Title
An anorexic lipid mediator regulated by feeding

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Oleylethanolamide (OEA) is a natural analogue of the endogenous cannabinoid anandamide. Like anandamide, OEA is produced in cells in a stimulus-dependent manner and is rapidly eliminated by enzymatic hydrolysis, suggesting a function in cellular signalling. However, OEA does not activate cannabinoid receptors and its biological functions are still unknown. Here we show that, in rats, food deprivation markedly reduces OEA biosynthesis in the small intestine. Administration of OEA causes a potent and persistent decrease in food intake and gain in body mass. This anorexic effect is behaviourally selective and is associated with the discrete activation of brain regions (the paraventricular hypothalamic nucleus and the nucleus of the solitary tract) involved in the control of satiety. OEA does not affect food intake when injected into the brain ventricles, and its anorexic actions are prevented when peripheral sensory fibres are removed by treatment with capsaicin. These results indicate that OEA is a lipid mediator involved in the peripheral regulation of feeding.

Fatty acid ethanolamides (FAEs) are unusual components of animal and plant lipids that are synthesized in response to a variety of physiological and pathological stimuli, including activation of neurotransmitter receptors in rat brain neurons and exposure to metabolic stressors in mouse epidermal cells. The primary mechanism underlying FAE generation in mammalian tissues involves two concerted biochemical reactions: cleavage of the membrane phospholipid N-acyl phosphatidylethanolamine (NAPE), catalysed by an unknown phospholipase D; and NAPE re-synthesis, mediated by an N-acyltransferase (NAT) that is regulated by calcium ions and cyclic AMP. After release, FAEs are transported back into cells and eventually broken down to fatty acid and ethanolamine by an intracellular fatty acid amide hydrolase (FAAH) and NAPE-acyltransferase (NAT). This animal cells release FAEs in a stimulus-dependent manner suggests that these compounds may participate in cell-to-cell communication. Further support for this idea comes from the discovery of the polyunsaturated FAE anandamide (arachidonyl-ethanolamide) as an endogenous ligand for cannabinoid receptors. However, the pharmacological effects of saturated or mono-unsaturated FAEs such as OEA cannot be accounted for by activation of any of the known cannabinoid receptor subtypes, and the biological roles of these compounds remain elusive.

Because anandamide may regulate feeding, we investigated the effects of OEA on food intake in rats. Systemic administration of OEA caused a dose- and time-dependent suppression of food consumption (Fig. 1). Under the same conditions anandamide and oleic acid had no effect, palmitylethanolamide was significantly less potent than OEA and elaidylethanolamide was similar in potency to OEA (Fig. 1a). These results indicate that OEA reduces eating in a structurally selective manner and suggest that the molecular requisites for this effect are distinct from those involved in the interaction of anandamide with recognized cannabinoid targets. In further support of this idea, CB1 and CB2 cannabinoid antagonists (SR141716A and SR144528, respectively) did not affect OEA hypophagia (data not shown).

It is possible that OEA reduced food intake by inducing a non-specific state of behavioural suppression. If this were the case, OEA should cause conditioned taste aversion, which can be readily provoked in rats by administration of lithium chloride (Fig. 1c). By contrast, a high dose of OEA had little effect in this assay, suggesting that the compound is not aversive (Fig. 1c). Furthermore, OEA did not produce anxiety-like symptoms, did not affect water intake. Asterisk, P < 0.05; n = 8–12 per group.

**Figure 1** OEA suppresses food intake in rats deprived of food for 24 h. a, Dose-dependent effects of i.p. OEA (open squares), elaidylethanolamide (open circles), palmitylethanolamide (triangles), oleic acid (filled squares) and anandamide (filled circles) after the first 60 min of food presentation. Food intake after injection of vehicle (70% DMSO in saline, 1 ml kg⁻¹ i.p.) was 7.1 ± 0.5 g per animal (100%). b, Time course of the hypogadic effects of i.p. OEA (20 mg kg⁻¹) (squares) or vehicle (diamonds) on food intake. c, Effects of i.p. vehicle (V, lithium chloride (LiCl); 0.4 M, 7.5 ml kg⁻¹) or OEA (20 mg kg⁻¹) on conditioned taste aversion. Open bars, water intake; solid bars, saccharin intake. d, Effects of i.p. vehicle (V) or OEA (5 or 20 mg kg⁻¹) on horizontal activity in an open field, assessed on habituated animals. Asterisk, P < 0.05; n = 8–12 per group.

**Figure 2** Effects of subchronic OEA administration on food intake and body mass. a, Effects of i.p. vehicle (V; 5% Tween 80 with 5% propylene glycol in sterile saline; open bars) or OEA (5 or 20 mg kg⁻¹), once per day; solid bars) on cumulative food intake. b, Time course of the effects of OEA (triangles) or vehicle (squares) on body mass gain. The arrow marks the administration of vehicle or OEA. c, Effects of vehicle or OEA on cumulative water intake. Asterisk, P < 0.05; n = 10 per group.
The pair-fed group received a total of 116 g of food, identical to the amount consumed by the OEA group.

"P<0.01 versus control.

Table 1 Effects of OEA and paired feeding on body mass gain

<table>
<thead>
<tr>
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<th>Cumulative mass gain (g)</th>
<th>Food intake (g)</th>
<th>Water intake (ml)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>15.6±2.3</td>
<td>143.2±10.3</td>
</tr>
<tr>
<td>OEA 11 mg/kg</td>
<td>11</td>
<td>115.8±4.2*</td>
<td>174.7±9.1</td>
</tr>
<tr>
<td>OEA 20 mg/kg</td>
<td>11</td>
<td>116</td>
<td>192.8±12.7</td>
</tr>
<tr>
<td>OEA 5 mg/kg</td>
<td>11</td>
<td>4.3±1.4†</td>
<td>115.7±12.7</td>
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<tr>
<td>Vehicle</td>
<td>10</td>
<td>15.6±2.3</td>
<td>143.2±10.3</td>
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<tr>
<td>Oleic acid</td>
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<td>115.8±4.2*</td>
<td>174.7±9.1</td>
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<tr>
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<td>115.7±12.7</td>
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Figure 3 Role of peripheral sensory fibres in OEA-induced hypophagia. Effects of i.p. vehicle (V), OEA (5 mg kg⁻¹), CCK-8 (10 μg kg⁻¹) and CP-93129 (1 mg kg⁻¹), a centrally active 5-HT₄ receptor agonist, on food intake in control rats (a–c) and capsaicin-treated rats (d–f). a, d, Food intake; b, e, Horizontal activity in an open field; c, f, Water intake. Asterisk, P<0.05; n=8–12 per group.

Figure 4 OEA increases c-fos mRNA expression in brain regions associated with feeding behaviour. a. Pseudocolour images of autoradiograms showing that i.p. OEA (right) elicits a profound and selective increase in c-fos mRNA expression in the paraventricular (PVN) and supraoptic (SO) hypothalamic nuclei, as assessed by in situ hybridization. A representative section from a vehicle-treated rat is shown (left). Labelling densities are indicated by colour: (low) blue–green–yellow–red (high). b. Quantification of c-fos mRNA labelling in forebrain regions (PVN, SO, arcuate (Arc), layer II piniform cortex (Pir), central nucleus of the amygdala (Ce) and S1 forelimb cortex (S1FL)) of rats treated with i.p. vehicle, OEA or oleic acid. c. Autoradiogram showing elevated c-fos mRNA expression in the NST in an OEA-treated rat. The inset shows c-fos antisense RNA labelling in the NST (red) identified by its localization relative to adjacent effferent nuclei (hypoglossal and dorsal motor nucleus of the vagus), which express choline acetyl transferase (ChAT) mRNA (purple). d. OEA increases c-fos mRNA expression in the NST, but not in the hypoglossal nucleus (HgN). Double asterisk, P<0.0001; n=5 per group.

Although potent when administered peripherally, OEA is ineffective after intracerebroventricular injection (see Supplementary Information), leading us to hypothesize that its primary sites of action are located outside the central nervous system (CNS). To test this idea, we destroyed peripheral sensory fibres by treating adult animals with capsicain. Capsaicin-treated rats failed to respond to systemically administered cholecystokinin-8 (CCK-8) (Fig. 3a, d) and drank more water than controls (Fig. 3c, f), two indications that the neurotoxin had removed sensory afferents. Capsaicin-treated animals also failed to become hypophagic in response to OEA (5 mg kg⁻¹ i.p.), but responded normally to the compound CP-93129, which targets brain 5-HT₁B receptors (Fig. 3a, d). These findings support the hypothesis that OEA reduces food intake by acting at a peripheral site and that sensory fibres are required for this effect. Interestingly, the ability of a high OEA dose (20 mg kg⁻¹ i.p.) to reduce motor activity was not affected by capsicain treatment (Fig. 3b, e), suggesting that OEA modulates eating and movement through distinct mechanisms.

Peripheral inputs related to appetite suppression recruit CNS structures such as the nucleus of the solitary tract (NST) in the
brainstem and the paraventricular (PVN) nucleus in the hypothalamus. To identify brain pathways engaged during OEA-evoked hypophagia, we mapped messenger RNA levels for the activity regulated gene c-fos by in situ hybridization after systemic administration of OEA, oleic acid or vehicle. When compared with controls, OEA (10 mg kg\(^{-1}\), i.p.) evoked a highly localized increase in c-fos mRNA levels in the PVN, supraoptic nucleus (Fig. 4a) and NST (Fig. 4c). This enhancement was specific, insofar as c-fos expression in other brain regions was not significantly affected by OEA (Fig. 4b, d). The stimulation of c-fos expression in the NST (which processes vagal sensory inputs to the CNS) and the PVN (a primary site for the coordination of central catabolic signals), is consistent with a role for OEA as a peripheral regulator of feeding behaviour.

The anorectic effects of OEA are reminiscent of those produced by gut peptides such as CCK. Because the release of CCK from duodenum is regulated by nutrients, we studied the impact of feeding behaviour. According to this model, food intake may decrease in rats deprived of food for 24 h (ref. 27) that were habituated to the experimental setting. We administered drugs 15 min before food presentation. For subchronic experiments, freely fed rats were deprived of food for two days. On day 3, we divided the animals into two equal groups and gave them daily injections of vehicle or OEA (5 mg kg\(^{-1}\) i.p.). Capsaicin was administered in 10% Tween 80, 10% ethanol and 80% saline; SR141716A, SR144528, CCK-8 and CP-93129 in 5% Tween 80, 5% propylene glycol and 90% saline (1 ml kg\(^{-1}\), i.p.).

**Enzyme assays**

In all biochemical experiments, rats were killed and tissues collected between 14:00 and 16:00. Microsome fractions were prepared as described. NA assays were performed using 1,2-dihexadecanoyl-sn-glycerophosphocholine as a substrate (100 mCi mmol\(^{-1}\), Amer sham) under conditions that were linear with time and protein concentrations. NA assays were performed under linear conditions according to ref. 24, except that [\(^{3}\)H]anandamide (arachidonyl-1-[\(^{3}\)H]ethanamide; 60 Ci mmol\(^{-1}\); ABC) was included as a substrate and radioactivity was measured in the aqueous phase after chloroform extraction.

**HPLC/MS analyses**

FAEs were extracted from tissues with a methanol–chloroform mixture and fractionated by column chromatography. FAEs were quantified by HPLC/MS, with an isotope dilution method.

**Blood chemistry**

We used commercial kits to measure glucose, insulin, leptin and other plasma metabolites and enzymes (Sigma and Linco Research). Plasma prolactin, corticosterone and luteinizing hormone were quantified by radioimmunoassay.

**Feeding experiments**

For acute experiments, we measured food intake in rats deprived of food for 24 h (ref. 27) that were habituated to the experimental setting. We administered drugs 15 min before food presentation. For subchronic experiments, freely fed rats received vehicle injections for two days. On day 3, we divided the animals into two equal groups and gave them daily injections of vehicle or OEA (5 mg kg\(^{-1}\) i.p.) for seven consecutive days, while measuring body mass, food intake and water intake. A third group of rats (pair fed) received an amount of food identical to that consumed by the OEA group. On day 9 of the study, the animals were killed and plasma was collected for biochemical analyses.

**Conditioned taste aversion**

Rats were deprived of water for 24 h and then accustomed to drinking from a graduated bottle during a 30-min test period for 4 days. On day 5, water was substituted with 0.1% saccharin solution and, 30 min later, the animals received injections of vehicle, OEA (20 mg kg\(^{-1}\)) or lithium chloride (0.4 M, 7.5 ml kg\(^{-1}\)). During the following 2 days, water consumption was recorded over 30-min test periods. The animals were then presented with water or saccharin, and drinking was measured.

**Operant responses for food**

Rats were trained to press a lever for food on a fixed ratio (FR) 1 schedule of reinforcement, while restricted to 20 g of food per rat per day. Once a stable response was achieved, the animals were trained to acquire an FR5, time-out 2-min schedule of food reinforcement and kept with limited access to food. When a stable baseline was obtained, the animals were used to test the effects of vehicle or OEA (5 or 20 mg kg\(^{-1}\)) administered 15 min before lever presentation. Tests lasted 60 min.

**Other behavioural assays**

The elevated plus maze test was conducted as described after the administration of vehicle or OEA (20 mg kg\(^{-1}\), i.p.). Horizontal activity in an open field and pain threshold in the hot plate test (35°C) were measured 15 min after injection of vehicle or OEA (20 mg kg\(^{-1}\)). Rectal temperature was measured with a digital thermometer.

In situ hybridization

We accustomed rats to the handling and injection procedure for 5 days. On day 6, we administered vehicle, OEA (10 mg kg\(^{-1}\), i.p.) or oleic acid (10 mg kg\(^{-1}\)) and killed the rats 60 min later by decapitation under anaesthesia. In situ hybridization analyses were conducted using [\(^{35}\)S]-labelled antisense RNA probes for c-fos and choline acetyl transferase. Average hybridization densities were determined from at least three tissue sections per rat. Statistical significance was evaluated using a one-way analysis of variance (ANOVA) followed by the Tukey–Kramer post-hoc test for paired comparisons.
Results are expressed as mean ± s.e.m. of n separate experiments. The significance of differences among groups was evaluated using ANOVA followed by a Student–Newman–Keuls post-hoc test, unless indicated otherwise.

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Epidemiological studies have documented a reduced prevalence of Alzheimer’s disease among users of nonsteroidal anti-inflammatory drugs (NSAIDs)1–3. It has been proposed that NSAIDs exert their beneficial effects in part by reducing neurotoxic inflammatory responses in the brain, although this mechanism has not been proved. Here we report that the NSAIDs ibuprofen, indomethacin and sulindac sulphide preferentially decrease the highly amyloidogenic Aβ42 peptide (the 42-residue isof orm of the amyloid-β peptide) produced from a variety of cultured cells by as much as 80%. This effect was not seen in all NSAIDs and seems not to be mediated by inhibition of cyclooxygenase (COX) activity, the principal pharmacological target of NSAIDs. Furthermore, short-term administration of ibuprofen to mice that produce mutant β-amyloid precursor protein (APP) lowered their brain levels of Aβ42. In cultured cells, the decrease in Aβ42 secretion was accompanied by an increase in the Aβ(1–38) isof orm, indicating that NSAIDs subtly alter γ-secretase activity without significantly perturbing other APP processing pathways or Notch cleavage. Our findings suggest that NSAIDs directly affect amyloid pathology in the brain by reducing Aβ42 peptide levels independently of COX activity and that this Aβ42-lowering activity could be optimized to selectively target the pathogenic Aβ42 species.

Increasing evidence suggests that a key event in the pathogenesis of Alzheimer’s disease is the altered production, aggregation and deposition of the Aβ peptide, a proteolytic fragment of 40–42 residues derived from APP. The longer isof orm, Aβ42, is selectively increased in all preclinical mutations analysed and in most mutant mutations that cause early-onset familial Alzheimer’s disease. Aβ42 is the Aβ species initially deposited in brain, and is particularly prone to aggregation in vitro. Therefore, Aβ42 is believed by many to be the main culprit in the pathogenesis of Alzheimer’s disease4. We examined whether NSAIDs alter APP processing and generation of Aβ, particularly the Aβ42 species. We treated cells with increasing concentrations of various NSAIDs, and analysed Aβ40 and Aβ42 levels in culture medium by sensitive sandwich enzyme-linked immunosorbent assay (ELISA) as described previously. The range of NSAID concentrations was chosen on the basis of tolerated plasma concentrations achieved in humans5. Multiple NSAIDs were examined in several studies, of them sharing similar activities (see below; an overview describing the cell lines and compounds studied is provided in the Supplementary Information). For brevity, the non-selective COX-inhibitor sulindac sulphide, the active metabolite of the pro-drug sulindac with well recognized antineoplastic