Digital Commons @ George Fox University

Faculty Publications - Department of Biology and Chemistry

Department of Biology and Chemistry

2003

The Anorexigenic Fatty Acid Synthase Inhibitor, C75, Is a Nonspecific Neuronal Activator

Kanji A. Takahashi

James L. Smart
George Fox University, jsmart@georgefox.edu

Hongyan Liu

Roger D. Cone

Follow this and additional works at: http://digitalcommons.georgefox.edu/bio_fac

Part of the Biology Commons, Chemistry Commons, and the Endocrinology Commons

Recommended Citation

Previously published in Endocrinology, 2003, vol. 145(1) http://press.endocrine.org/doi/full/10.1210/en.2003-0337

This Article is brought to you for free and open access by the Department of Biology and Chemistry at Digital Commons @ George Fox University. It has been accepted for inclusion in Faculty Publications - Department of Biology and Chemistry by an authorized administrator of Digital Commons @ George Fox University. For more information, please contact arolfe@georgefox.edu.

The Anorexigenic Fatty Acid Synthase Inhibitor, C75, Is a Nonspecific Neuronal Activator

Kanji A. Takahashi, James L. Smart, Hongyan Liu, and Roger D. Cone

Address all correspondence and requests for reprints to: Roger Cone, Vollum Institute L-474, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Portland Oregon 97239. E-mail: .

Abstract

C75, a recently derived compound that potently suppresses feeding and induces weight loss, has been proposed to act mainly by inhibiting fatty acid synthase (FAS) in central neurons that control feeding. For example, normal, fasting-associated, hypothalamic increases in neuropeptide Y (NPY)/Agouti-related protein (AGRP) expression and decreases in proopiomelanocortin (POMC)/cocaine and amphetamine regulated transcript (CART) expression were reported to be blocked by C75. Using loose-patch extracellular recording in acute slices, we tested the effect of C75 on anorexigenic POMC neurons and orexigenic NPY neurons of the hypothalamic arcuate nucleus, which were identified by promoter-driven GFP expression, as well as on feeding-unrelated cerebellar Purkinje neurons. We expected C75 to activate POMC neurons, inhibit NPY neurons, and have no effect on Purkinje neurons. Instead, C75 activated all cell types, suggesting that it lacks target specificity. This activation was probably not caused by FAS inhibition, because the classical FAS inhibitor, cerulenin, did not have this effect when tested on POMC and NPY neurons. Nonspecific neuronal activation and resulting neurological effects might contribute to the decreased feeding reported to follow centrally administered C75. Injection, ip, of C75 induced severe loosening or liquefaction of stools, weight loss, and decreased food intake in both wild-type and melanocortin-4 receptor knockout mice. In contrast, cerulenin failed to loosen stools, even at a molar dose over 9-fold greater than C75, and had a much smaller effect on body weight. FAS inhibitory activity, by itself, seems to be insufficient to reproduce all of the effects of ip-injected C75.

OBESITY IS A MAJOR and rapidly growing public health issue (1, 2, 3). However, nonsurgical treatments for obesity have had little long-term success, largely because of homeostatic mechanisms that oppose decreases in adiposity (4, 5, 6). Early results using C75, a novel fatty acid synthase (FAS) inhibitor, to induce weight loss were extremely promising. Originally developed for its antitumor activity, C75 was designed to be a less reactive (and therefore potentially safer) form of the classical FAS inhibitor, cerulenin (7). C75 powerfully suppressed feeding and induced rapid weight loss not only in lean mice but also in mice that were obese because of leptin-deficiency (*ob/ob*) or consumption of a high-fat diet (8, 9). Moreover, whereas regular administration of low doses of C75 produced rapid tachyphylaxis in lean animals, obese animals remained sensitive, a characteristic of potentially great clinical utility (10). Because feeding was inhibited by both central and peripheral administration of C75 (8, 10) and because C75 seemed to block fasting-induced changes in neuropeptide expression (11), a central mechanism of action was proposed. These data were consistent with the critical role of the central melanocortin system in regulation of both long-term energy homeostasis (12, 13) and satiety (14). These results also met with great scientific interest, because they seemed to address a hypothesis that high intracellular malonyl-CoA concentration, as an indicator of high cellular energy charge, modulates neuronal activity so as to inhibit further feeding (8, 15). Malonyl-CoA is a key intermediate in fatty acid synthesis.

When fuel is abundant, cytosolic ATP, acetyl-CoA, and citrate are plentiful. In the committed step in fatty acid synthesis, acetyl-CoA carboxylase adds a carboxyl group to acetyl-CoA to form malonyl-CoA in a reaction driven by ATP hydrolysis

(Fig. 1). Subsequently, FAS serially adds two-carbon units to the growing fatty acid chain, a process usually beginning with acetyl-CoA (C_2) and ending with palmitate (C_{16}). Feeding increases cytosolic malonyl-CoA concentration, both by increasing its precursors and by increasing cytoplasmic citrate, which is an allosteric activator of acetyl-CoA carboxylase. Elevated [malonyl-CoA] inhibits carnitine palmitoyltransferase I (CPT I, also carnitine acyltransferase I), the enzyme that translocates long-chain fatty acids into mitochondria and makes their oxidation possible. In this way, malonyl-CoA also serves as a signaling molecule that couples recent digestion of carbohydrate and/or protein to decreased fatty acid oxidation (16).

It has been hypothesized that elevated [malonyl-CoA] might act to signal the fed state in specialized cell types, causing increased insulin secretion in pancreatic β-cells and modulating the activity of feeding-related neurons so as to inhibit further feeding (8, 15, 17). According to this hypothesis, malonyl-CoA might act directly, or by inhibiting CPT I it might increase cytoplasmic long-chain free fatty acids and diacylglycerol, both of which are potential signaling molecules or precursors. Inhibiting FAS could increase [malonyl-CoA] both by substrate accumulation and by reducing cytosolic palmitoyl-CoA, a molecule that normally opposes formation of malonyl-CoA by inhibiting translocation of citrate from mitochondria to cytosol, and by blocking the activation of acetyl-CoA carboxylase by citrate (16) (Fig. 1). As predicted, C75 administered after a bolus of glucose increased [malonyl-CoA] in liver 1 h later. It was then proposed that C75 suppresses feeding by increasing [malonyl-CoA] in key feeding-related neurons, as predicted by the malonyl-CoA hypothesis (8).

Proopiomelanocortin (POMC)/cocaine and amphetamine regulated transcript (CART) neurons of the hypothalamic arcuate nucleus inhibit feeding (13, 18). Fasting causes POMC and CART expression to fall in the arcuate nucleus (19, 20, 21, 22). Also, certain manipulations that decrease feeding, such as leptin (21, 22, 23, 24) or LPS administration (25), increase POMC/CART expression or cFOS expression in POMC/CART neurons. The POMC knockout mouse is obese (26), as are POMC-deficient humans (27). The secreted POMC cleavage product that is most important in feeding is α-MSH, and the relevant receptor knockouts, MC4-RKO (melanocortin 4 receptor) (13) and MC3-RKO (28, 29), are obese. It has been estimated that over 4% of cases of severe pediatric obesity result from MC4-R haploinsufficiency (30, 31, 32).

Neuropeptide Y (NPY)/Agouti-related protein (AGRP) neurons, also located in the hypothalamic arcuate nucleus, are thought to promote feeding. Fasting causes hypothalamic NPY and AGRP expression to increase greatly (33, 34), and both NPY (35) and AGRP (36) cause profound increases in feeding when administered intracerebroventricularly. AGRP antagonizes the anorexigenic effect of α-MSH at melanocortin receptors (37); and POMC and NPY/AGRP neurons send projections to many of the same sites (38, 39). Furthermore, there is evidence of cross-talk between these two classes of arcuate neurons (40); NPY/AGRP neurons are thought to provide GABAergic input to POMC neurons (12).

It has been reported that single peripheral doses of C75, although almost completely inhibiting feeding, were not followed by the normal effects of fasting on neuropeptide expression. Instead of decreasing, POMC and CART expression increased slightly, and the usual fasting-induced increases in NPY and AGRP expression were severely blunted (11). This seemed to suggest that modulation of the activity of anorexigenic POMC/CART neurons and orexigenic NPY/AGRP neurons contributed to the observed inhibition of feeding. However, contradictory results were obtained when C75 injections were continued for several days (10) and when *obese* mice were used (11). We tested the *in vitro* electrophysiological effect of C75 on POMC and NPY neurons, identified by their expression of fluorescent transgenes, in acute hypothalamic slice preparations. In characterizing the actions of hormones and neuropeptides on arcuate neuronal activity, results reported using this preparation have been highly consistent with *in vivo* results (12, 41, 42, 43). As a control, C75 was also tested on cerebellar Purkinje neurons, which are unrelated to feeding. The importance of metabolic processes to the proposed mechanism of C75 made it necessary to record from neurons without intracellular disruption. Therefore, cellular activity was monitored by loose-patch extracellular recording.

Materials and Methods

Animals

Arcuate nucleus POMC neurons were studied in mice hemizygous for a transgene in which the POMC promoter drives expression of enhanced green fluorescent protein (EGFP) (12) (POMC-EGFP, 33–47 d old in single-cell experiments, 30–38 d old in preincubation experiments). These mice were also used to study Purkinje cells in cerebellar vermis and paravermis (19–23 d old). Arcuate nucleus NPY neurons were studied in mice hemizygous or homozygous for a transgene in which the NPY promoter drives expression of a fusion protein of tau and sapphire, a green fluorescent protein variant (44, 45) (NPY-SAP, 31–37 d old). Within the arcuate nucleus, all sapphire fluorescent neurons seem to express AGRP mRNA (Dr. Lora Heisler, Beth Israel Deaconess Medical Center and Harvard Medical School, personal communication). Both transgenes were expressed in a C57BL/6J background. Both male and female mice were used to study each cell type, and procedures were approved by the Oregon Health and Science University Animal Care and Use Committee.

Electrophysiology

Coronal slices of hypothalamus and sagittal slices of cerebellum were prepared 200-μm thick with a vibrating slicer (Leica VT1000S) using standard techniques. Subsequently, slices were maintained in a submersion-type holding chamber at 34–35 C for the first 30 m, then at room temperature for at least another 30 min before use. After transferring slices to a recording chamber (30 C), at least 10 m were allowed for equilibration before recording. Saline used for slice preparation and maintenance contained (in mM): 126.18 NaCl, 3.1 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 10 glucose, and 16.24 sucrose; 320 mosmol/kg, pH 7.39 when gassed with 95% O_2-5% CO_2. In saline used for recording, sucrose was omitted and replaced with (in mM): 1 kynurenic acid (ionotropic glutamate receptor antagonist), 1.05 NaOH, 0.1 picrotoxin ($GABA_A$ receptor antagonist), and 14.09 (0.1%) total dimethylsulfoxide (DMSO). The recording chamber was approximately 0.7 ml in vol and was perfused at approximately 2.3 ml/m.

Loose-patch extracellular recordings (46) were performed by filling a patch pipette ($R_{pipette}$ 1.4–1.8 MΩ) with recording saline and loosely attaching it to an identified neuron (R_{seal} 2.1–8 MΩ) using epifluorescence and IR-DIC optics (Carl Zeiss, Jena GmbH, Germany). The looseness of the seal helped to prevent spontaneous rupture of the patched membrane. To counter the tendency for the patched membrane to be drawn further into the pipette and potentially destabilize over time, a small amount of positive pressure from a short column of water was applied via tubing to the pipette holder. With this technique, in contrast to whole-cell patch clamp and sharp microelectrode recording, cytosolic contents and processes are undisturbed, and neuronal activity is affected by neither seal strength nor current injection through the pipette. This allows unbiased measurements of basal spike frequency to be made. Because loose-patch recordings required a small amount of membrane to be drawn into the tip of the patch pipette, recorded neurons could be unambiguously identified. Recordings were conducted in current clamp mode at 100× gain and filtered at 5 kHz. Data were acquired at 10 kHz using an Axopatch 1D amplifier and Clampex 8.2 software (Axon Instruments, Union City, CA). Data were analyzed using Clampfit 8.2 (Axon Instruments), Mini Analysis Program 5.3.8 (Synaptosoft, Decatur, GA), GraphPad Prism 2.0 (Graphpad Software, Inc., San Diego, CA), and Excel 2000 (Microsoft Corp., Bellevue, WA). Spike frequency was calculated in 60-sec bins; 100 mM C75, 100 mM cerulenin, and 1000 mMcerulenin stock solutions in DMSO were stored in small aliquots at −78 C. C75 was a kind gift of Dr. M. Daniel Lane, Johns Hopkins University, who indicated that the compound was more than 98% pure, based on HPLC, mass spectrometry, and nuclear magnetic resonance data.

The basal spike frequency of individual POMC neurons ranged from 0 to over 3 Hz. A few neurons with basal frequencies from 0.16–3.2 Hz were used to test the effect of C75 on single cells in long-term recordings. After at least 20 min of baseline recording, the perfusion saline was switched to either C75 or a matched control. Baseline was calculated from the mean frequency 0–20 min before drug/control application, and this was subtracted from each data point. Results for each cell were quantified by measuring the mean change in spike frequency 34–44 min after drug/control application, and the slope of the change in spike frequency (by linear regression) from 10–44 min after drug/control application.

In preincubation experiments, single hypothalamic or cerebellar slices were transferred from the room temperature holding chamber to a 30-C preincubation chamber containing C75, cerulenin, or vehicle in 100 ml of continuously gassed recording saline. After 60 min, the slice was transferred to a recording chamber and perfused with control (drug-free) recording saline. After a 10-min equilibration, recordings of basal spike frequency were obtained from as many morphologically normal neurons as possible for the next 50 min. Spike frequency was calculated from the first 120 sec of each recording. In the few cases where aberrant spike patterns seemed to indicate failing cell health, recordings were rejected.

Feeding experiments

All *in vivo* experiments were conducted using singly housed mice. To determine the effect of C75 on normal mice, 2- to 3-month-old, female, POMC-EGFP(Tg/+) and NPY-SAP(Tg/+) mice were distributed by age and genotype into control and test groups, which differed in mean initial body weight by less than 1.7% (control, 19.9 ± 0.7 g; test, 19.6 ± 0.4 g). Because of results from a pilot experiment, a paper towel was laid on the bedding of each cage to facilitate examination of feces. The same frozen stock of C75 used for electrophysiological experiments was diluted in RPMI Medium 1640 (Invitrogen Corp., Carlsbad, CA) and ip injected into mice by a blinded investigator at a dose of 15 mg/kg in a total vol of 20 μl/g body weight. Control mice were injected with RPMI containing an equal concentration of DMSO (2.95%). Injections were performed approximately 1 h before lights out. Similar procedures were used for *in vivo* cerulenin experiments. The same DMSO concentration and injection volume were used, and both injections and subsequent measurements were performed by a blinded investigator. Cerulenin experiments used 1.5- to 2-month-old, male and female, C57BL/6J, POMC-EGFP/+ and NPY-SAP/+ mice, distributed by age, gender, and genotype into four equivalent groups, which differed in initial body weight by less than 1.3% (0, 30, 60, 120 mg/kg: 19.2 ± 1.2, 19.0 ± 1.2, 19.2 ± 0.8, 19.2 ± 1.0 g). C75 was also tested on 1-month-old, male and female MC4-RKO mice (13) with procedures modified as follows. Because these mice are acutely sensitive to stress (47), mice were conditioned with daily ip vehicle injections for 5 d before injection with 15 mg/kg C75. Injection vol was 11.8 μl/g body weight, and DMSO concentration was 5%. In cerulenin and MC4-RKO experiments, bedding consisted only of several layers of paper towels to facilitate examination of feces.

Statistical analysis

In all descriptions of results and in figures, data are presented as mean ± SEM. Results of statistical tests are described in *Results* and in figure legends. The significance level of all tests was set at $P = 0.05$.

Results

C75 induced a delayed activation of anorexigenic arcuate nucleus POMC neurons

Because C75 was hypothesized to act by causing an increase in cytosolic [malonyl-CoA], it was important to record from neurons without perturbing their metabolic processes. Therefore, extracellular recordings of action potentials were made from loose-patched POMC neurons of the hypothalamic arcuate nucleus. In all recordings, after a delay, C75 induced a steady increase in spike frequency (Fig. 2). Especially at 100 μM C75, this increase seemed to be preceded by a small, transient decrease in spike frequency. Consistent with the lipophilicity of C75, the increase in spike frequency was resistant to washout (data not shown). C75-induced stimulation of POMC cells, whether measured as net increase in spike frequency or as the slope of that increase, was statistically significant at both 50-μM and 100-μM doses ($P < 0.001$). The increases at 50 μM and 100 μM C75 were not significantly different from each other. Results (mean ± SEM) were as follows (change in spike frequency, slope of change, number of cells): control, 0.22 ± 0.09 Hz, 0.0046 ± 0.0051 Hz/m, 8 cells; 50 μM C75, 2.05 ± 0.27 Hz, 0.077 ± 0.0079 Hz/m, 3 cells; 100 μM C75, 2.46 ± 0.62 Hz, 0.079 ± 0.018 Hz/m, 3 cells.

Preincubation in C75 increased the basal firing rate of arcuate nucleus POMC neurons

Taking advantage of the resistance of the effect of C75 to washout, we switched to a preincubation protocol to conserve our limited supply of the compound (see *Materials and Methods*). Consistent with our earlier data, 60 min of preincubation with C75 greatly increased the basal spike frequency of POMC neurons in the hypothalamic arcuate nucleus (Fig. 3). Although C75 is known to inhibit FAS (7), its specificity for this enzyme is unclear. Another known FAS inhibitor, cerulenin, failed to cause a significant increase in spike frequency, even at a dose 8-fold higher (9.1× higher in moles) than an effective dose of C75. Results (mean ±SEM) were as follows (spike frequency, elapsed time after start of 60-min preincubation, number of cells): control, 1.20 ± 0.16 Hz, 91.2 ± 1.6 min, 77 cells; 25 μM C75, 2.80 ± 0.38 Hz, 94.5 ± 2.2 min, 42 cells; 100 μM C75, 4.09 ± 0.45 Hz, 94.6 ± 2.3 min, 41 cells; 25 μMcerulenin, 1.08 ± 0.21 Hz, 93.6 ± 2.1 min, 45 cells; 100 μM cerulenin, 1.25 ± 0.30 Hz, 89.1 ± 2.3 min, 36 cells; 200 μM cerulenin, 0.86 ± 0.23 Hz, 94.9 ± 3.1 min, 22 cells.

Preincubation in C75 increased the basal firing rate of orexigenic arcuate nucleus NPY neurons

NPY/AGRP neuronal activation is normally associated with increased hunger. Therefore, if C75 reduces feeding through normal physiological pathways, any C75 influence on arcuate NPY activity should be inhibitory. Unexpectedly, C75 greatly increased the basal spike frequency of arcuate NPY neurons in a dose-dependent manner (Fig. 4). Here also, cerulenin did not cause a significant increase in spike frequency. Results (mean ± SEM) were as follows (spike frequency, elapsed time after start of 60-m preincubation, number of cells): control, 0.55 ± 0.10; 92.8 ± 1.5, 85; 25 μM C75, 2.35 ± 0.32, 93.8 ± 2.7, 28; 100 μM C75, 3.53 ± 0.37, 90.8 ± 2.5, 29; 100 μM cerulenin, 0.73 ± 0.17, 93.4 ± 2.3, 38; 200 μM cerulenin, 0.47 ± 0.18, 92.4 ± 2.9, 22.

Preincubation in C75 caused activation of cerebellar Purkinje neurons

Assuming that [malonyl-CoA] varies with feeding state in all neurons, because normal variations in feeding state do not cause global neuronal modulation, neurons unrelated to feeding should be relatively insensitive to changes in [malonyl-CoA]. Therefore, if C75 inhibits feeding by boosting [malonyl-CoA], C75 should have no effect on neurons that are unrelated to feeding. To test this idea, C75 was applied to cells with no hypothesized role in the regulation of hunger, cerebellar Purkinje neurons.

Under our conditions, Purkinje neurons had relatively high spike frequencies at rest (48). As Purkinje neurons are depolarized, they switch from a steady firing mode with constant spike amplitude to a bursting mode in which bursts of action potentials are separated by silent pauses (49). Toward the end of each burst, spike frequency is so high that spike amplitude shrinks because of inactivation.

Cerebellar slices were preincubated as before in control or C75 solutions. Purkinje cells were examined for 180 sec each to determine whether firing was steady (Fig. 5A) or bursting (Fig. 5B) during that time. Mean spike frequency was determined for nonbursting cells from 120 sec of recording. C75 greatly increased the proportion of Purkinje cells in bursting mode (Fig. 5C). For example, exposure to 100 μM C75 increased the proportion of Purkinje cells in bursting mode from 1 of 34 (2.94%) to 11 of 14 (78.6%). Under control conditions, many Purkinje cells had relatively low spike frequencies, but this group was no longer present after C75 incubation (Fig. 5D). C75 seemed to shift the frequency distribution upward, with the most depolarized cells entering the bursting mode. Results (mean ± SEM) were as follows (proportion of cells exhibiting a bursting firing pattern, spike frequency of nonbursting cells, time after start of 60 m preincubation): control, 1 of 34 cells (2.94%), 37.76 ± 3.11 Hz, 90.9 ± 2.4 min; 25 μM C75, 11 of 20 cells (55.0%), 46.34 ± 3.31 Hz, 91.3 ± 2.9 min; 50 μM C75, 7 of 10 cells (70.0%), 44.19 ± 2.33 Hz, 97.3 ± 4.5 min; 100 μM C75, 11 of 14 cells (78.6%), 62.93 ± 14.18 Hz, 87.9 ± 3.3 min.

In vivo effects of C75

To confirm previous results (8, 10, 11) and the *in vivo* efficacy of our stock of C75, mice were injected with 15 mg/kg C75 or a vehicle control. Four of five C75-treated mice ate much less and lost weight relative to vehicle-treated controls (Fig. 6). Unexpectedly, these same four mice exhibited a diarrhea-like loosening of stools, which began within an hour of ip injection and then worsened. Symptoms ranged from very soft, watery stools to feces the consistency of a slightly thickened liquid. Stools were so soft that, after deposition in bedding, they were absorbed and lost within a few hours, perhaps explaining the absence of previous reports of this effect. Detection depended on frequent postinjection observation and stool deposition on food containers and on paper towels laid on the bedding for this purpose. The fifth C75-treated mouse ate robustly, lost no weight, and displayed little, if any, softening of stool. Feces and weight of all vehicle-treated mice seemed to be unaffected. C75 also disrupted normal nest-building behavior. By 18 h after injection, all mice unaffected by loosened stools had begun to shred the paper towels left in their cages, whereas paper towels in the cages of the affected mice were completely intact, consistent with visceral illness. Overall, at 24 h post injection, percent initial body weight was significantly less in C75-treated mice (91.3 ± 2.4%, mean ± SEM, n = 5) than in vehicle-treated controls (100.1 ± 0.8%, $P = 0.005$, n = 6). In the 24 h after injection, C75-treated mice ate only half as much (1.4 ± 0.48 g) as vehicle-treated mice (2.7 ± 0.33 g, mean ± SEM, $P < 0.05$).

To determine whether the loose/liquefied stools, weight loss, and decreased feeding produced by C75 were attributable to inhibition of FAS, groups of four mice were injected with vehicle or cerulenin at 30, 60, and 120 mg/kg (Fig. 7). Even though in moles, doses of cerulenin (MW 223.3) were 2.3×, 4.6×, and 9.1× the amount of C75 (MW 254.32) in a 15-mg/kg dose; cerulenin did not induce loose stools at any dose. The effects of cerulenin on body weight and food consumption were also small compared with C75. Only the highest dose of cerulenin (120 mg/kg) produced a significant decrease in body weight, and the corresponding decrease in food consumption did not reach significance. Results (mean ± SEM) were as follows (percent initial body weight, cumulative food consumption at 24 h post injection): control, 101.9 ± 1.2%, 2.8 ± 0.4 g; 30 mg/kg, 99.8 ± 0.8%, 2.8 ± 0.3 g; 60 mg/kg, 100.5 ± 0.8%, 2.5 ± 0.3 g; 120 mg/kg, 96.9 ± 1.3%, 1.7 ± 0.5 g.

It has been proposed that C75's effects are mediated, in part, through activation of anorexigenic POMC/CART neurons of the hypothalamic arcuate nucleus (11). The melanocortin-4 receptor (MC4-R) is thought to be the receptor most important in mediating the effects of POMC on feeding. Therefore, we examined the role of MC4-R in mediating the actions of C75 using MC4-RKO mice (13). Four MC4-RKO mice were conditioned with daily ip injections of vehicle for 5 d, during which they steadily gained weight (Fig. 8). On the 6th day, all mice were injected with 15 mg/kg C75. In all mice, treatment with C75 was followed by abundant very soft or liquefied stool, decreased weight, and decreased food intake (Fig. 8). Soft stool was first observed within 10, 17, 25, and 36 min of C75 injection. C75 caused a 24-h decrease in body weight of 12.4 ± 0.67% (mean ± SEM), compared with a 2.0 ± 0.19% increase the day before ($P = 0.000276$). C75 caused a 66% drop in 24 h food intake, from 2.17 ± 0.23 g the day before injection to 0.73 ± 0.09 g the day after ($P = 0.0053$). These results were consistent with results described above in wild-type mice. Although body weight decreased by a small amount in the 3 h after each vehicle injection (−1.9 ± 0.31% the day before C75), this decrease was much greater 3 h after C75 injection (−6.0 ± 0.27%, $P = 0.0031$; significance in all MC4-RKO experiments was evaluated using two-tailed, paired *t* tests).

Discussion

In vitro

C75, a potent inducer of weight loss, was applied to anorexigenic arcuate POMC neurons, orexigenic arcuate NPY neurons, and feeding-unrelated cerebellar Purkinje neurons, all of which were activated. Two lines of evidence suggest that this activation was direct and not synaptic. First, to reduce synaptic input, antagonists of $GABA_A$ and ionotropic glutamate receptors were included in all preincubations and recordings. Second, a direct, nonsynaptic effect is the simplest explanation for the uniformity of response in two functionally antagonistic classes of hypothalamic neurons and in an unrelated type of cerebellar neuron. This nonspecificity suggests that C75 has the potential to activate many other types of neurons, also. However, *in vivo*, the effect of C75 on any given neuron would be the sum of direct effects and effects on presynaptic neurons, making the net effect on any particular neuron hard to predict. A disproportionately large effect on inhibitory inputs could potentially counterbalance C75-mediated excitation in certain neurons. Still, the final result would be network dysregulation. When C75 is given centrally (8, 50), the neurological consequences of general neuronal activation, even if mild, might explain the resulting inhibition of feeding. This hypothesis contradicts earlier conclusions about the mechanism by which centrally administered C75 induces anorexia (51, 52). When given peripherally, because of limited central nervous system (CNS) penetration (8), a dose of C75 sufficient to inhibit feeding might not be enough to cause obvious centrally mediated behavioral deficits.

Early results with C75 met with great scientific interest because of the elegance of its proposed mechanism of action, in which activity of feeding-related neurons is modulated by the increase in [malonyl-CoA] that results from FAS inhibition. Although C75 activation of anorexigenic POMC neurons is consistent with this model, C75 activation of orexigenic

NPY/AGRP neurons and cerebellar Purkinje neurons contradicts it. Even more convincing is the failure of even high concentrations of cerulenin to mimic the effects of C75 on POMC and NPY neurons, which strongly suggests that C75-induced neuronal activation is not a consequence of FAS inhibition. Finally, if increased [malonyl-CoA] caused the observed activation, then normal postfeeding increases in [malonyl-CoA] would cause excitation of POMC, NPY, and Purkinje neurons and, given C75's apparent lack of target-specificity, perhaps even widespread neuronal excitation; but this does not occur.

Cerulenin is known to inhibit not only fatty acid synthesis (53) but also protein acylation (54,55, 56) and other processes involving a similar reaction mechanism (53, 57) (although unlikely, it remains formally possible that these additional properties of cerulenin block or compensate for the *in vitro* and *in vivo* effects that would otherwise be caused by its FAS inhibitory activity). Because neither inhibition of FAS nor other cerulenin-like properties can account for our observations, C75 must have an additional activity. There are no data to preclude the existence of additional activities, and potential targets are hard to predict. For example, it has recently been reported (58) that C75 activates CPT I, an enzyme required for fatty acid metabolism, whereas indirect inhibition of CPT I had been predicted.

The extremely slow onset of C75-induced somatic excitation suggests gradual modulation of membrane conductances rather than direct channel block. Possible mechanisms include reduced K^+ channel activity or enhancement of depolarizing conductances such as I_h (59) or subthreshold Na^+ conductances (49, 60). Alternatively, if intracellular Ca^{2+} were decreased (*i.e.* by decreasing Ca^{2+} channel activity, increasing Ca^{2+} extrusion, or other actions), the consequent reduction in K_{Ca} activity could potentially trigger bursting or depolarization. Earlier work in Purkinje neurons suggests that such a mechanism is possible (61, 62, 63). Finally, interference with ion transport could cause a gradual depolarization of membrane potential and could also be involved in loosening of stools *in vivo*.

The concentrations of C75 (25–100 μM) used in these *in vitro* experiments were probably not unreasonably high. For comparison, in previous studies, peak inhibition of feeding was obtained with 15 mg/kg ip (59.0 μmol/kg). 82% inhibition of feeding was obtained with intracerebroventricular injection of 2 μl of 5.0 μg/μl (19.66 nmol/μl) C75 (8); and this translates into 39.3 μM, assuming even distribution into 1 ml of brain tissue, and a much higher initial concentration in CSF and circumventricular areas such as the arcuate nucleus, which straddles the third ventricle. The ineffectiveness of cerulenin was probably not attributable to insufficient drug. Cerulenin was used *in vitro* at concentrations similar to those used effectively in other systems (53, 56, 64, 65, 66). Even though cerulenin has been shown to be more potent than C75 in inhibiting growth of fibroblasts and a breast cancer cell line (7), cerulenin was still ineffective at 8× (200 μM) the effective concentration of C75 (25 μM).

Loose-patch extracellular recording from POMC, NPY, and Purkinje neurons seems to be a useful way to test potential anorexigenic drugs for selectivity. Because small changes in membrane potential can have large effects on neuronal output (action potentials), extracellular recording is a sensitive and reliable method for testing compounds with potentially weak somatic effects.

In vivo

When given ip, C75 rapidly caused stool to become extremely loose or liquid, and this was accompanied by weight loss, decreased food intake, and inhibition of normal paper-shredding behavior. All of these effects are consistent with visceral illness. It has been reported that peripheral, but not central, administration of C75 induces visceral illness in rats (Ref. 50 , but see Ref. 67), although loose stools have not been reported. In contrast, cerulenin did not induce loose stools, even at a molar dose over 9-fold that of C75, suggesting that FAS inhibition was not responsible for this effect. Cerulenin also decreased food intake and body weight much less effectively than C75.

It is possible that loosening of stools and weight loss/anorexia are both manifestations of a C75-induced visceral illness (50). If so, then the inability of cerulenin to loosen stools would suggest that it does not induce a C75-type of visceral illness, and this would explain the relative weakness of cerulenin's anorexigenic activity. But if loose stools and anorexia are both parts of a visceral illness that cannot be reproduced by cerulenin, then it follows that these effects do not result from FAS inhibition. This conclusion contradicts current hypotheses about the mechanism of C75-induced weight loss (8, 51, 52). On the other hand, the inability of cerulenin to reproduce the actions of C75 could result from low penetrance into the CNS, rapid inactivation, or rapid clearance relative to C75. It is also possible that the extreme loosening of stools and the weight loss/anorexia induced by ip C75 are unrelated.

Our results with cerulenin are consistent with an earlier report in rat in which, even after extended administration, the effect of cerulenin was very modest (68), although a more substantial effect was seen in a different report in mice (8). Cerulenin interferes with numerous physiological processes (64, 65, 69, 70), and where mechanism has been determined, it has often been found to result from inhibition of protein acylation (55, 56, 66,71, 72) rather than fatty acid synthesis (73). Given this nonspecificity, and the extremely high doses typically required, the mechanism by which cerulenin induces weight loss is unclear.

In all treated MC4-RKO mice, C75 induced abundant very soft or liquefied stools, weight loss, and decreased food intake, and results were similar to those in wild-type mice. Therefore, C75 does not require the receptor most responsible for the influence of POMC on feeding. This is consistent with an earlier report showing that in *ob/ob* mice, the effect of C75 was undiminished, despite a lack of effect on hypothalamic POMC expression (11).

Given its *in vitro* nonspecificity, C75 might cause visceral illness by activating neurons of the area postrema (AP) or enteric nervous system. Diarrhea usually results from decreased absorption of electrolytes and water and/or increased active secretion of anions into the intestinal lumen (74). To this end, C75 could directly affect ion transport in the intestinal epithelium. Alternatively, C75 could activate intestinal smooth muscle, another excitable cell type, in such a way as to cause hypermotility and reduce time available for water uptake (74). However, our data do not address these possibilities. It is also possible that the AP, a critical CNS sensor of circulating toxins that is involved in nausea and vomiting (75), interprets C75 as a toxin. Part of the adjacent nucleus tractus solitarius (NTS) is also involved in regulating feeding (76, 77). Both are especially attractive potential CNS targets of C75, because the AP and part of the NTS lie outside the blood brain barrier (75, 78). It has been reported recently that ip administration of C75 is followed rapidly by induction of c-Fos expression in the AP and NTS (52). Although only a small proportion of ip-injected C75 reaches the rest of the brain (8), a direct C75 effect there cannot be excluded, especially at other sites where the blood-brain barrier is weak or absent.

This work was supported by Grants DK55819 (to R.D.C.) and HG00201 (to J.L.S.).

Abbreviations: AGRP, Agouti-related protein; AP, area postrema; CART, cocaine and amphetamine regulated transcript; CoA, coenzyme A; CNS, central nervous system; CPT I, carnitine palmitoyltransferase I; DMSO, dimethylsulfoxide; EGFP, enhanced green fluorescent protein; FAS, fatty acid synthase; GABA, γ-aminobutyric acid; NPY, neuropeptide Y; NTS, nucleus tractus solitarius; POMC, proopiomelanocortin; RKO, receptor knockout; SAP, sapphire fluorescent protein.

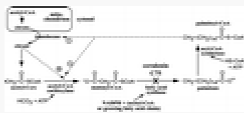


Fig. 1. Fatty acid synthesis. Inhibition of FAS causes malonyl-CoA concentration to increase, probably by both decreasing its utilization and increasing its synthesis. Normally, palmitoyl-CoA inhibits both citrate translocation and allosteric activation of acetyl-CoA carboxylase by citrate; but by blocking palmitate production, C75 could release this inhibition, resulting in increased synthesis of malonyl-CoA.

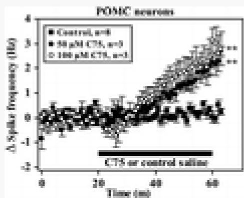
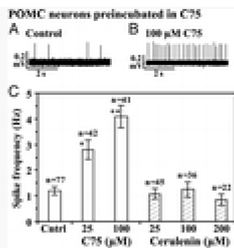


Fig. 2. After a 10-min delay, C75 induced a steady increase in the spike frequency of arcuate POMC neurons that continued even after perfusion with control saline was restored. This increase seemed to be preceded by a small, transient decrease in firing rate, especially at 100 μM C75. Data are plotted as mean ± SEM in 60-sec bins. **, Significantly different from control ($P < 0.001$); ANOVA was followed by Student-Newman-Keuls multiple-comparison post test.

Fig. 3. Preincubation in C75 increased the basal firing rate of arcuate POMC neurons. A and B, Representative traces from recordings



of POMC neurons after preincubation in control saline or 100 µM C75. C, Preincubation in C75, but not cerulenin, greatly increased the basal spike frequency of POMC neurons. Preincubations and recordings in all experiments were conducted in the presence of 1 mM kynurenic acid and 100 µM picrotoxin to block ionotropic glutamate receptors and $GABA_A$ receptors. Data are plotted as mean ±SEM. *, Significantly different from control ($P < 0.01$); **, significantly different from control ($P < 0.001$); Kruskal-Wallis test was followed by Dunn's multiple-comparison post test; 25 µMand 100 µM C75 results were not significantly different from each other.

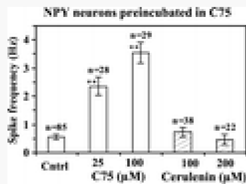


Fig. 4. Preincubation in C75, but not cerulenin, greatly increased the basal spike frequency of arcuate nucleus NPY neurons. **, Significantly different from control ($P < 0.001$); Kruskal-Wallis test was followed by Dunn's multiple-comparison post test; 25 µMand 100 µM C75 results were not significantly different from each other. Data are plotted as mean ± SEM.

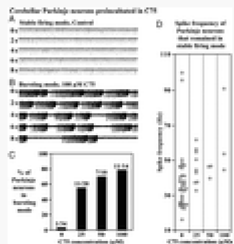


Fig. 5. Preincubation in C75 changed the firing mode and frequency distribution of cerebellar Purkinje cells. A, Stable firing mode in a control Purkinje cell. B, Bursting mode in a Purkinje cell preincubated in 100 µM C75. C, C75 induced a dose-dependent increase in the proportion of cells in bursting mode that was statistically significant by χ^2 test ($P < 0.0001$). D, Spike frequencies of all nonbursting Purkinje cells. Cells with low spike frequencies are rare after exposure to C75. C75 incubation seemed to shift cells either to higher spike frequencies or into bursting mode.

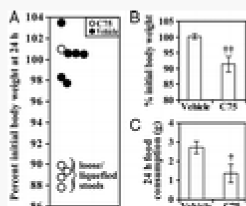


Fig. 6. Injection ip of 15 mg/kg C75 induced weight loss, a large decrease in food consumption, and very loose/liquefied stools. Vehicle, n = 6. C75, n = 5. A, Weight of individual mice, 24 h after injection with C75 or vehicle, displayed as percent initial weight. C75-induced weight loss was associated with

very loose or liquefied stools. B, Percent initial weight of C75-treated mice was significantly lower than that of vehicle-treated mice 24 h post injection. C, C75-treated mice consumed half as much food as vehicle-treated mice in the 24-h period after injection. B and C: †, $P < 0.05$; ††, $P = 0.005$; unpaired, two-tailed, homoscedastic t test. Data are plotted as mean ± SEM.

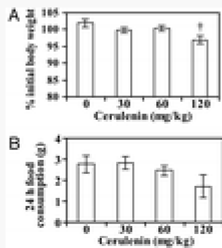


Fig. 7. Injection ip of cerulenin at 0, 30, 60, and 120 mg/kg did not induce loose stools and had a much smaller effect on body weight than C75 (n = 4 in each group). Data are plotted as mean ± SEM. A, Percent initial body weight 24 h after injection. B, Cumulative food consumption 24 h after injection. †, Significantly different from control ($P < 0.05$); ANOVA was followed by Dunnett's multiple-comparison test.

Fig. 8. In all MC4-RKO mice, ip injection of 15 mg/kg C75 robustly induced very loose/liquefied stools, weight loss, and decreased food intake. Because MC4-RKO mice are sensitive to stress, mice were acclimated to the protocol by weighing just before and 3 h after a daily ip injection of vehicle. A, Weight of individual mice over time. B, Percent change in body weight over the preceding 24 h. Data are plotted as mean ± SEM. C, Food consumed over the preceding 24 h for each mouse. ††, $P = 0.005$; †††, $P = 0.0003$, significantly different from the previous day by two-tailed, paired t test.

We thank Wei Fan for help and advice on *in vivo* experiments, Lora Heisler (Beth Israel Deaconess Medical Center and Harvard Medical School) for communication of unpublished results, Malcolm J. Low (Vollum Institute, Oregon Health and Science University) for use of POMC-EGFP transgenic mice, Jeffrey M. Friedman (Rockefeller University) for use of NPY-SAP transgenic mice, and colleagues of the Cone Laboratory for helpful discussion.

1. **Flegal KM, Carroll MD, Ogden CL, Johnson CL** 2002 Prevalence and trends in obesity among US adults, 1999–2000. *JAMA* 288:1723–1727 CrossRef, Medline
2. **Wolf AM, Colditz GA** 1998 Current estimates of the economic cost of obesity in the United States. *Obese Res* 6:97–106 CrossRef, Medline
3. **Pi-Sunyer FX** 1993 Medical hazards of obesity. *Ann Intern Med* 119:655–660 CrossRef, Medline
4. **Mun EC, Blackburn GL, Matthews JB** 2001 Current status of medical and surgical therapy for obesity. *Gastroenterology* 120:669–681 CrossRef, Medline
5. **Rosenbaum M, Leibel RL** 1998 The physiology of body weight regulation: relevance to the etiology of obesity in children. *Pediatrics* 101:525–539 Medline
6. **Rosenbaum M, Murphy EM, Heymsfield SB, Matthews DE, Leibel RL** 2002 Low-dose leptin administration reverses effects of sustained weight reduction on energy expenditure and circulating concentrations of thyroid hormones. *J Clin*

Endocrinol Metab 87:2391–2394Abstract, Medline
7.**Kuhajda FP, Pizer ES, Li JN, Mani NS, Frehywot GL, Townsend CA** 2000 Synthesis and antitumor activity of an inhibitor of fatty acid synthase. *Proc Natl Acad Sci USA*97:3450–3454 CrossRef, Medline
8.**Loftus TM, Jaworsky DE, Frehywot GL, Townsend CA, Ronnett GV, Lane MD, Kuhajda FP** 2000 Reduced food intake and body weight in mice treated with fatty acid synthase inhibitors. *Science* 288:2379–2381 CrossRef, Medline
9.**Thupari JN, Landree LE, Ronnett GV, Kuhajda FP** 2002 C75 increases peripheral energy utilization and fatty acid oxidation in diet-induced obesity. *Proc Natl Acad Sci USA* 99:9498–9502 CrossRef, Medline
10.**Kumar MV, Shimokawa T, Nagy TR, Lane MD** 2002 Differential effects of a centrally acting fatty acid synthase inhibitor in lean and obese mice. *Proc Natl Acad Sci USA*99:1921–1925 CrossRef, Medline
11.**Shimokawa T, Kumar MV, Lane MD** 2002 Effect of a fatty acid synthase inhibitor on food intake and expression of hypothalamic neuropeptides. *Proc Natl Acad Sci USA* 99:66–71 CrossRef, Medline
12.**Cowley MA, Smart JL, Rubinstein M, Cerdan MG, Diano S, Horvath TL, Cone RD, Low MJ** 2001 Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* 411:480–484 CrossRef, Medline
13.**Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, Gu W, Kesterson RA, Boston BA, Cone RD, Smith FJ, Campfield LA, Burn P, Lee F** 1997Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* 88:131–141CrossRef, Medline
14.**Butler AA, Marks DL, Fan W, Kuhn CM, Bartolome M, Cone RD** 2001 Melanocortin-4 receptor is required for acute homeostatic responses to increased dietary fat. *Nat Neurosci*4:605–611 CrossRef, Medline
15.**Ruderman NB, Saha AK, Vavvas D, Witters LA** 1999 Malonyl-CoA, fuel sensing, and insulin resistance. Am J Physiol 276:E1–E18
16.**Stryer L** 1995 Biochemistry. 4th ed. New York: W. H. Freeman and Company; 614–628
17.**Brun T, Roche E, Assimacopoulos-Jeannet F, Corkey BE, Kim KH, Prentki M** 1996Evidence for an anaplerotic/malonyl-CoA pathway in pancreatic β-cell nutrient signaling.*Diabetes* 45:190–198 CrossRef, Medline
18.**Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD** 1997 Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature* 385:165–168 CrossRef, Medline
19.**Brady LS, Smith MA, Gold PW, Herkenham M** 1990 Altered expression of hypothalamic neuropeptide mRNAs in food-restricted and food-deprived rats. *Neuroendocrinology* 52:441–447 CrossRef, Medline
20.**Bergendahl M, Wiemann JN, Clifton DK, Huhtaniemi I, Steiner RA** 1992 Short-term starvation decreases POMC mRNA but does not alter GnRH mRNA in the brain of adult male rats. *Neuroendocrinology* 56:913–920 CrossRef, Medline
21.**Schwartz MW, Seeley RJ, Woods SC, Weigle DS, Campfield LA, Burn P, Baskin DG**1997 Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. *Diabetes* 46:2119–2123 CrossRef, Medline
22.**Kristensen P, Judge ME, Thim L, Ribel U, Christjansen KN, Wulff BS, Clausen JT, Jensen PB, Madsen OD, Vrang N, Larsen PJ, Hastrup S** 1998 Hypothalamic CART is a new anorectic peptide regulated by leptin. *Nature* 393:72–76 CrossRef, Medline
23.**Thornton JE, Cheung CC, Clifton DK, Steiner RA** 1997 Regulation of hypothalamic proopiomelanocortin mRNA by leptin in *ob/ob* mice. *Endocrinology* 138:5063–5066 Abstract,Medline
24.**Elias CF, Aschkenasi C, Lee C, Kelly J, Ahima RS, Bjorbaek C, Flier JS, Saper CB, Elmquist JK** 1999 Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. *Neuron* 23:775–786 CrossRef, Medline
25.**Marks DL, Butler AA, Turner R, Brookhart G, Cone RD** 2003 Differential role of melanocortin receptor subtypes in cachexia. *Endocrinology* 144:1513–1523 Abstract, Medline
26.**Yaswen L, Diehl N, Brennan MB, Hochgeschwender U** 1999 Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. *Nat Med*5:1066–1070 CrossRef, Medline
27.**Krude H, Biebermann H, Luck W, Horn R, Brabant G, Gruters A** 1998 Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. *Nat Genet* 19:155–157 CrossRef, Medline
28.**Butler AA, Kesterson RA, Khong K, Cullen MJ, Pelleymounter MA, Dekoning J, Baetscher M, Cone RD** 2000 A unique metabolic syndrome causes obesity in the melanocortin-3 receptor-deficient mouse. *Endocrinology* 141:3518–3521 Abstract, Medline
29.**Chen AS, Marsh DJ, Trumbauer ME, Frazier EG, Guan XM, Yu H, Rosenblum CI, Vongs A, Feng Y, Cao L, Metzger JM, Strack AM, Camacho RE, Mellin TN, Nunes CN, Min W, Fisher J, Gopal-Truter S, MacIntyre DE, Chen HY, Van der Ploeg LH** 2000Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. *Nat Genet* 26:97–102 CrossRef, Medline
30.**Farooqi IS, Yeo GS, Keogh JM, Aminian S, Jebb SA, Butler G, Cheetham T, O'Rahilly S** 2000 Dominant and recessive inheritance of morbid obesity associated with melanocortin 4 receptor deficiency. *J Clin Invest* 106:271–279 CrossRef, Medline
31.**Cone RD** 2000 Haploinsufficiency of the melanocortin-4 receptor: part of a thrifty genotype? *J Clin Invest* 106:185–

187 CrossRef, Medline
32. **Vaisse C, Clement K, Durand E, Hercberg S, Guy-Grand B, Froguel P** 2000Melanocortin-4 receptor mutations are a frequent and heterogeneous cause of morbid obesity.*J Clin Invest* 106:253–262 CrossRef, Medline
33. **Chua Jr SC, Brown AW, Kim J, Hennessey KL, Leibel RL, Hirsch J** 1991 Food deprivation and hypothalamic neuropeptide gene expression: effects of strain background and the diabetes mutation. *Brain Res Mol Brain Res* 11:291–299 CrossRef, Medline
34. **Mizuno TM, Mobbs CV** 1999 Hypothalamic agouti-related protein messenger ribonucleic acid is inhibited by leptin and stimulated by fasting. *Endocrinology* 140:814–817 Abstract,Medline
35. **Stanley BG, Kyrkouli SE, Lampert S, Leibowitz SF** 1986 Neuropeptide Y chronically injected into the hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity.*Peptides* 7:1189–1192 CrossRef, Medline
36. **Rossi M, Kim MS, Morgan DG, Small CJ, Edwards CM, Sunter D, Abusnana S, Goldstone AP, Russell SH, Stanley SA, Smith DM, Yagaloff K, Ghatei MA, Bloom SR**1998 A C-terminal fragment of Agouti-related protein increases feeding and antagonizes the effect of α-melanocyte stimulating hormone *in vivo*. *Endocrinology* 139:4428–4431 Abstract,Medline
37. **Lu D, Willard D, Patel IR, Kadwell S, Overton L, Kost T, Luther M, Chen W, Woychik RP, Wilkison WO, Cone RD** 1994 Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. *Nature* 371:799–802 CrossRef, Medline
38. **Haskell-Luevano C, Chen P, Li C, Chang K, Smith MS, Cameron JL, Cone RD** 1999Characterization of the neuroanatomical distribution of agouti-related protein immunoreactivity in the rhesus monkey and the rat. *Endocrinology* 140:1408–1415 Abstract, Medline
39. **Bagnol D, Lu XY, Kaelin CB, Day HE, Ollmann M, Gantz I, Akil H, Barsh GS, Watson SJ** 1999 Anatomy of an endogenous antagonist: relationship between Agouti-related protein and proopiomelanocortin in brain. J Neurosci 19:RC26:1–7
40. **Cowley MA, Pronchuk N, Fan W, Dinulescu DM, Colmers WF, Cone RD** 1999Integration of NPY, AGRP, and melanocortin signals in the hypothalamic paraventricular nucleus: evidence of a cellular basis for the adipostat. *Neuron* 24:155–163 CrossRef, Medline
41. **Cowley MA, Smith RG, Diano S, Tschop M, Pronchuk N, Grove KL, Strasburger CJ, Bidlingmaier M, Esterman M, Heiman ML, Garcia-Segura LM, Nillni EA, Mendez P, Low MJ, Sotonyi P, Friedman JM, Liu H, Pinto S, Colmers WF, Cone RD, Horvath TL** 2003The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron* 37:649–661 CrossRef, Medline
42. **Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, Wren AM, Brynes AE, Low MJ, Ghatei MA, Cone RD, Bloom SR** 2002 Gut hormone PYY(3–36) physiologically inhibits food intake. *Nature* 418:650–654 CrossRef, Medline
43. **Heisler LK, Cowley MA, Tecott LH, Fan W, Low MJ, Smart JL, Rubinstein M, Tatro JB, Marcus JN, Holstege H, Lee CE, Cone RD, Elmquist JK** 2002 Activation of central melanocortin pathways by fenfluramine. *Science* 297:609–611 CrossRef, Medline
44. Deleted in proof
45. **Pollok BA, Heim R** 1999 Using GFP in FRET-based applications. *Trends Cell Biol* 9:57–60 CrossRef, Medline
46. **Roberts WM, Almers W** 1992 Patch voltage clamping with low-resistance seals: loose patch clamp. *Methods Enzymol* 207:155–176 CrossRef, Medline
47. **De Souza J, Butler AA, Cone RD** 2000 Disproportionate inhibition of feeding in A(y) mice by certain stressors: a cautionary note. *Neuroendocrinology* 72:126–132 CrossRef, Medline
48. **Hausser M, Clark BA** 1997 Tonic synaptic inhibition modulates neuronal output pattern and spatiotemporal synaptic integration. *Neuron* 19:665–678 CrossRef, Medline
49. **Llinas R, Sugimori M** 1980 Electrophysiological properties of in vitro Purkinje cell somata in mammalian cerebellar slices. *J Physiol* 305:171–195 CrossRef, Medline
50. **Clegg DJ, Wortman MD, Benoit SC, McOsker CC, Seeley RJ** 2002 Comparison of central and peripheral administration of C75 on food intake, body weight, and conditioned taste aversion. *Diabetes* 51:3196–3201 CrossRef, Medline
51. **Wortman MD, Clegg DJ, D'Alessio D, Woods SC, Seeley RJ** 2003 C75 inhibits food intake by increasing CNS glucose metabolism. *Nat Med* 9:483–485 CrossRef, Medline
52. **Gao S, Lane MD** 2003 Effect of the anorectic fatty acid synthase inhibitor C75 on neuronal activity in the hypothalamus and brainstem. *Proc Natl Acad Sci USA* 100:5628–5633 CrossRef, Medline
53. **Omura S** 1981 Cerulenin. *Methods Enzymol* 72:520–532 CrossRef, Medline
54. **Schlesinger MJ, Malfer C** 1982 Cerulenin blocks fatty acid acylation of glycoproteins and inhibits vesicular stomatitis and Sindbis virus particle formation. *J Biol Chem* 257:9887–9890Medline
55. **Jochen AL, Hays J, Mick G** 1995 Inhibitory effects of cerulenin on protein palmitoylation and insulin internalization in rat adipocytes. *Biochim Biophys Acta* 1259:65–72 CrossRef,Medline
56. **Lawrence DS, Zilfou JT, Smith CD** 1999 Structure-activity studies of cerulenin analogues as protein palmitoylation

inhibitors. *J Med Chem* 42:4932–4941 CrossRef, Medline
57. **Wall L, Meighen E** 1989 Covalent reaction of cerulenin at the active site of acyl-CoA reductase of Photobacterium phosphoreum. *Biochem Cell Biol* 67:163–167 CrossRef, Medline
58. **Thupari JN, Pinn ML, Kuhajda FP** 2001 Fatty acid synthase inhibition in human breast cancer cells leads to malonyl-CoA-induced inhibition of fatty acid oxidation and cytotoxicity. *Biochem Biophys Res Commun* 285:217–223 CrossRef, Medline
59. **Robinson RB, Siegelbaum SA** 2003 Hyperpolarization-activated cation currents: from molecules to physiological function. *Annu Rev Physiol* 65:453–480 CrossRef, Medline
60. **Do MT, Bean BP** 2003 Subthreshold sodium currents and pacemaking of subthalamic neurons. Modulation by slow inactivation. *Neuron* 39:109–120 CrossRef, Medline
61. **Womack M, Khodakhah K** 2002 Active contribution of dendrites to the tonic and trimodal patterns of activity in cerebellar Purkinje neurons. *J Neurosci* 22:10603–10612 Medline
62. **Womack MD, Khodakhah K** 2002 Characterization of large conductance Ca2+-activated K+ channels in cerebellar Purkinje neurons. *Eur J Neurosci* 16:1214–1222 CrossRef, Medline
63. **Womack MD, Khodakhah K** 2003 Somatic and dendritic small-conductance calcium-activated potassium channels regulate the output of cerebellar purkinje neurons. *J Neurosci*23:2600–2607 Medline
64. **Falo Jr LD, Benacerraf B, Rothstein L, Rock KL** 1987 Cerulenin is a potent inhibitor of antigen processing by antigen-presenting cells. *J Immunol* 139:3918–3923 Medline
65. **Ueno K** 2000 Involvement of fatty acid synthase in axonal development in mouse embryos. *Genes Cells* 5:859–869 CrossRef, Medline
66. **Hurley JH, Cahill AL, Currie KP, Fox AP** 2000 The role of dynamic palmitoylation in Ca2+ channel inactivation. *Proc Natl Acad Sci USA* 97:9293–9298 CrossRef, Medline
67. **Kim EK, Miller I, Landree LE, Borisy-Rudin FF, Brown P, Tihan T, Townsend CA, Witters LA, Moran TH, Kuhajda FP, Ronnett GV** 2002 Expression of FAS within hypothalamic neurons: a model for decreased food intake after C75 treatment. Am J Physiol Endocrinol Metab 283:E867–E879
68. **Makimura H, Mizuno TM, Yang XJ, Silverstein J, Beasley J, Mobbs CV** 2001Cerulenin mimics effects of leptin on metabolic rate, food intake, and body weight independent of the melanocortin system, but unlike leptin, cerulenin fails to block neuroendocrine effects of fasting. *Diabetes* 50:733–739 CrossRef, Medline
69. **Yajima H, Komatsu M, Yamada S, Straub SG, Kaneko T, Sato Y, Yamauchi K, Hashizume K, Sharp GW, Aizawa T** 2000 Cerulenin, an inhibitor of protein acylation, selectively attenuates nutrient stimulation of insulin release: a study in rat pancreatic islets. *Diabetes* 49:712–717 CrossRef, Medline
70. **Yamada S, Komatsu M, Aizawa T, Sato Y, Yajima H, Yada T, Hashiguchi S, Yamauchi K, Hashizume K** 2002 Time-dependent potentiation of the β-cell is a Ca2+-independent phenomenon. *J Endocrinol* 172:345–354 CrossRef, Medline
71. **Oda T, Wu HC** 1993 Cerulenin inhibits the cytotoxicity of ricin, modeccin, Pseudomonas toxin, and diphtheria toxin in brefeldin A-resistant cell lines. *J Biol Chem* 268:12596–12602 Medline
72. **Straub SG, Yajima H, Komatsu M, Aizawa T, Sharp GW** 2002 The effects of cerulenin, an inhibitor of protein acylation, on the two phases of glucose-stimulated insulin secretion. Diabetes 51(Suppl 1):S91–S95
73. **Kuhajda FP, Jenner K, Wood FD, Hennigar RA, Jacobs LB, Dick JD, Pasternack GR**1994 Fatty acid synthesis: a potential selective target for antineoplastic therapy. *Proc Natl Acad Sci USA* 91:6379–6383 CrossRef, Medline
74. **Semrad CE, Chang EB** 2000 Approach to the patient with diarrhea. In: Humes HD, ed. Kelley's textbook of internal medicine. Philadelphia: Lippincott Williams, Wilkins; 738–755
75. **Miller AD, Leslie RA** 1994 The area postrema and vomiting. *Front Neuroendocrinol*15:301–320 CrossRef, Medline
76. **Schwartz GJ, Moran TH** 2002 Leptin and neuropeptide y have opposing modulatory effects on nucleus of the solitary tract neurophysiological responses to gastric loads: implications for the control of food intake. *Endocrinology* 143:3779–3784 Abstract, Medline
77. **Schwartz GJ** 2000 The role of gastrointestinal vagal afferents in the control of food intake: current prospects. *Nutrition* 16:866–873 CrossRef, Medline
78. **Gross PM, Wall KM, Pang JJ, Shaver SW, Wainman DS** 1990 Microvascular specializations promoting rapid interstitial solute dispersion in nucleus tractus solitarius. Am J Physiol 259:R1131–R1138