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CALL FOR PAPERS | *Neural Integration of Peripheral Signals Implicated in the Control of Energy Homeostasis and Metabolism*

Targeted enhancement of oleoylethanolamide production in proximal small intestine induces across-meal satiety in rats

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Fu J, Kim J, Oveisi F, Astarita G, Piomelli D. Targeted enhancement of oleoylethanolamide production in proximal small intestine induces across-meal satiety in rats. *Am J Physiol Regul Integr Comp Physiol* 295: R45–R50, 2008. First published April 23, 2008; doi:10.1152/ajpregu.00126.2008.—Pharmacological administration of the natural lipid amide, oleoylethanolamide (OEA), inhibits food intake in free-feeding rodents by prolonging latency to feed and postmeal interval. This anorexic effect is mediated by activation of type- α peroxisome proliferator-activated receptors (PPAR- α). Food intake stimulates mucosal cells in duodenum and jejunum to generate OEA, suggesting that this lipid-derived messenger may act as a local satiety hormone. As a test of this hypothesis, here, we examined whether targeted enhancement of OEA production in the small intestine affects feeding behavior in rats. We constructed an adenoviral vector (Ad-NPLD) that directs overexpression of the enzyme *N*-acylphosphatidylethanolamine (NAPE)-phospholipase D (PLD), which catalyzes the hydrolysis of NAPE to generate OEA. Intraduodenal injection of the Ad-NPLD vector resulted in a time-dependent increase in NAPE-PLD expression and OEA production, which was restricted to the proximal small intestine. No such effect was observed after administration of a control adenoviral vector. Enhanced OEA production in Ad-NPLD-injected animals was temporally associated with increased expression of two PPAR- α target genes (PPAR- α and CD36) and with decreased food intake. The hypophagic phenotype of Ad-NPLD-injected rats was attributable to increase feeding latency and postmeal interval, rather than decreased meal size. The results suggest that localized changes in OEA production in the small intestine, such as those produced by food intake, are sufficient to induce in rats a state of across-meal satiety similar to that elicited by systemic administration of exogenous OEA.

N-acylphosphatidylethanolamine-phospholipase D; peroxisome proliferator-activated receptor- α ; adenovirus

THE GASTROINTESTINAL TRACT plays a critical role in the control of food intake, regulating multiple aspects of this process through both neuronal and hormonal mechanisms. Among the signals released by the gut, peptides such as CCK or glucagon-like peptide-1 (GLP-1) have received the most attention (4, 6), but evidence indicates that lipid-derived mediators may also be involved. For example, food ingestion stimulates cells in the mucosal layer of the duodenum and jejunum to produce the endogenous lipid amide oleoylethanolamide (OEA) (3, 8, 18),

suggesting that this compound may participate in the induction of satiety. In support of this idea, pharmacological studies have shown that administration of exogenous OEA reduces feeding in rats and mice (2, 9, 13, 15, 18). Behavioral analyses in free-feeding rodents indicate that the anorexic effects of OEA cannot be attributed to malaise, anxiety, stress, or motor inhibition (17, 18) but rather to a prolongation of latency to feed and postmeal interval, which is suggestive of enhanced across-meal satiety (10, 15). Thus, the anorexic properties of OEA appear to be distinct from those of gut peptides such as CCK and GLP-1, which reduce food intake by decreasing meal size and duration (4, 6).

Several lines of evidence indicate that peroxisome proliferator-activated receptor- α (PPAR- α), a nuclear receptor involved in the regulation of energy balance (19), mediates the anorexic actions of OEA. First, OEA binds with high affinity to the purified ligand-binding domain of PPAR- α (dissociation constant, $K_D \approx 40$ nM) and activates with high potency PPAR- α -driven transactivation in heterologous expression systems (half-maximal effective concentration, $EC_{50} \approx 100$ nM) (2, 9). Second, synthetic PPAR- α agonists exert anorexic effects that are behaviorally identical to those produced by OEA (2, 9). Third, targeted deletion of the PPAR- α gene in mice abolishes the hypophagic actions of OEA and synthetic PPAR- α agonists (9). Finally, the concentrations reached by endogenous OEA in the rat small intestine mucosa after feeding (200–400 nM) (8) are sufficient to fully activate PPAR- α , which is highly expressed in murine small intestine (5) but not to affect other putative OEA receptors, such as GPR119 ($EC_{50} \approx 3$ μ M) (16) and TRPV-1 ($EC_{50} \approx 2$ μ M) (1).

The findings outlined above suggest that food-induced OEA production in the proximal small intestine may regulate satiety through a local autocrine or paracrine mechanism. A corollary of this hypothesis is that experimental manipulations that selectively elevate OEA formation in the small intestine should mimic the anorexic effects elicited by systemic administration of exogenous OEA. To test this prediction, in the present study, we constructed an adenoviral vector that directs overexpression of the enzyme *N*-acylphosphatidylethanolamine (NAPE)-phospholipase D

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Fig. 1. Characterization of the NAPE-PLD-overexpressing adenoviral vector (Ad-NPLD) in HeLa cell cultures. Effects of infecting HeLa cells with control (open bars) or Ad-NPLD (closed bars) vector (10^8 pfu, 36 h) on (A) NAPE-PLD mRNA levels; (B) NAPE-PLD protein levels, as assessed by immunoblot analysis; (C) NAPE-PLD protein levels, as assessed by immunocytochemistry (top panel, Ad-NPLD; bottom panel, control vector); (D) Cellular levels of OEA, PEA and anandamide (AEA), as assessed by liquid chromatography/mass spectrometry. Results are expressed as mean $\pm$ SEM; $**P < 0.01$, $***P < 0.001$; $n = 8$.

(PLD) (14, 23, 24), which catalyzes the hydrolysis of NAPE to produce OEA. Injection of this viral vector into the duodenum of rats resulted in local increases in NAPE-PLD expression and OEA production, which were accompanied by a significant enhancement of across-meal satiety.

MATERIALS AND METHODS

Animals. Adult male Wistar rats (250–300 g) were purchased from Charles River (Wilmington, MA). A 12-h light-dark cycle was set with lights on at 5:30 AM. Water and standard chow pellets (Prolab RMH 2500; PMI Nutrition International, Brentwood, MO) were available ad libitum. All procedures met the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Lipid analyses. Rats were killed with halothane, and tissues were rapidly collected and snap-frozen in liquid N_2 . Frozen tissues were weighed and homogenized in methanol containing internal standards (8). Cells were harvested in 50% methanol in PBS containing internal standards. OEA and other fatty-acid ethanolamides (palmitoylethanolamide, anandamide), as well as their NAPE precursors, were quantified after lipid extraction by isotope-dilution liquid chromatography/mass spectrometry, as described (8).

Adenovirus preparation. An *EcoRI/NheI* fragment containing full-length rat brain NAPE-PLD was cloned into the pCD515 vector (Microbix, Toronto, Ontario, Canada). Plasmids were cotransfected with adenoviral genomic plasmid into low-passage human embryonic kidney 293 (HEK293) cells using the Superfect transfection reagent (Qiagen, Valencia, CA). The shuttle plasmid pCD515 was used as a control. Cotransfected cells were harvested and subjected to three cycles of freezing and thawing. The recombinant adenovirus was recovered by centrifugation at 1500 g for 10 min. Viral particles were purified by 50,000 g ultracentrifugation for 2.5 h using cesium chloride gradient. The viral band was collected by puncture, and the purified virus was dialyzed against 3% sucrose/phosphate buffer (0.1 M). Stock virus was titrated by plaques in HEK293 cell.

Intraduodenal injections. Animals were fasted for 24 h before surgery and anesthetized by intraperitoneal injection of ketamine-xylazine (9%:1%; 1 ml/kg). A 2- to 3-cm incision was made into the ventral midline skin and abdominal muscle wall beneath the xiphoid process. The mesentery was gently retracted with sterile gauze to

reveal the duodenum. The duodenum was lifted, and an adenovirus suspension (10^{12} pfu/1 ml PBS) was injected into the intestinal lumen ~ 2 cm below the pylorus using a 27-gauge needle. The ends of the scalp incision were sutured; recovering animals were kept on a heated pad at 30°C until they awoke.

Feeding behavior. Food intake was recorded in free-feeding rats by using an automated monitoring system (Scipro, New York, NY), as described previously (10). The system consists of 24 cages equipped with food baskets connected to weight sensors. The following feeding parameters were analyzed: total food intake (g/kg)—the amount of food consumed during 24 h; feeding latency (min)—the time interval from dark onset to the first eating episode; meal size (g/kg)—the amount of food consumed during the meal; postmeal interval (min)—the time interval between end of a meal and beginning of next meal; and satiety ratio ($\text{min} \cdot \text{g}^{-1} \cdot \text{kg}^{-1}$)—the ratio between postmeal interval and meal size.

Quantitative PCR. Total RNA was extracted from tissue and cells with TRIzol (Invitrogen, Carlsbad, CA), and cDNA was synthesized by using SuperscriptII RNase H-reverse transcriptase (Invitrogen) following the manufacturer's instructions. Real-time quantitative PCR was performed in an Mx 3000P system (Stratagene, La Jolla, CA)

Fig. 2. Intraduodenal injections of Ad-NPLD enhance NAPE-PLD mRNA expression in rat jejunum. D5–D12, day after virus injection. Open bars, control adenoviral vector; closed bars, Ad-NPLD vector. Results are expressed as mean $\pm$ SEM; $*P < 0.05$, $**P < 0.01$; $n = 6$ –8.

Table 1. Effects of duodenal injections of Ad-NPLD or Ad-mock on NAPE-PLD mRNA levels in various rat tissues

Day of Injection	Organs	Stomach	Colon	Liver	Fat	Pancreas	Kidney
7th day	Ad-Mock	0.97±0.17	0.55±0.07	0.13±0.006	3.92±0.19	36.11±3.18	0.9±0.21
	Ad-NPLD	1.07±0.18	0.74±0.21	0.109±0.01	2.82±0.4	23.24±5.3	0.99±0.07
10th day	Ad-Mock	0.877±0.18	0.667±0.14	0.125±0.01	2.89±0.1	27.6±3.18	1.28±0.15
	Ad-NPLD	1.05±0.15	0.77±0.1	0.126±0.01	3.63±0.15	40.27±7.5	1.08±0.05
12th day	Ad-Mock	0.99±0.12	0.729±0.14	0.163±0.01	3.32±0.08	27.02±5.3	0.89±0.04
	Ad-NPLD	0.93±0.16	0.73±0.17	0.146±0.02	3.12±0.65	32.67±3.3	1.03±0.05

Results, in arbitrary units, are expressed as means ± SE; $n = 6-8$. Ad-Mock, control adenoviral vector.

using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The primer/probe sequences were NAPE-PLD, forward primer: TGGCTGGGACACGCG; reverse primer: GG-GATCCGTGAGGAGGATG; probe: CGCTGATGGTGAAATG-GACGAGC; PPAR- α , forward primer: TGGTGGACCTCCGGCA; reverse primer: TCTTCTTGATGACCTGCACGA; probe: CTGGT-CACGGAGCATGCGCAGT; CD36, forward primer: CGGCGAT-GAGAAAGCAGAA; reverse primer: CAACCAGGCCAGGAGC; probe: tgttcagaaccaagtaccgggaaataa; GAPDH, forward primer: AAGTATGATGACATCAAGAAGGTGGT; reverse primer: AGC-CCAGGATGCCCTTTAG; and probe: AAGCAGGCGGCCGAG-GGC. mRNA expression levels were normalized by using GAPDH as an internal standard and were calculated as described (20).

Antibody generation. We constructed a glutathione-S-transferase-linked peptide comprising residues 38–53 of rat NAPE-PLD. The DNA fragment was amplified using primers incorporated with *Bam*HI and *Xho*I restriction sites on the 5' and 3' end of amplicon, respectively. After digestion with *Bam*HI and *Xho*I, the amplicon was subcloned into vector pGEX-4T-1 (Amersham Biosciences, Piscataway, NJ). The fusion protein was expressed in *Escherichia coli* BL-21 cells (Novagen, Madison, WI) and purified using glutathione sepharose 4B (Amersham) following the manufacturer's instructions. The polyclonal NAPE-PLD antibody was raised in rabbits and characterized using standard procedures (8, 22).

Protein analyses. Hela cells were harvested in Tris·HCl buffer (50 mM, pH 8.0) containing a protease inhibitor cocktail (EMD Chemicals, San Diego, CA) after 36 h infection with 10^8 pfu of Ad-NPLD or control virus. After sonication, proteins were subjected to SDS-gel electrophoresis (4–15%) and transferred to Hybond-P membranes (Amersham Biosciences). Immunoblot analyses were conducted using a previously characterized NAPE-PLD antiserum (8) (1:3,000 dilution) using ECL-Plus detection kit (Amersham).

Immunocytochemistry. Hela cells were fixed in 4% paraformaldehyde and blocked with 3% normal goat serum for 2 h at room temperature, rinsed with PBS (0.1 M, PBS), and incubated with NAPE-PLD antiserum (1:2,000 dilution) overnight at 4°C. After rinsing with PBS, we incubated the cells with anti-rabbit IgG Alexa Fluor 488 (1:5,000 dilution; Invitrogen) for 1 hr at room temperature.

Fluorescent images were captured using the SpotFlex digital imaging system (Diagnostic Instruments, Sterling Heights, MI).

Statistical analyses. Results are expressed as means ± SE. Statistical significance was evaluated using the Student's *t*-test or, when appropriate, one-way ANOVA followed by the Dunnett's post hoc test. Analyses were conducted using GraphPad Prism (GraphPad Software, San Diego, CA), and differences were considered significant if $P < 0.05$.

RESULTS

We first verified that the adenoviral vector Ad-NPLD directs NAPE-PLD expression in intact cells. Infection of HeLa cell cultures with Ad-NPLD (10^8 pfu, 36 h) resulted in a significant increase in NAPE-PLD mRNA levels, whereas infection with a control vector had no such effect (Fig. 1A). Heterologous NAPE-PLD expression was confirmed by both immunoblot analysis, which revealed the presence in Hela cell extracts of an appropriately sized protein band (≈ 46 kD) (Fig. 1B), and immunocytochemistry (Fig. 1C). The characterization of the NAPE-PLD antibody used in these experiments is reported elsewhere (8). As shown in Fig. 1D, NAPE-PLD expression was accompanied by an elevation in the cellular levels of OEA and its analog palmitoylethanolamide (PEA). By contrast, we observed only a small nonsignificant change in cellular anandamide content, confirming the limited role of NAPE-PLD in the production of this endogenous cannabinoid ligand (14, 21).

Intraduodenal injections of Ad-NPLD (10^{12} pfu), but not control vector, resulted in a time-dependent elevation in jejunal NAPE-PLD mRNA content, which reached maximal levels 8 to 10 days after virus administration (Fig. 2). Confirming that this effect was restricted to the small intestine, no increase in NAPE-PLD mRNA was noted in a broad series of visceral organs and tissues, including stomach, colon, liver, epididymal fat, pancreas, and kidney (Table 1). Jejunal changes in NAPE-PLD expression were temporally associated with an increase in OEA and PEA levels (Fig. 3, A and B), but only with small

Fig. 3. NAPE-PLD overexpression increases OEA production in rat jejunum. Time-course of the effects of Ad-NPLD or control vector on the levels of OEA (A), PEA (B), and anandamide (AEA) (C). D7–D12, day after virus injection. Open bars, control adenoviral vector; closed bars, Ad-NPLD vector. Results, in pmol/g wet tissue, are expressed as mean ± SEM; * $P < 0.05$, ** $P < 0.01$; $n = 6-8$.

Table 2. Effects of duodenal injections of Ad-NPLD or Ad-mock on the OEA precursor, NOPE, (m/z 1030.8 > 744.8), and the PEA precursor, NPPE, (m/z 1004.8 > 718.8) in rat jejunum

	NOPE		NPPE	
	Ad-Mock	Ad-NPLD	Ad-Mock	Ad-NPLD
7th day	208.57 ± 34.8	172.33 ± 30.5	256.46 ± 54.6	245.18 ± 47.5
8th day	233.52 ± 53	135.91 ± 34.9	355.3 ± 66.9	174.61 ± 51.6*
9th day	250.21 ± 45.09	105.46 ± 19.9*	303.82 ± 72.1	117.22 ± 26.8*
10th day	230.12 ± 45.09	126.83 ± 10.5*	303.03 ± 67.6	175.13 ± 20.7*
12th day	219.7 ± 37.3	186.76 ± 52.4	235.67 ± 53.1	247.35 ± 24.2

Results, in pmol/g of wet tissue, are expressed as means ± SE; $n = 3$. Ad-NPLD, *N*-acylphosphatidylethanolamine (NAPE)-phospholipase D; NOPE, 1-stearoyl-2-arachidonoyl-*sn*-glycero-phosphoethanolamine-*N*-oleoyl; NPPE, 1-stearoyl-2-arachidonoyl-*sn*-glycero-phosphoethanolamine-*N*-palmitoyl. * $P < 0.05$; $n = 3$.

nonsignificant alteration in anandamide levels (Fig. 3C). As expected, NAPE-PLD overexpression was also accompanied by a decrease in the jejunal levels of the OEA precursor, 1-stearoyl-2-arachidonoyl-*sn*-glycero-phosphoethanolamine-*N*-oleoyl, and the PEA precursor, 1-stearoyl-2-arachidonoyl-*sn*-glycero-phosphoethanolamine-*N*-palmitoyl (Table 2).

To determine whether virally induced overproduction of endogenous OEA is sufficient to activate PPAR- α in rat jejunum, we measured the expression of two representative PPAR- α target genes, those encoding for PPAR- α itself and for the fatty-acid transporter CD36 (9). Quantitative PCR analyses showed that, concomitantly with OEA overproduction (Fig. 3A), Ad-NPLD injection resulted in a significant elevation in jejunal PPAR- α (Fig. 4A) and CD36 mRNA (Fig. 4B). Administration of a control adenoviral vector had no effect on PPAR- α or CD36 mRNA levels (Fig. 4).

The intraduodenal injection of control vector did not alter free-feeding behavior (Figs. 5 and 6). By contrast, injection of the Ad-NPLD virus resulted in a reversible reduction in total food intake (Fig. 5), which overlapped with the time of maximal NAPE-PLD expression (Fig. 2) and OEA elevation (Fig. 3A). Meal pattern analyses on days 7–12 after virus injections revealed that this temporary hypophagia was due to a prolongation of the

Fig. 5. NAPE-PLD overexpression reduces food intake in free-feeding rats. Time-course of the effects of Ad-NPLD or control vector on daily food consumption. D5–D12, day after virus injection. Open bars, control adenoviral vector; closed bars, Ad-NPLD vector. Results, in g/kg of body weight, are expressed as mean ± SEM; * $P < 0.05$, $n = 11$ –12.

latency to feed (Fig. 6A) and postmeal interval (Fig. 6B), with no detectable change occurring in meal size (Fig. 6C). The expected increase in satiety ratio (postmeal interval/meal size) associated with this response pattern is illustrated in Fig. 6D. Notably, these alterations in feeding behavior were only observed during the first hours of nocturnal feeding (Fig. 7). Later nocturnal food intake or diurnal food intake were not affected by NAPE-PLD overexpression (data not shown).

DISCUSSION

In the present study, we examined whether artificial rises in OEA levels in the small intestine, quantitatively similar to those naturally caused by food ingestion (8), can alter feeding behavior in rats. To increase local OEA production, we constructed an adenoviral vector that directs expression of the OEA-synthesizing enzyme NAPE-PLD and injected this vector into the rat duodenum. This resulted in parallel increases in NAPE-PLD expression, PPAR- α activation, and OEA production, which were restricted to the small intestine and were

Fig. 4. Intraduodenal injections of Ad-NPLD induce expression of PPAR- α -target genes in rat jejunum. Time-course of the effects of Ad-NPLD or control vector on PPAR- α mRNA (A), and CD36 mRNA (B). D7–D12, day after virus injection. Open bars, control adenoviral vector; closed bars, Ad-NPLD vector. Results are expressed as mean ± SEM; * $P < 0.05$, ** $P < 0.001$; $n = 6$ –8.

Fig. 6. NAPE-PLD overexpression increases across-meal satiety in free-feeding rats. Meal pattern analyses were performed on day 7–12 (D7–D12) after injection of Ad-NPLD or control vector. (A) Latency to feed after dark onset; (B) First postmeal interval (PMI) (left panel) and average PMI for first 3 nocturnal meals (right panel); (C) First meal size (left panel) and average meal size for first 3 nocturnal meals (right panel); (D) Satiety ratio for first nocturnal meal (left panel) and average satiety ratio for first 3 nocturnal meals (right panel). Open bars, control adenoviral vector; closed bars, Ad-NPLD vector. Results are expressed as mean $\pm$ SEM; * P < 0.05, n = 11–12.

temporally associated with a significant reduction in food intake. Importantly, the reversible hypophagia associated with NAPE-PLD overexpression was behaviorally identical to that elicited by exogenous OEA (10, 15), as it was due to a prolongation of feeding latency and postmeal interval rather than to a decrease in meal size. Also, of interest is the finding that the anorexic effect induced by NAPE-PLD overexpression was restricted to the first hours of nocturnal feeding, when rats eat the first and largest of their daily meals. These results support the hypothesis that feeding-induced fluctuations in small-intestinal NAPE-PLD activity and OEA production are sufficient to influence across-meal satiety (3, 8).

Fig. 7. The effects of NAPE-PLD overexpression are limited to the early hours of nocturnal feeding. Time-course of the effect of Ad-NPLD or control vector on satiety ratio, assessed on day 10 after vector injection. Open bars, control adenoviral vector; closed bars, Ad-NPLD vector. m1–m3, meals one to three. Results are expressed as mean $\pm$ SEM; * P < 0.05, n = 11–12.

Previous studies have shown that food ingestion stimulates OEA mobilization in the mucosal layer of the duodenum and jejunum but exerts no such effect in the serosal layer from the same intestinal segments, in other sections of the gastrointestinal tract (stomach, ileum, colon) or in a broad series of internal organs and tissues (e.g., liver and brain) (8, 18). Furthermore, food intake does not alter OEA concentrations in portal or cardiac blood (8, 18). These observations suggest that OEA may act as a local—that is, autocrine or paracrine—satiety signal rather than as a blood-borne hormone. The present experiments show that a localized increase in OEA mobilization in the small intestine can both activate PPAR- α —as evidenced by the induction of two key PPAR- α target genes (PPAR- α itself and CD36)—and reduce food intake, thus providing a critical piece of evidence in support of a local messenger role of OEA. Further support for this hypothesis comes from studies showing that surgical resection of the vagus nerve below the diaphragm or pharmacological ablation of peripheral sensory fibers each abrogates the anorexic effects elicited by intraperitoneal administration of OEA (18).

Our experiments raise several questions, which remain to be answered. The first pertains to the identity of the fatty-acid ethanolamides involved in the regulation of feeding behavior. Both food intake (3, 8) and NAPE-PLD overexpression (present results) increase the levels of multiple fatty-acid ethanolamides along with OEA. At present, the functional roles played by each member of this family of compounds, if any, remain unclear. Because both OEA and PEA activate PPAR- α and reduce feeding (11, 18), the possibility that PEA contributes to the hypophagic effect of NAPE-PLD overexpression should be taken into consideration. The second question relates to the cell types in the small intestine (5) responsible for OEA biosynthesis. Our viral approach allowed us to restrict NAPE-PLD expression to the upper gastrointestinal tract, but not to direct it to a specific cellular locale. Therefore, additional

experiments using cell type-specific deletion or overexpression of the NAPE-PLD gene (or those encoding other OEA-metabolizing enzymes) are necessary to fully elucidate the functional anatomy of OEA signaling in the upper gut. Another question concerns the possible roles of OEA in aspects of intestinal physiology other than feeding regulation. Both exogenous OEA administration (18) and endogenous OEA overproduction (present results) stimulate expression of the PPAR- α -regulated protein CD36. This multifunctional cell-membrane glycoprotein is highly expressed in the small intestine and has been implicated in the transport of fatty acids across the enterocyte membrane (7, 12), confirming the suggestion that OEA signaling at PPAR- α regulates lipid absorption along with food intake (25). A final consideration is the time course of the anorexic effects of OEA overproduction. The finding that such effects are limited to the first hours of the night, when rodents consume a significant proportion of their daily food, suggests that OEA signaling might be controlled by circadian regulators, which remain to be discovered.

Despite these open questions, the present results provide important new evidence for a role of OEA as a local satiety messenger in the small intestine and further validate the use of viral technology as a tool for the local delivery of pharmacologically active agents to organs and tissues.

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