA on transit do not appear to involve the cannabinoid CB<sub>1</sub> or CB<sub>2</sub> receptors or the TRPV1 receptor. While GPR119 and GPR55 are activated by OEA, further studies are required to determine whether these receptors are mediating the action of OEA on GI transit.

## ACKNOWLEDGMENTS

We thank Winnie Ho for the genotyping of the cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptor gene-deficient mice and Nancy Buckley for supplying the breeding stock of cannabinoid CB<sub>2</sub> receptor gene-deficient mice. These studies were supported by a grant from Canadian Institutes of Health Research (to KAS). KAS is an Alberta Heritage Foundation for Medical Research Medical Scientist and the Crohn's and Colitis Foundation of Canada Chair in Inflammatory Bowel Disease Research.

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## SUPPORTING INFORMATION

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

**Figure S1.** PPAR $\alpha$  immunoreactivity in the myenteric plexus (MP) and submucosal plexus (SMP) of the duodenum of the guinea pig (top row) and rat (bottom row). Scale bar: 50  $\mu$ m.

**Figure S2.** PPAR $\alpha$  and PGP9.5 immunoreactivity in the myenteric plexus of the mouse ileum. Note that the nuclei of neuronal cell bodies were brightly stained while weaker staining was observed in the cytoplasm. Scale bar: 50  $\mu$ m.

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