Vagal afferents are not necessary for the satiety effect of the gut lipid messenger oleylethanolamide (OEA)

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Abstract:

The endogenous lipid messenger OEA inhibits eating and modulates fat metabolism supposedly through the activation of PPAR-\(\alpha\) and vagal sensory fibers. We tested in adult male rats whether OEA stimulates fatty acid oxidation (FAO) and ketogenesis and whether it increases plasma levels of the satiating gut peptides glucagon-like peptide-1 (GLP-1) and peptide tyrosine-tyrosine (PYY). We also explored whether OEA still inhibits eating after subdiaphragmatic vagal deafferentation (SDA). We found that intraperitoneally (IP) injected OEA (10 mg/kg body weight = BW) reduced \((P < 0.05)\) food intake mainly by increasing meal latency and that this effect was stronger in rats fed a 60% high-fat diet (HFD) than in chow-fed rats. OEA increased \((P < 0.05)\) postprandial plasma non-esterified fatty acids and \(\beta\)-hydroxybutyrate (BHB) in the hepatic portal vein (HPV) and vena cava (VC) 30 min after injection, which was more pronounced in HFD- than in chow-fed rats. OEA also increased the protein expression of the key-ketogenetic enzyme, mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase, in the jejunum of HFD-fed rats, but not in the liver or duodenum of either diet group. Furthermore, OEA decreased GLP-1 and PYY concentrations \((Ps < 0.05)\) in the HPV and VC 30 min after administration. Finally, OEA reduced food intake in SDA and sham-operated rats similarly. Our findings indicate that neither intact abdominal vagal afferents nor prandial increases in GLP-1 or PYY are necessary for the satiety effect of OEA. The enhanced FAO and ketogenesis raise the possibility of an involvement of intestinal-derived BHB in OEA’s satiety effect under certain conditions.

**Keywords:** Ketogenesis, small intestine, food intake, fatty acid oxidation, gut peptides
Introduction

The small intestine plays a key role in the regulation of energy balance and fat homeostasis (1, 34). Digestion, absorption and intracellular processing of dietary fat in the small intestine trigger the release of gut hormones that inhibit eating, such as cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), peptide tyrosine-tyrosine (PYY), and apolipoprotein A-IV (Apo AIV) (1, 68). The intestine also releases other signaling substances that are implicated in the control of eating (26, 48), i.e., fatty acid ethanolamides (FAEs) such as anandamide, palmitoylethanolamide, linolethanolamide and oleoylethanolamide (OEA). OEA, the amide of ethanolamine and oleic acid, potently reduces food intake without inducing visceral illness or anxiety, suggesting that it has a specific inhibitory effect on eating (44, 46, 48). The exact mechanism by which OEA inhibits eating is still poorly understood, but the effect seems to depend on the feeding state of the animals: in *ad libitum*-fed rats, OEA delays the onset of eating and decreases meal frequency without altering meal size, indicating that its hypophagic effect is mainly due to a prolongation of satiety, i.e., the state of non-eating between meals. In food-deprived rats, however, OEA also reduces meal size (20, 24), suggesting that it can also affect satiation (= meal termination).

Intraperitoneal (IP) injections of OEA stimulate c-Fos expression in the nucleus of the solitary tract (NST) and in peptide-secreting neurons of the paraventricular (PVN) and supraoptic nuclei (SON) of the hypothalamus (23, 48). Moreover, OEA increases the expression and release of oxytocin (OT) in both PVN and SON (23, 49), and intracerebroventricular (ICV) infusion of a selective OT receptor antagonist abolished the satiety effect of IP OEA, suggesting that it is mediated by the central release of OT (23).
Independent of its central nervous system mediation, several lines of evidence indicate that the satiety effect of OEA is linked to an activation of peroxisome proliferator-activated receptor alpha (PPAR-α) (6, 20, 21, 38, 58). This effect appears to occur in the periphery because OEA failed to reduce food intake after ICV administration (48). As a key transcriptional regulator of enzymes involved in fatty acid oxidation (FAO) and lipid metabolism, PPAR-α is expressed in many tissues, in particular in the liver and the small intestine (11). OEA and the synthetic PPAR-α agonist Wy-14643 increase the expression of PPAR-α and its target genes in the liver and the small intestine (20, 70), and stimulate FAO. In fact, similar to synthetic PPAR-α agonists, OEA increased circulating β-hydroxybutyrate (BHB), an indicator of enhanced peripheral FAO and ketogenesis, in vivo, and FAO in isolated hepatocytes (25). Hepatic FAO has long been implicated in the control of food intake (36, 55), but this hypothesis has recently been questioned (34). Also, we showed that the hypophagic effect of Wy-14643 in rats was associated with a stimulation of FAO and ketogenesis specifically in the jejunum (30). It is therefore possible that OEA’s hypophagic effect is also related to a stimulation of intestinal FAO and ketogenesis, which may contribute to the inhibition of eating (34).

We here investigated the effects of IP OEA injection on food intake and meal patterns in rats fed either chow or high-fat diet (HFD) as well as on peripheral FAO and ketogenesis via monitoring BHB levels in blood collected from the hepatic portal vein (HPV) and the inferior vena cava (VC). We also explored whether the eating-inhibitory effect of OEA is associated with an activation of ketogenesis in liver and intestine by measuring the protein expression of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mHMG-CoAS2), the rate-limiting enzyme in ketogenesis (28), in both tissues. Further, we examined the effect of OEA on the circulating levels of gut peptides (i.e., PYY and GLP-1) that might contribute to OEA’s satiety.
effect (37, 46). Finally, as it has been reported that OEA’s satiety effect was abolished in rats after treatment with the neurotoxin capsaicin or after a total subdiaphragmatic vagotomy (20, 48, 63), we examined the role of abdominal vagal afferents in OEA’s satiety effect by IP injecting OEA in rats after subdiaphragmatic vagal deafferentation (SDA) or sham surgery. SDA is a surgical procedure that eliminates all abdominal vagal afferents, but leaves about 50% of the vagal efferents intact; it is considered to be the most complete and selective abdominal vagal deafferentation technique available (43, 64).

Materials and methods

Animals and housing

Male Sprague Dawley rats (Charles Rivers, Sulzfeld, Germany), weighing 180–200g upon arrival, were individually housed in acrylic infusion cages under a 12:12 h dark/light cycle in a climate-controlled room (22 ± 2 °C and 60% relative humidity). Animals were fed *ad libitum* standard chow (N3433, 12.6% of energy from fat, caloric density: 3.1 Kcal/g, ProvimiKliba SA, Switzerland) or high-fat diet (HFD, 60% of energy from fat, caloric density: 5.2 Kcal/g, No. E15742, SSNIFF GmbH, Germany) unless otherwise stated. The animals were adapted to housing and diet conditions for at least 10 days prior to catheter implantation (see below). All procedures were approved by the Veterinary Office of the Canton of Zurich.

Catheter assembly

Catheters with custom-made headsets were manufactured as described before (59, 62). Briefly, the catheters consisted of silicone tubing (Dow Corning, Midland, MI; inner diameter
(ID) x outer diameter (OD), 0.51 x 0.91 mm) connected to a polished L-shaped needle (Sterican, B. Braun, Germany). We used 20 cm tubing and 22 gauge needles for the IP catheters and 23 cm silicone tubing with 22 gauge needles for the VC and HPV catheters, respectively. The connections between tubing and needles were shielded with 3 mm (ID x OD, 0.76 x 1.65 mm) and 2.2 cm (ID x OD, 1.02 x 2.18mm) long pieces of silicone tubing as inner and outer layers, respectively.

**Surgery preparations**

All surgeries were performed under aseptic conditions. Instruments were autoclaved and the catheters sterilized (Kodan® Rote Farblos, Schulke, Switzerland) prior to use. Three hours prior to surgery, rats received a subcutaneous (SC) injection of antibiotics (4 mg/kg body weight (BW) of trimethoprim and 20 mg/kg BW of sulfadoxine, Borgal 24%; Intervet/Shering-Plough Animal Health, Kenilworth, NJ) for infection prophylaxis. Fifteen min before surgery an IP injection of atropine (0.05 mg/kg; Sintetica, Mendrisio, Switzerland) was given followed by an injection of anesthetics (1.2 ml/kg BW), which consisted of a mix of 80 mg/kg BW ketamine (Ketasol-100; Dr. E. Gräub AG, Bern, Switzerland) and 4.0 mg/kg BW xylazine (Rompun; Bayer, Leverkusen, Germany). Body temperature was maintained at 37–38 °C throughout surgery.

**IP catheter implantation**

All animals were equipped with IP catheters for substance administrations. The IP catheters were implanted under isoflurane anesthesia as described previously (30).
end of the catheter was led SC from the neck to a 4 cm midline incision in the abdomen and inserted in the abdominal cavity through a puncture hole. IP catheters ended in the peritoneal cavity and were anchored on the left side of the abdominal wall with silk sutures. The abdominal muscle wall and skin were closed with absorbable sutures (3-0 and 5-0 Vicryl®, respectively; Ethicon, Norderstedt, Germany). Five mg/kg BW carprofen (Rimadyl; E. Gräub, Bern, Switzerland) and 4 mg/kg Borgal 24% were injected for 2 days after surgery for analgesia and infection prophylaxis, respectively. To keep the catheters patent they were flushed every 2-3 days with 0.5 ml 0.9% sterile saline. All rats were allowed to recover from surgery for at least 2 weeks before the experiments started.

**Experiment 1: Effect of OEA on energy intake and meal patterns in rats fed chow or HFD**

About 2 weeks after IP catheter implantation, 26 rats fed either HFD (n = 13) or chow (n = 13) were adapted to the experimental procedure by receiving IP saline bolus injections for 3 days. Cumulative food intake was recorded automatically every 30 s during 24 h with an automated monitoring system as previously described (7). In brief, food cups were placed on balances (XS4001S, Mettler-Toledo, Switzerland) connected to a computer with custom-designed software allowing for meal pattern analysis. Meals were defined as food removals of 0.3 g or more, with the intervals between any two removals (inter-meal interval) being 15 min or more. OEA (Department of Physiology and Pharmacology, Sapienza University of Rome, Rome, Italy) was dissolved in sterile saline/polyethylene glycol/tween 80 (90/5/5, v/v). Food cups were closed 30 min prior to dark onset, and freshly prepared vehicle (2 ml, sterile saline/polyethylene glycol/tween 80 (90/5/5, v/v) or OEA (10 mg/2ml/kg BW) solutions were injected via the IP catheter (Fig. 1). The food cups were opened shortly before dark onset, and the program for
cumulative food intake and meal pattern recordings was started. Vehicle and OEA were administered in a within-subject cross-over design with two intervening days between trials. Continuous food intake recordings after the first trial showed that two intervening days were sufficient to avoid carry-over effects.

Experiment 2: Effect of OEA on plasma metabolite and gut peptide concentrations in HPV and VC

Rats were equipped with VC and HPV catheters for blood samplings in addition to the IP catheter. The VC and HPV catheter headsets were led SC from 2 cm midline interscapular incisions to puncture wounds 1 cm rostral to the incision and the metal ends were exteriorized. The distal ends of the catheters were led subcutaneously to a 4 cm incision in the midline of the abdomen. The HPV catheter was inserted into the ileocolic vein, advanced into the HPV so that it ended 1 to 2 cm distal from the liver, then fixed to the ileocolic vein with sutures and histoacryl glue (Braun, Tuttlingen, Germany), and anchored to the inside of the abdominal wall with silk suture (Silkam, 3/0; Braun, Tuttlingen, Germany). For the VC catheter implantation, the inferior VC was exposed and its surface just rostral to the renal veins was grasped with forceps and lifted a few millimeters. The caudal aspect of the resulting tent-shape was pierced with a 21-gauge syringe needle, and the tip of the tubing inserted 3-4 cm into the vein, ensuring that it ended distal from the liver veins. The tubing was then fixed to the posterior psoas muscle near its entry site with non-absorbable sutures (3-0 Silkam black; Braun, Tuttlingen, Germany), and the abdominal muscle wall and skin were closed with absorbable sutures (3-0 and 5-0 Vicryl, respectively). Immediately after the surgery and for 3 and 2 days thereafter rats were injected SC with 5 mg/kg/day carprofen and 4 mg/kg Borgal 24%, respectively. On the following days, all
catheters were flushed regularly with 0.5 ml 0.9% sterile saline and were filled with 150 µl of 50% heparinized glycerol (200 IU heparin/ml; Heparin, Braun) daily for one week after surgery and every second day thereafter to maintain patency. Catheters were considered patent as long as blood could be withdrawn. After surgery, rats were adapted to either chow (n = 12) or HFD (n = 11) for at least 2 weeks before the start of the experiment. The rats were gradually acclimated to the experimental procedure (13 h food deprivation prior to dark onset and consumption of a test meal within 10 min) at least 3 days prior to the start of experiment. On experimental days, isocaloric test meals (3 g of HFD or 6 g of chow) were offered 30 min before dark onset to 13 h food-deprived rats, and vehicle (2 ml) or OEA solution (10 mg/2ml/kg BW) were freshly prepared and administered via the IP catheter. The size of the test meal was chosen based on previous findings indicating that under these conditions all animals would eat the whole meal within less than 20 min(57). Blood samples (350 µl from each vein at each time point) were taken simultaneously from the HPV and the VC catheters 45 min prior to OEA or vehicle injection (baseline) and 30, 60 and 120 min thereafter. The rats did not have access to food until after the last blood sampling. Blood samples were immediately transferred into tubes containing EDTA (Titriplex 3, Merck, Germany) and Aprotinin (Sigma-Aldrich, Buchs, Switzerland) and then centrifuged (10 min, 10,000 rpm, 4 °C) within 20 min. Plasma samples were aliquoted and stored at −20 °C for later analysis. At the end of the blood-sampling period a blood donor rat, which was fed the same diet as the experimental rats, was anesthetized with isoflurane, and about 9 ml blood was taken by heart puncture. About 1.5 ml blood was given to each experimental rat. This procedure did not affect several metabolites and plasma cortisol in our previous experiments (3). The experiment followed a within-subject cross-over design in which each trial was balanced for all experimental conditions (type of diet and treatment) and with two
intervening days between trials. Plasma levels of non-esterified fatty acids (NEFA) and β-
hydroxybutyrate (BHB) were determined using an enzymatic reaction and measuring the
absorbance of the resulting metabolite using a spectrophotometer (33) (Cobas Mira auto
analyzer, Hoffman La Roche, Switzerland).

The circulating plasma levels of PYY and active GLP-1 were determined using an
immunoassay (Meso Scale Discovery, Gaithersburg, USA) according to the manufacturer’s
protocol. All samples were analyzed in duplicates within one assay, and quality control samples
(pooled plasma) were used in the beginning and the end of each plate. The mean intra- and inter-
assay coefficients of variation were 5.6 and 4.3%, respectively.

Experiment 3: Effect of OEA on mHMG-CoAS2 in the liver and small intestinal mucosa

Ad libitum-fed rats received vehicle or OEA (10 mg/2ml/kg BW) injections via the IP
catheter shortly before dark onset and were sacrificed by decapitation 30 min later without prior
access to food. The left lobe of the liver was removed and immersed immediately in liquid
nitrogen. The intestinal mucosa from the duodenum and jejunum segments was scraped off with
a glass slide and immediately frozen in liquid nitrogen. The liver and small intestinal mucosa
samples were homogenized in lysis buffer (1% Triton X-100, 100 mM Tris pH 7.4, 400 mM
NaCl, 10 mM EDTA) containing a protease inhibitor mixture (EDTA-free protease inhibitor
cocktail tablets, Roche), and protein concentrations were measured with the Bio-Rad protein
assay (Bio-Rad DC protein assay kit, Germany). Twenty to 50 µg of homogenate proteins were
loaded on a 10% SDS-PAGE gel and transferred electrophoretically onto nitrocellulose
membranes (pore size 0.2 µm; Protran, Whatman). Western blot analyses were conducted using
antibodies against mHMG-CoAS2 (1:200; Santa Cruz Biotechnology, sc-33828) and β-actin
(1:3000; Sigma-Aldrich, A2228). Protein bands were visualized using enhanced chemiluminescence (ECL), and the intensity of bands was quantified by densitometry using Image J software. In addition, trunk blood samples were collected from these animals, transferred into EDTA containing tubes, and centrifuged (10 min, 10,000 rpm, 4 °C) to measure BHB and NEFA concentrations as described above.

**Experiment 4: Effect of SDA on the eating-inhibitory effect of OEA**

Twenty-seven rats were subjected to SDA (n = 15) or sham (n = 12) surgery. They were offered unsweetened condensed milk (Migros, Zurich, Switzerland) and moistened chow mash (sweetened milk + powdered chow, 2:3) in addition to ground chow for at least 2 days prior to surgery. The left dorsal (afferent) vagal rootlets at the level of the brain stem and the dorsal (left) esophageal trunk of the vagus in the abdomen were visualized and sectioned as previously described (43, 64). This procedure results in a complete elimination of all vagal afferents from below the diaphragm, while leaving approximately half of the abdominal vagal efferents intact. The sham procedure consisted of similarly exposing the vagal rootlets and abdominal vagus without manipulating them. Five ml of warm Ringer lactate solution (Ri-Lac; B. Braun Medical AG, Sempach, Switzerland) were injected IP after closing the abdomen. The IP catheter was implanted in the same surgery as described above. Carprofen was injected once daily for 3 days for postsurgical analgesia. Solid food was withheld for 12 h after surgery, and only unsweetened condensed milk and moistened chow mash (see above) were offered for 1 week after surgery to facilitate recovery (4).
Sham and SDA rats (BW 450 ± 12 and 442 ± 8 g means ± SEM, on the experimental day) were fed chow *ad libitum* or were food deprived for 16 h prior to OEA administration. Freshly prepared vehicle (2ml) or OEA (10 mg/2ml/kg BW) solutions were injected via the IP catheter 30 min prior to dark onset as described above, in a within-subject cross-over design with two intervening days between trials. Cumulative food intake was recorded for 24 h after administration and meal patterns were analyzed as described (Fig. 1).

At the end of the experiment, SDA was verified functionally by the loss of cholecystokinin (CCK)-induced satiation as previously described (4, 40). After 4 h food deprivation, 4 µg/kg of CCK-8 (Bachem, Budendorf, Switzerland) or saline were injected via the IP catheter shortly before dark onset in a within-subject cross-over design. Chow was offered at dark onset, and 30-min food intake was measured. In sham-operated rats, CCK-8 reduced 30-min food intake 52.1 ± 6% (mean ± SEM) compared to saline. Based on previous studies (4), a minimum of 30% food intake reduction by CCK was set as threshold for complete SDA surgeries. All SDA animals met this criterion.

**Statistical analyses**

Statistical analyses were performed using the SPSS statistical software (SPSS Inc., version 19.0, IL, USA). ANOVA assumption of normality and equal variance were checked and data sets that did not meet these criteria were transformed to square root or logarithms to improve normality. Non-parametric statistical tests were performed for data sets that did not pass the normality criteria even after transformation. Cumulative food intake and meal pattern data were analyzed using ANOVA for repeated-measures with either diet (chow vs. HFD) or surgery (sham
vs. SDA) as between-subject and treatment (vehicle vs. OEA) as within-subject factors as appropriate. Student’s t-test was used for separate comparisons of the percent reductions between two groups at single time points. Also, results from plasma metabolite and gut peptide concentrations were analyzed using a $2 \times 4 \times 2$ (treatment $\times$ time-points$\times$ vein) repeated-measures design; mixed ANOVA for the blood sampling routes, and $2 \times 4 \times 2$ (treatment $\times$ time-points $\times$ diet) for the diet groups. If appropriate, post-hoc pairwise comparisons were performed using the Hochberg variation of the Bonferroni-Holm method (29). All data are expressed as means ± SEM. In all analyses, differences between means were considered significant when $P < 0.05$.

Results

Experiment 1: Effects of OEA on energy intake and meal patterns in rats fed chow or HFD

Confirming previous results (20, 24), IP OEA (10 mg/2ml/kg BW) reduced energy intake 0.5 and 1 h after injection compared with vehicle (Wilcoxon Signed Rank Test, Fig. 2A) in both chow- and HFD-fed rats. The reduction in energy intake after OEA treatment in HFD-fed rats (93.8%) tended to be greater than in chow-fed rats (72.4%) at 0.5 h ($P = 0.07$) and was greater (91.3% vs. 50.2% in HFD- and chow-fed rats, respectively) at 1 h after injection ($P < 0.005$, Fig. 2A). In both diet groups the hypophagic effect of OEA had disappeared 2 h after administration, and there was no further significant treatment difference for the entire duration of the measurements (all $Ps > 0.05$).

The meal pattern analysis revealed that OEA’s eating-inhibitory effect in both diet groups was mainly due to an increase in the latency to eat (Wilcoxon Signed Rank Test, Fig. 2B). Other
meal parameters such as first meal size, first meal duration and first inter-meal interval (the time between the first and the second meal) were not significantly affected by OEA (Mixed ANOVA, $P > 0.05$, Fig. 2B). Moreover, OEA had no effect on meal sizes at later time points or on meal frequency and average 12 or 24 h meal parameters (Data not shown).

Experiment 2: Effects of OEA on plasma metabolite and gut peptide concentrations in HPV and VC

IP OEA injection increased plasma BHB and NEFA levels (all $P_s < 0.05$) compared to vehicle 30 and 60 min after injection in both HPV and VC (Mixed ANOVA, no significant treatment × vein interaction). The increase in plasma BHB level in the HPV was greater in HFD-fed rats than in chow-fed rats at 30 min and in the VC at 30 and 60 min after OEA injection (Bonferroni-Holm test, $P < 0.05$, Fig. 3A), whereas there was no significant difference between diet groups in the plasma NEFA concentration after OEA injection (all $P_s > 0.05$, Fig. 3B). The treatment × diet interaction for the plasma NEFA level was significant ($P < 0.05$) at 30 and 60 min, but the main effect of the diet was not. The ANOVA results for the main effects of OEA, diet, and their interaction were: $F (1, 27) = 26.37$, $P < 0.001$, $F (1, 27) = 1.18$, $P > 0.05$, and $F (1, 27) = 5.26$, $P < 0.05$, respectively. In addition, post-hoc comparisons (Bonferroni-Holm test) showed that OEA produced a substantial increase (81 and 86% in VC and 79 and 84% in HPV, in chow and HFD-fed rats, respectively; all $P_s < 0.05$ vs. baseline; Fig. 3A) in plasma BHB concentrations from baseline to a peak at 30 min in both diet groups. This was followed by a sharp decline to below baseline levels at 120 min. Similarly, HPV and VC plasma NEFA levels increased (all $P_s < 0.05$) from baseline to 30 min and decreased to below baseline levels within
120 min after OEA injection in both diet groups (Fig. 3B). The baseline plasma NEFA and BHB levels did not differ between diet groups ($P > 0.05$).

IP OEA injection decreased HPV plasma PYY concentration (Mixed ANOVA, $P < 0.05$, Fig. 4A) compared to vehicle at 30 and 60 min irrespective of diet, but did not significantly affect VC plasma PYY levels. In chow-fed, but not in HFD-fed rats, the OEA-induced decrease in the HPV PYY level at 30 and 60 min was also significant compared to baseline. Baseline VC and HPV PYY levels in chow-fed rats were higher ($P < 0.05$) than in HFD-fed rats. OEA also reduced the HPV plasma GLP-1 concentration compared to vehicle at 30 and 60 min in HFD-fed rats, but the reduction did not reach statistical significance in the VC (Fig. 4B). In chow-fed rats, however, OEA blunted the increase in HPV and VC plasma GLP-1 level only 30 min after injection. Moreover, in chow-fed, but not in HFD-fed rats, IP OEA injection reduced the further increase in HPV plasma GLP-1 level observed at 30 min compared to baseline (-45 vs. 30 min, $P < 0.05$) (Fig. 4B). In general, plasma PYY and GLP-1 concentrations were lower in the VC than in the HPV (treatment × vein interaction, $Ps < 0.05$) at all time points.

**Experiment 3: Effects of OEA on mHMG-CoAS2 in the liver and small intestinal mucosa**

IP OEA injection increased the protein expression levels of mHMG-CoAS2 in the jejunum of HFD-fed rats (Fig. 5, $P < 0.05$), but not in the jejunum of chow-fed rats or in the duodenum and liver of both diet groups (all $Ps > 0.05$). HFD feeding itself increased the protein expression of mHMG-CoAS2 as compared with chow feeding in the jejunum, but not in the liver ($P < 0.05$). Furthermore, IP OEA injection substantially increased plasma BHB concentration in trunk blood samples collected from *ad libitum* HFD- but not chow-fed rats ($P < 0.005$, Table 1). OEA did not significantly affect plasma NEFA levels in both diet groups ($P > 0.05$).
**Experiment 4: Effect of SDA on the eating-inhibitory effect of OEA**

IP OEA injection reduced food intake similarly in sham and SDA rats, in both *ad libitum*-fed and 16-h food-deprived animals (Wilcoxon Signed Rank Test, Ps<0.05 at various time points; Fig. 6A and B). In *ad libitum*-fed SDA and sham rats OEA reduced food intake primarily during the first hour (Ps < 0.05); thereafter the cumulative food intake difference between OEA and vehicle treated rats was still present to some degree between 3 and 6 hours, but had disappeared in both surgical groups at 24 h after injection (data not shown). In sham and SDA animals, OEA initially (until 2 h) reduced food intake more potently after 16-h food deprivation than in the corresponding *ad libitum*-fed rats (percent food intake reduction in food deprived versus *ad libitum*-fed rats, Student’s *t*-test, sham: 0.5 h: *t* = 4.92, *P* = 0.001; 1 h: *t* = 3.69, *P* = 0.001, SDA: 0.5 h: *t* = 3.48, *P* = 0.002; 1 h: *t* = 3.29, *P* = 0.003). Again, the resulting treatment difference was visible to varying degrees until 6 h after injection in sham rats and until 24 h in SDA rats (data not shown). The meal pattern analysis revealed that OEA reduced food intake in all groups primarily by prolonging the latency to eat with *ad libitum* access to food (Wilcoxon Signed Ranks Test, sham: *Z* = 2.8, *P* = 0.004; SDA: *Z* = 2.6, *P* = 0.008)(Fig. 7A) as well as after 16-h food deprivation (Wilcoxon Signed Ranks Test, sham: *Z* = 2.8, *P* = 0.005; SDA: *Z* = 3.05, *P* = 0.002). In the latter case the effect was more pronounced in SDA than in sham rats (*P* = 0.01, Fig. 7B). In addition, IP OEA reduced the first meal size and the duration of the first inter-meal interval only in 16-h food deprived sham and SDA rats, and in this case the effect was weaker in SDA rats than in sham rats. Moreover, SDA rats treated with OEA showed a longer first meal duration than sham rats treated with OEA after food deprivation (Wilcoxon Signed Rank test, *P* < 0.05, Fig. 7B), but no differences were observed for the same parameter in *ad libitum*-feeding
rats. All other meal pattern parameters were not significantly affected by OEA in either surgical
group (Mixed ANOVA, no significant surgery × treatment interaction, Fig. 7A, B). The analysis
of meal patterns beyond 2h did not reveal significant differences in any meal pattern parameters
between vehicle and OEA treated rats in both surgery groups.

Discussion

In the present study, we examined the effects of IP-injected OEA on 1) energy intake in
rats fed either chow or HFD, 2) indicators of peripheral FAO and ketogenesis, 3) the meal-
induced release of GLP-1 and PYY, and 4) the role of abdominal vagal afferents in mediating the
observed inhibition of eating by OEA.

Consistent with earlier studies (24, 48), we found that OEA reduced food intake in chow-
and HFD-fed rats mainly by delaying meal onset rather than by reducing meal size. The observed
increase in circulating BHB levels indicates that OEA stimulated FAO and ketogenesis, and the
enhanced expression level of mitochondrial HMG-CoAS2 in the jejunum of HFD-fed rats
suggests that some of this stimulation of FAO and ketogenesis might occur in the small intestine.
Previous findings suggest that intestinal FAO and ketogenesis can affect eating (30, 57). Our
results are therefore consistent with the idea that a stimulation of intestinal FAO and ketogenesis
may also contribute to the satiety effect of OEA (34), at least under certain conditions. In
addition to inhibiting eating, OEA counteracted the meal-induced release of GLP-1 and PYY in
both diet groups, which indicates that these hormones are not involved in mediating OEA’s
satiety effect. Finally, in contrast to previous reports (48, 63), we found that intact abdominal
vagal afferents are not necessary for the satiety effect of OEA under our experimental conditions.
In ad libitum-fed rats, the eating-inhibitory effect of OEA was more pronounced with the HFD than with chow. This is remarkable because prolonged consumption of HFD decreases the eating-inhibitory effect of several gut peptides (54, 66). Interestingly, we recently found that HFD-feeding altered the sensitivity of the central nervous system (CNS) response to IP administered OEA in a site-specific fashion. While hindbrain activation by IP OEA was reduced in HFD-fed rats, the activation of the hypothalamic PVN was enhanced (50). The latter effect may be related to OEA’s stronger eating-inhibitory effect in HFD-fed rats observed here because PVN OT has been implicated in the inhibition of eating by OEA (23). It has been shown that chronic HFD consumption can also reduce the levels of endogenous OEA in the small intestine (5, 16). Perhaps the reduced endogenous levels caused a compensatory increase in the sensitivity to OEA, which contributed to the enhanced PVN activation. Rapid degradation of OEA in plasma and proximal intestine, where OEA-generating and hydrolyzing enzymes, i.e., N-acyl-phosphatidylethanolamine phospholipase (NAPE-PLD) and fatty-acid amide hydrolase (FAAH), respectively, are highly expressed (44), may contribute to the short duration of OEA’s inhibition of eating observed in the first experiment of the present study. The observed variability in the duration of the OEA-induced reduction in cumulative food intake (short duration in Experiment 1 vs. longer duration in Experiment 4) is consistent with the variable durations of the OEA effect in ad libitum-fed animals reported before (short duration: (24, 46); long duration: (20, 48)) and may be one example of the generally observed variability of food intake data in response to exogenous substances given under different experimental conditions (69).

Consistent with previous reports (24, 48), OEA reduced food intake in rats fed chow or a HFD ad libitum by delaying the onset of the first meal without affecting its size, indicating that OEA enhanced satiety (i.e., delayed the occurrence of hunger) rather than satiation (i.e.,
promoting meal termination). The additional reduction of the first meal size by OEA in food-deprived rats suggests, however, that OEA can also affect meal size, perhaps when larger meals are consumed as after food deprivation. In fact, food deprivation almost doubled meal size in control animals compared to ad libitum feeding (5.9 ± 0.4 vs. 3.2 ± 0.6 g). Interestingly, FAO inhibitors or stimulators usually affect eating in rodents by changing meal frequency rather than meal size (30, 36, 57). The observed OEA-induced changes in meal patterns are therefore consistent with a role of enhanced FAO and ketogenesis in the satiety effect of OEA.

Higher doses of OEA (20-25 mg/kg BW) can evoke visceral pain in mice through activation of the capsaicin receptor, transient receptor potential vanilloid-1 (TRPV1) (2, 65). This raises the possibility that the eating-inhibitory effect of OEA might be due to visceral pain. Using the lower OEA dose (10 mg/kg BW), however, OEA reduced rather than enhanced nociceptive responses (61) and, when administered to mice with a genetic deletion of TRPV1, OEA still inhibited eating (38). These observations suggest that an induction of visceral pain is probably not crucial for the eating-inhibitory effect of OEA reported here.

OEA has been shown to stimulate lipolysis and peripheral FAO by activating PPAR-α (25, 70), but the site where OEA acts to stimulate FAO is unclear. To further investigate this aspect we measured plasma metabolites from blood collected from the HPV and VC. Regardless of the blood vessel, plasma BHB and NEFA levels increased 30 and 60 min after OEA injection in both chow- and HFD-fed rats, and the increase was more pronounced in HFD-fed rats. Fasting triggers lipolysis in adipocytes and, hence, leads to an increase in circulating NEFA and glycerol, which fuel oxidative metabolism in energy demanding tissues such as the liver. With access to food, however, circulating NEFA, glycerol and ketone bodies usually decrease, mainly due to the
inhibitory action of insulin on lipolysis and the concomitant stimulation of lipogenesis, which in turn inhibits FAO (41, 45). Therefore, the increase in circulating NEFA levels observed after OEA injection and access to a test meal in our study is surprising and supports previous assumptions of a stimulatory action of OEA on lipolysis (25, 70). Catecholamines might be involved in the lipolytic effect of OEA (32), but to our knowledge, no evidence is available regarding the effect of OEA on circulating catecholamines. The HPV drains blood from the intestine, whereas the VC collects blood from several organs including the liver. The surgical procedure employed ensured that the tips of the VC catheters were located distal from the entry of the hepatic veins into the VC, i.e., the VC blood samples also contained the outflow from the liver. Therefore, the parallel and similar increase in HPV and VC BHB levels after OEA injection does not disclose the site of origin of the circulating BHB. The observed increase in the protein expression of the key-ketogenetic enzyme, the mitochondrial HMG-CoAS2 (28), specifically in the jejunum and not in the liver or other parts of the small intestine suggests, however, that at least some of the BHB originated from the jejunum. The increases in circulating BHB levels and in the expression of mHMG-CoAS2 were greater in HFD-fed rats than in chow-fed rats. This might reflect an increase in intestinal oxidation of dietary fatty acids and, hence, enhanced intestinal ketogenesis with HFD feeding. In line with this assumption, we previously showed that IP administration of the potent synthetic PPAR-α agonist, Wy-14643, enhanced FAO and the formation of ketone bodies mainly in the jejunum and not in the liver of HFD-fed rats (30).

The changes in the circulating BHB levels and enzyme protein expression following OEA injection, both, occurred within the same time frame as the eating response and with a significantly stronger effect of OEA in HFD-fed rats than in chow-fed rats. Because the
inhibition of FAO has been shown to be associated with a stimulation of eating (56), and because peripheral (35) or central (15, 52) administration of BHB reduced food intake in rats, an increase in peripheral FAO and ketogenesis may inhibit eating. Moreover, infusion of BHB into the third ventricle reduced food intake by prolonging the inter-meal interval rather than reducing meal size (52). It seems therefore reasonable to hypothesize that the increase in circulating ketone bodies, independent of their origin, could contribute to the eating-inhibitory effect of OEA in HFD-fed rats. Further studies are necessary to critically examine whether there is such a causal relationship.

It should also be noted that BHB levels in the HPV and VC were measured in animals that had access to a test meal after 13 h food deprivation. In contrast, the analysis of mHMG-CoAS2 expression was performed in rats fed ad libitum and sacrificed for tissue collection and BHB measurements from trunk blood 30 min after OEA administration. In this situation OEA increased the plasma BHB levels only in HFD-fed animals, but had no effect in chow-fed rats. Therefore, the failure of OEA to affect BHB levels in chow-fed rats without prior test meal may be related to the absence of an increase in mHMG-CoAS2 expression observed in the jejunum under the same feeding condition. Thus, OEA seems to limit food intake in chow-fed rats independent of an overt stimulation of intestinal FAO and ketogenesis. Therefore, the exact peripheral mechanisms underlying the inhibition of eating by OEA under different conditions still remain to be identified.

We measured plasma levels of PYY and GLP-1 to examine whether these peptides might contribute to the inhibition of eating by OEA. The higher postprandial PYY and GLP-1 levels in both diet groups were expected because these peptides are released in response to a meal (13). Also, the PYY and GLP-1 levels were generally higher in the HPV than in the VC because the
HPV drains the blood from the intestine, where these peptides are secreted by the enteroendocrine L-cells. Interestingly, IP injection of OEA decreased the meal-induced release of PYY and active GLP-1, suggesting that GLP-1 and PYY do not contribute to the eating-inhibitory effect of OEA. This interpretation is further strengthened by the finding that OEA delayed the onset of eating (satiety effect), whereas GLP-1 and PYY usually reduce meal size (satiation effect) (51, 60). In fact, the lack of effect of OEA on meal size in *ad libitum*-fed rats might partly be due to the inhibitory effect of OEA on the release of gut peptides. OEA has been reported to induce the release of GLP-1 in an enteroendocrine cell model (GLUTag cells) (37) and also following intraluminal OEA administration in anesthetized rats (12, 37). In another study (46), OEA had no effect on gut peptide levels in trunk blood plasma from freely-feeding rats at different time points following OEA administration. The discrepant findings are likely due to methodological differences between these studies. We collected blood at various time points from two different veins of non-anesthetized animals offered a test meal, whereas in the other studies blood samples were collected either from the carotid artery or whole body and, at times, in anesthesia. Also, consistent with the inhibitory effect of OEA on the meal-induced release of GLP-1 *in vivo*, preliminary results of cell culture experiments in GLUTag cells showed that OEA inhibited oleic acid-induced GLP-1 release *in vitro* (Clara et al., in preparation). Overall, the feeding state of the animal seems to be crucial for the effect of OEA on GLP-1 release. Although the mechanisms of the effect of OEA on GLP-1 and PYY release are unknown, it is worth mentioning that norepinephrine inhibits GLP-1 secretion (27). It is therefore possible that OEA attenuated GLP-1 and PYY release through an increase in circulating catecholamine levels. Further experiments will be necessary to clarify the exact mechanism and generality of this effect.
Probably the most important finding of our study in the light of previous reports is that OEA did not require intact abdominal vagal afferents to reduce food intake. This is the first report of the effect of peripherally administered OEA in SDA rats. It has been reported that total subdiaphragmatic vagotomy (TVX) as well as capsaicin pre-treatment prevented the satiety effect of OEA, which prompted the interpretation that OEA’s effect on eating is mediated by vagal sensory fibers (20, 48). We used SDA because it eliminates all abdominal vagal afferents while sparing approximately half of the efferents (43, 64). Unlike TVX, the SDA procedure therefore allows to distinguish between the specific effects of afferent versus efferent signaling. Moreover, SDA has fewer side effects that might interfere with the normal control of food intake or affect the results of the OEA challenge. Because in TVX both vagal afferents and efferents are removed, the normal release of peripheral humoral signals to the CNS changes, and it is difficult to differentiate between the sensory mechanisms and the gastrointestinal secretory and motor dysfunctions induced by TVX (22). On the contrary, in SDA behavioral and physiological disturbances including altered gastric function and malnutrition that may interfere with normal eating behavior in TVX rats are minimal (14, 31). We functionally confirmed the completeness of the SDA lesion by the loss of CCK satiation at the end of the experiments, which makes it very unlikely that the observed results were due to an incomplete surgical disconnection of vagal afferents. Moreover, we took great care during the post-surgical period to minimize any factors that might affect normal eating behavior, and did not perform the experiment before the animals had completely recovered from surgery. The success of the total vagotomy procedure or lack of regeneration of vagal afferents were not reported in the previous study by Fu and colleagues (20). Also, because the TVX animals were purchased and not operated in the investigators’ laboratory, detailed information regarding the TVX animals’ post-operative body weight
development and well-being could not be provided. Thus, small procedural differences or surgical side effects might have contributed to the previously reported antagonism of the eating-inhibitory effect of OEA by TVX. There is one report in which TVX failed to block the eating-inhibition by OEA (42), but in this study the animals consumed a 15% sucrose solution instead of solid food, which makes a direct comparison questionable because of potentially confounding factors such as hedonics. Surgical side effects, however, cannot explain the lack of eating-inhibitory effect of OEA after capsaicin lesions of small, unmyelinated afferents (20, 48). Systemic capsaicin treatment selectively removes only unmyelinated visceral sensory neurons in vagal and spinal afferents without an effect on efferent neurons (9, 17), i.e., the lesion is not specific for vagal afferents. Also, because up to 20% of visceral neurons are myelinated (8, 47), capsaicin leaves a significant number of afferents intact. Probably the best method for selective long-term removal of vagal sensitive fibers by capsaicin is a localized application near the peripheral vagal trunks. In the reported studies capsaicin administration to eliminate afferent sensory fibers is unclear and depending on the administration route, capsaicin can damage somatosensory neurons in addition to viscerosensory fibers (10, 53). Thus, one interpretation accommodating our present SDA and the previous capsaicin data is that unmyelinated spinal visceral afferents mediate the eating-inhibitory effect of OEA.

Interestingly, a recent study reported that surgical sympathetic superior mesenteric ganglionectomy (SGX) attenuated the food-induced synthesis of OEA in the jejunum (19). Also, SGX increased average meal frequency and decreased average meal size, and peripheral OEA administration normalized the meal patterns in SGX animals. These findings indicate that the normal meal patterns are partly controlled by the sympathetic nervous system in the intestine, as is the release of OEA, but that OEA can alter meal patterns independent of an intact sympathetic
innervation. Unfortunately the cumulative food intake after OEA administration in SGX rats was not reported, but the most parsimonious interpretation of these findings together with the here reported failure of SDA to block the satiety effect of OEA is that OEA reduces food intake either by direct action in the brain or by triggering the release of another peripheral humoral mediator. Although it was shown that OEA does not reduce food intake when administered by ICV infusion in rats (48), this does not necessarily exclude an action on circumventricular organs such as the area postrema (AP). In fact, we recently reported that IP OEA causes a significant activation of the AP and the subpostremal nucleus of the solitary tract (50). As previous studies showed that higher doses of OEA may cause nonspecific side effects (46), we performed our experiments with 10 mg/kg, a dose that still reliably inhibits eating (24, 48). IP administration of this dose (10 mg/kg) increased the OEA plasma level to 0.13 µg/ml (325.5 pmol/ml) in rats (6). In contrast, the plasma OEA level in response to a meal was only 5.9 pmol/ml (18). Therefore, it is possible that this supraphysiological circulating levels of OEA after IP administration may reach the AP and induce some or all of the c-fos activation in this region. Further experiments should therefore critically examine the possible role of the AP as a site of action for peripherally administered OEA. On the other hand, the fact that dietary fat increases the OEA level only in the small intestine suggests a local action of OEA in the intestine (18). As alluded to above, other humoral signals, perhaps related to an increase in peripheral FAO, may contribute to the satiety effect of OEA at least with fat-enriched diets.

In general, the effect of OEA on food intake observed in this experiment mirrored the effects reported before (20, 24), i.e., it was more pronounced after food deprivation than in ad libitum-fed animals. Also the effects of OEA on meal patterns in SDA rats were very similar to the effects observed in sham rats and in Experiment 1, with two notable exceptions after 16 h
food deprivation: 1) in this situation the effect of OEA on the latency to eat was more
pronounced in SDA rats than in sham rats, indicating that SDA removed some eating-stimulatory
signal that normally counteracted the satiety effect of OEA in food-deprived sham rats. The
nature of this signal remains to be identified. 2) The effect of OEA on first meal size after food
depprivation was less pronounced in SDA rats than in sham rats. As discussed above, this may be
due to the greater meal size in sham versus SDA rats, i.e., to the fact that the potency of OEA to
reduce meal size may be directly related to meal size.

Perspectives and Significance

Our results indicate that neither GLP-1 nor PYY or abdominal vagal afferent signaling is
crucial for the satiety effect of peripherally administered OEA in rats. Whether OEA acts directly
on the brain (e.g., on the AP) to reduce food intake or whether other humoral mediators are
involved requires further investigations. Such humoral mediators may be related to a stimulation
of intestinal oxidation of dietary fat-derived fatty acids. In this context, it is interesting that BHB
can regulate sympathetic nervous system activity directly by binding to the G-protein-coupled
receptor 41 (GPR41) expressed in celiac-superior mesenteric ganglia (67). Moreover, the
expression of PPAR-α in the dorsal root ganglia (DRG) that contain the cell bodies of sensory
neurons in the spinal cord even raises the possibility that OEA may directly activate neuronal
PPAR-α to regulate the expression of genes encoding FA metabolism enzymes (39). Clearly,
further studies are necessary to clarify the exact mechanisms of OEA’s action.
References


Figure Legends:

**Fig. 1:** Experimental design of the food intake recording experiment. Rats had *ad libitum* access to chow or HFD. Thirty min before injection (10:00) all food cups were closed to be refilled and to start the automatic recording program. At 10:30, rats received an IP OEA (10 mg/2 ml/kg BW) or vehicle (saline/polyethylene glycol/tween 80) injection. Food access was reestablished immediately after injections (shortly before dark onset) and energy intake was recorded for 24 h.

**Fig. 2:** IP OEA injection reduced energy intake in chow- and HFD-fed rats. (A) Effect of OEA on energy intake. Data were analyzed for individual time points by the Wilcoxon Signed Rank test, and significant differences between diet groups were followed-up by the Mann-Whitney U test (*P > 0.05*, not significant). (B) Effect of OEA on meal patterns. Data for latency to eat were analyzed with the Wilcoxon Signed Rank test; other meal pattern parameters were analyzed with a 2 × 2 mixed ANOVA (with diet groups as between-subject and treatment as within-subject factors). *P < 0.05, **P < 0.005* vs. vehicle in the same diet group, ∂P < 0.05 vs. chow in the OEA treatment group. Data are presented as means ± SEM.

**Fig. 3:** IP OEA injection increased β-hydroxybutyrate (BHB) (A) and non-esterified fatty acid (NEFA) (B) levels in chow- and HFD-fed rats in hepatic portal vein (HPV) and vena cava (VC). Rats were food deprived for 13 h and re-fed before injections for 10 min with a test meal of either 6 g chow- or 3 g HFD. IP OEA or vehicle was injected just prior to dark onset. Data were analyzed with a 2 × 4 × 2 mixed ANOVA with treatment (OEA or vehicle) and time points (-45, 30, 60 and 120 min) as within-subject factors, and diet (chow or HFD) as between-subject factor. Significant differences were followed-up by Bonferroni-Holm *post-hoc* comparisons. $P < 0.05$
Fig. 4: IP OEA injection reduced HPV plasma PYY (A) and HPV and VC plasma GLP-1 (B) levels in chow- and HFD-fed rats. OEA reduced plasma PYY levels at 30 and 60 min after injection in chow- and HFD-fed rats (left panel) in the HPV, but not in the VC (right panel). Also, OEA reduced HPV plasma GLP-1 levels at 30 min in chow-fed rats and at 30 and 60 min in HFD-fed rats (left panel). Finally, OEA reduced the VC plasma GLP-1 level only in chow-fed rats at 30 min after injection (right panel). Significant differences were followed-up by Bonferroni-Holm post-hoc comparisons. *P < 0.05 vs. vehicle in the same diet group, #P < 0.05 vs. chow. Data are expressed as means ± SEM.

Fig. 5: IP OEA injection increased the expression of the key ketogenic enzyme mHMG-CoA S2 in the jejunum. The bottom panels show a representative western blot of the liver and intestinal mucosa from chow and HFD-fed rats 30 min after IP OEA (10 mg/2 ml/kg BW) injection. The proteins were immunoblotted with anti-mHMG-CoA S2 antibody and were normalized to β-actin (as control). The values presented in the histograms are relative to the vehicle chow condition (100%). *P < 0.05 vs. vehicle in the same diet group, #P < 0.05 vs. chow in the same treatment group. Data are expressed as means ± SEM.

Fig. 6: IP OEA injection reduced food intake in sham (n = 12, left panels) and SDA (n = 15, right panels) rats during the first hour following injection with both ad libitum-feeding (A) and 16-h food-deprivation (B). The reduction in cumulative food intake was still present to some degree between 3 and 6 hours in both feeding conditions. Data were analyzed for individual time points by the Wilcoxon Signed Rank test. No significant differences in the food intake reduction
by OEA were observed between SDA and Sham rats (Mann-Whitney U test). *P < 0.05, **P < 0.005 vs. vehicle in the same surgery group; #P < 0.05 vs. 16-h food-deprived rats in the same treatment group. Data are presented as means ± SEM.

**Fig. 7:** IP OEA injection increased the latency to eat in both sham and SDA rats compared with vehicle injection with *ad-libitum*-feeding (A) and after 16-h food-deprivation, where it also reduced the first meal size and the duration of the first inter-meal interval (B). Data were analyzed with the Wilcoxon Signed Rank test, and significant differences between surgery groups (sham and SDA) were followed-up by Mann-Whitney U test (P > 0.05, not significant). *P < 0.05, **P < 0.005 vs. vehicle-injected rats of the same surgery group; #P < 0.05 vs. sham-operated rats that received the same treatment. Data are presented as means ± SEM.
**Table 1:** Effect of IP OEA injection on plasma levels of β-hydroxybutyrate (BHB) and non-esterified fatty acids (NEFA)

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<td>(µmol/L)</td>
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Ad libitum-fed rats (n = 6/diet) received OEA (10 mg/2 ml/kg) or vehicle (2 ml/kg) 30 min prior to dark onset. Food cups were closed and trunk blood samples were taken 30 min after injection. Data are expressed as means ± SEM. **P < 0.005 vs. vehicle in the same diet group (Wilcoxon Signed Rank test); #P < 0.05 vs. chow in the same treatment group (Mann-Whitney U test).
Acknowledgement:

This work was supported by Swiss National Science Foundation grant 31003A_130665 (W.L.)
Dark Phase

Veh or OEA infusion

11:00

Light Phase

11:00

Food access

10:00

Closing food cups

10:30

Food intake recording
(A) Cumulative food intake (g) over time after infusion (hours) in the Ad libitum state.

(B) Cumulative food intake (g) over time after infusion (hours) in the Food-deprived state.

- Sham-Veh
- Sham-OEA
- SDA-Veh
- SDA-OEA

Significance levels:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001

Notations:
- # p < 0.1
- ⬤ p < 0.001
(A)

Latency to eat (min)

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Inter meal interval (min)

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(B)

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Inter meal interval (min)

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* P = 0.059