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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[1]. However, OEA does not activate cannabinoid receptors and its biological functions are still unknown[2]. 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[3–5] that are synthesized in response to a variety of physiological and pathological stimuli, including activation of neurotransmitter receptors in rat brain neurons[1,6] and exposure to metabolic stressors in mouse epidermal cells[7]. 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[8,9]. After release, FAEs are transported back into cells[10] and eventually broken down to fatty acid and ethanolamine by an intracellular fatty acid amide hydrolase (FAAH)[11,12]. That 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 that the polyunsaturated FAE anandamide (arachidonylethanolamide) serves as an endogenous ligand for cannabinoid receptors[13]. 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[2], and the biological roles of these compounds remain elusive.

Because anandamide may regulate feeding[14], 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

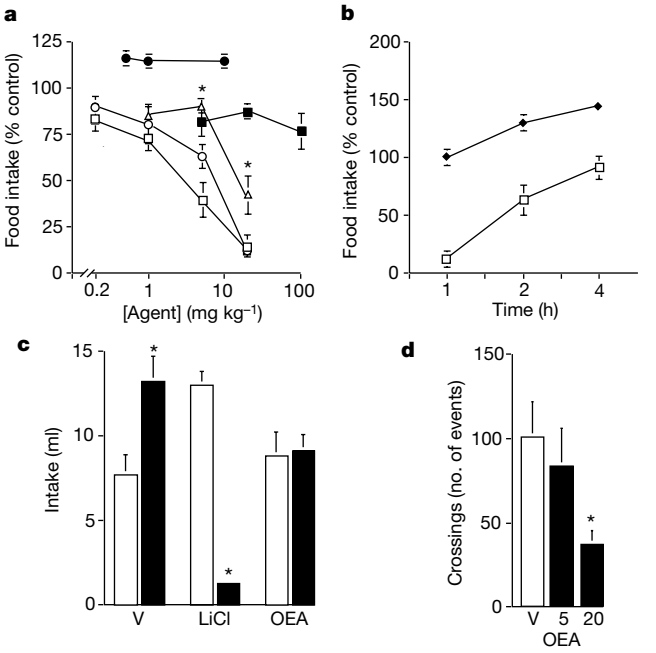

Figure 1 OEA suppresses food intake in rats deprived of food for 24 h. **a**, Dose-dependent effects of i.p. OEA (open squares), elaidylethanolamide (open circles), palmitylethanolamide (triangles), oleic acid (filled squares) and anandamide (filled circles) after the first 60 min of food presentation. 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 hypophagic 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.

targets[15]. 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

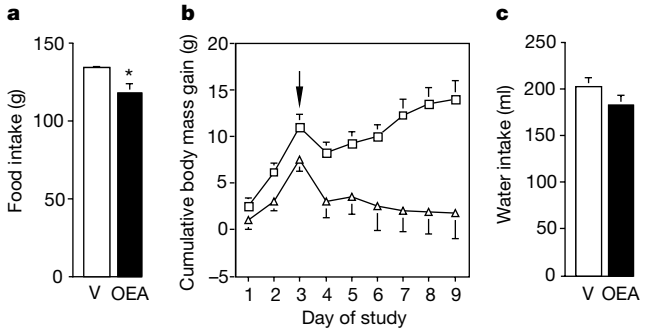

Figure 2 Effects of subchronic OEA administration on food intake and body mass. **a**, Effects of i.p. vehicle (V; 5% Tween 80 with 5% propylenglycol in sterile saline; open bars) or OEA (5 or 20 mg kg^{-1}, once per day; solid bars) on cumulative food intake. **b**, Time course of the effects of OEA (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.

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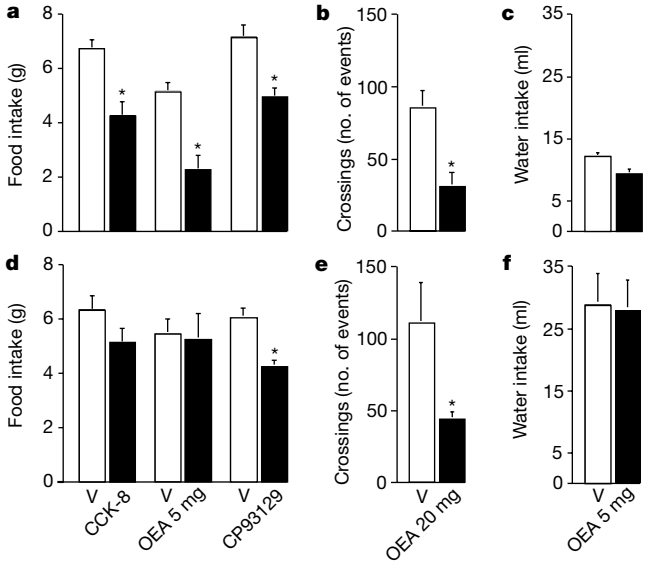

Figure 3 Role of peripheral sensory fibres in OEA-induced hypophagia. Effects of i.p. vehicle (V), OEA (5 mg kg^{-1}), CCK-8 (10 μg kg^{-1}) and CP-93129 (1 mg kg^{-1}), a centrally active 5-HT$_{1B}$ receptor agonist, on food intake in control rats (**a**–**c**) and capsaicin-treated rats (**d**–**f**). **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.

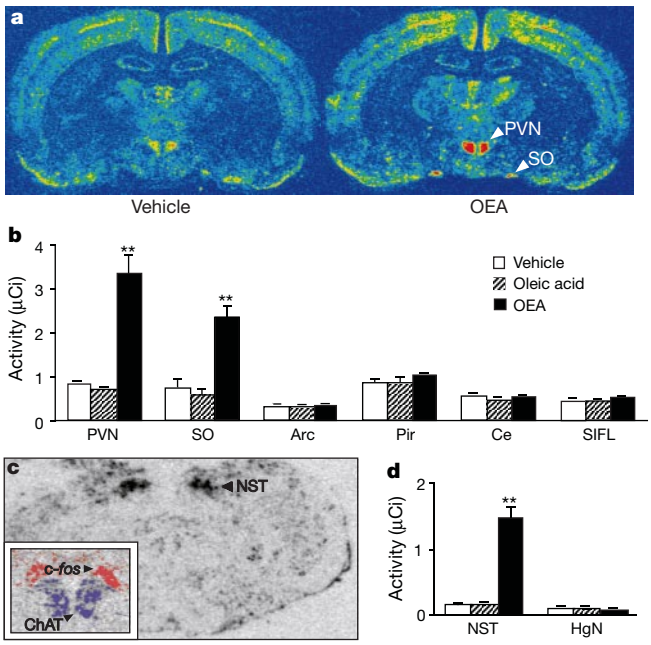

Figure 4 OEA increases c-*fos* mRNA expression in brain regions associated with feeding behaviour. **a**, Pseudocolour images of autoradiograms showing that i.p. OEA (right) elicits a profound and selective increase in c-*fos* mRNA expression in the paraventricular (PVN) and supraoptic (SO) hypothalamic 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 piriform 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 efferent 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.

activity of the hypothalamus–pituitary–adrenal (HPA) axis, did not change body temperature, pain threshold, plasma glucose levels (vehicle: 1.11 ± 0.04 mg ml^{-1}; OEA (5 mg kg^{-1} intraperitoneally, i.p.): 30 min, 1.10 ± 0.03 mg ml^{-1}; 60 min, 0.99 ± 0.02 mg ml^{-1}; 120 min, 1.08 ± 0.07 mg ml^{-1}), insulin levels (vehicle: 1.7 ± 0.3 ng ml^{-1}; OEA (5 mg kg^{-1} i.p.): 30 min, 1.5 ± 0.1 ng ml^{-1}; 60 min, 1.6 ± 0.1 ng ml^{-1}; 120 min, 1.6 ± 0.2 ng ml^{-1}; $n = 5$ or 6) or leptin levels (vehicle: 13.3 ± 2.4 ng ml^{-1}; OEA (5 mg kg^{-1} i.p.): 30 min, 10.5 ± 0.4 ng ml^{-1}; 60 min, 11.4 ± 1.1 ng ml^{-1}; 120 min, 11.6 ± 0.8 ng ml^{-1}; $n = 5$) (see also Supplementary Information). Although a high OEA dose reduced motor activity (Fig. 1d), this effect may not contribute to the compound's anorexic actions, for two reasons: the same dose of OEA did not affect water intake (see Supplementary Information); and removal of sensory fibres by capsaicin treatment abrogated the effects of OEA on food intake, but not those on movement (see below). This pharmacological profile differentiates OEA from other appetite suppressants such as amphetamine and glucagon-like peptide 1 (the effects of which include aversion and anxiety), and from cannabinoids such as anandamide (which stimulates food intake in pre-satiated animals, reduces pain and activates the HPA axis)[16].

To test whether tolerance develops to the hypophagic actions of OEA, we administered it subchronically in rats. Daily injections of OEA (5 mg kg^{-1} i.p.) resulted in a small but significant decrease in cumulative food intake (Fig. 2a), which was accompanied by a marked inhibition of body mass gain (Fig. 2b). By contrast, subchronic OEA administration had no effect on water intake (Fig. 2c) or on plasma levels of various metabolites and liver enzymes (see Supplementary Information). Pair-feeding experiments suggest that decreased food consumption is sufficient to account for the mass-reducing actions of OEA (Table 1). We cannot exclude, however, the possible contribution of other factors to this effect, such as stimulation of energy expenditure or inhibition of energy accumulation, as suggested by the significantly lower triglyceride levels in OEA-treated rats than in controls (see Supplementary Information).

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

	n	Cumulative mass gain (g)	Food intake (g)	Water intake (ml)
Control	10	15.6 ± 2.3	143.2 ± 10.3	184.3 ± 12.1
OEA	11	4.1 ± 1.4†	115.9 ± 4.2*	174.7 ± 9.1
Pair fed	11	4.3 ± 1.4†	116	192.8 ± 12.7

OEA (5 mg kg^{-1} i.p.) or vehicle (5% propylenglycol, 5% Tween 80, 90% sterile saline) were administered once per day for 7 days, after 3 days of baseline measurements. The pair-fed group received a total of 116 g of food, identical to the amount consumed by the OEA group.
*$P < 0.05$ versus control.
†$P < 0.01$ versus control.

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 rats with capsaicin[17]. 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[18]. 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-HT$_{1B}$ receptors (Fig. 3a, d)[19]. 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 capsaicin 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

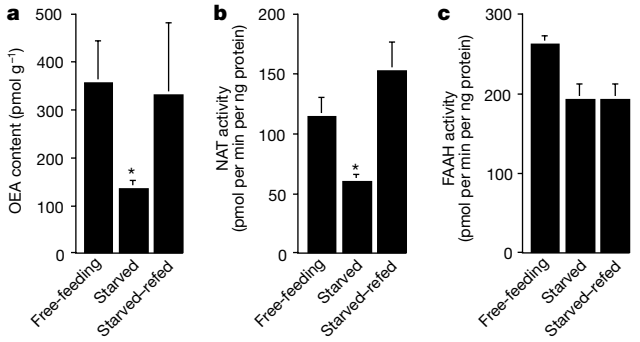

Figure 5 Feeding regulates OEA biosynthesis in small intestine. Effects of free feeding, starvation and starvation–refeeding on OEA levels (**a**), NAT activity levels (**b**) and FAAH activity levels (**c**). Asterisk, $P < 0.05$; $n = 3$–5 per group.

brainstem and the paraventricular (PVN) nucleus in the hypothalamus[20]. To identify brain pathways engaged during OEA-evoked hypophagia, we mapped messenger RNA levels for the activity regulated gene c-*fos*[21] 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)[20], is consistent with a role for OEA as a peripheral regulator of feeding behaviour.

The anorexic 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[22], we studied the impact of feeding on intestinal OEA biosynthesis. Analyses with high-performance liquid chromatography and mass spectrometry (HPLC/MS) revealed that small-intestinal tissue from free-feeding rats contains substantial amounts of OEA (354 ± 86 pmol g^{-1}, $n = 3$). Intestinal OEA levels were markedly decreased after food deprivation, but returned to baseline after refeeding (Fig. 5a). By contrast, no such changes were observed in stomach (control, 210 ± 20 pmol g^{-1}; starvation, 238 ± 84 pmol g^{-1}; starvation–refeeding, 239 ± 60 pmol g^{-1}; $n = 3$). Variations in intestinal OEA levels were accompanied by parallel alterations in NAT activity, which participates in OEA formation[4], but not in FAAH activity, which catalyses OEA hydrolysis (Fig. 5b, c)[11,12]. These findings suggest that starvation and feeding reciprocally regulate OEA biosynthesis in small intestine. In agreement with an intra-abdominal source of OEA, we found that plasma OEA levels in starved rats are higher in portal than in caval blood (porta, 14.6 ± 1.8 pmol ml^{-1}; cava, 10.3 ± 2.8 pmol ml^{-1}; $n = 5$). The contribution of other intra-abdominal tissues to OEA formation cannot be excluded at present.

Our results suggest a hypothetical model for the role of OEA in feeding behaviour. According to this model, food intake may stimulate NAT activity, enhancing OEA biosynthesis in the small intestine and possibly other intra-abdominal tissues. Newly produced OEA may activate local sensory fibres, which may in turn inhibit feeding by engaging brain structures such as the NST and PVN. A number of questions remain, such as what physiological stimuli initiate and terminate OEA biosynthesis, whether there is a functional relationship between OEA and other nutritional signals, and what the molecular targets of OEA are. Irrespective of the answers, our results reveal an unexpected role for OEA in the peripheral regulation of feeding, and provide a framework to develop medicines for the treatment of eating disorders. □

Methods

Animals

We used male Wistar rats (200–350 g). All procedures met the National Institutes of Health guidelines for the care and use of laboratory animals, and the European Communities directive 86/609/EEC regulating animal research.

Chemicals

FAEs and [$^2H_4$] FAEs were synthesized in the laboratory[23]; 1,2-dioleyl-*sn*-glycero-phosphoethanolamine-*N*-oleyl was purchased from Avanti Polar Lipids; SR141716A was provided by RBI as part of the Chemical Synthesis Program of the National Institute of Mental Health; SR144528 was a gift of Sanofi Recherche; all other drugs were from Tocris or Sigma. FAEs were dissolved in dimethylsulphoxide (DMSO) and administered in 70% DMSO in sterile saline (acute treatments) or 5% Tween 80 with 5% propylenglycol in sterile saline (subchronic treatments) (1 ml 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% propylenglycol 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[24]. NAT assays were performed using 1,2-di[^{14}C]palmityl-*sn*-glycerophosphocholine as a substrate (108 mCi mmol^{-1}, Amersham) under conditions that were linear with time and protein concentrations[9]. FAAH assays were performed under linear conditions according to ref. 24, except that [^{3}H]anandamide (arachidonyl-[1-^{3}H]ethanolamide; 60 Ci mmol^{-1}; ARC) 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[23]. FAEs were quantified by HPLC/MS, with an isotope dilution method[25].

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[26].

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} at 19:00) 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 graded bottle during a 30-min test period for 4 days. On day 5, water was substituted with a 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[26] 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 (55 °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*[28] and choline acetyl transferase[29]. 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.

Data analysis

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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A subset of NSAIDs lower amyloidogenic Aβ42 independently of cyclooxygenase activity

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Epidemiological studies have documented a reduced prevalence of Alzheimer's disease among users of nonsteroidal anti-inflammatory drugs (NSAIDs)[1–5]. 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 isoform 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[6]. 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) isoform, 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 isoform, Aβ42, is selectively increased in all presenilin mutations analysed and in most APP 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 disease[7].

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-link immunosorbent assay (ELISA) as described previously[8]. The range of NSAID concentrations was chosen on the basis of tolerated plasma concentrations achieved in humans[9]. Multiple NSAIDs were examined in this study, several 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