

Fatty Acid Synthase Inhibitors Modulate Energy Balance via Mammalian Target of Rapamycin Complex 1 Signaling in the Central Nervous System

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OBJECTIVE—Evidence links the hypothalamic fatty acid synthase (FAS) pathway to the regulation of food intake and body weight. This includes pharmacological inhibitors that potently reduce feeding and body weight. The mammalian target of rapamycin (mTOR) is an intracellular fuel sensor whose activity in the hypothalamus is also linked to the regulation of energy balance. The purpose of these experiments was to determine whether hypothalamic mTOR complex 1 (mTORC1) signaling is involved in mediating the effects of FAS inhibitors.

RESEARCH DESIGN AND METHODS—We measured the hypothalamic phosphorylation of two downstream targets of mTORC1, S6 kinase 1 (S6K1) and S6 ribosomal protein (S6), after administration of the FAS inhibitors C75 and cerulenin in rats. We evaluated food intake in response to FAS inhibitors in rats pretreated with the mTOR inhibitor rapamycin and in mice lacking functional *S6K1* (*S6K1*^{-/-}). Food intake and phosphorylation of S6K1 and S6 were also determined after C75 injection in rats maintained on a ketogenic diet.

RESULTS—C75 and cerulenin increased phosphorylation of S6K1 and S6, and their anorexic action was reduced in rapamycin-treated rats and in *S6K1*^{-/-} mice. Consistent with our previous findings, C75 was ineffective at reducing caloric intake in ketotic rats. Under ketosis, C75 was also less efficient at stimulating mTORC1 signaling.

CONCLUSIONS—These findings collectively indicate an important interaction between the FAS and mTORC1 pathways in the central nervous system for regulating energy balance, possibly via modulation of neuronal glucose utilization. *Diabetes* 57: 3231–3238, 2008

Energy balance is achieved when caloric intake is matched to expenditure. A complex neuroendocrine system underlies this process and regulates energy homeostasis in mammals. In addition to sensing hormonal signals of stored fuels, such as leptin (1), specific populations of neurons in the central nervous system (CNS), and particularly within the hypothalamus, have the ability to sense locally available nutrients, including glucose (2), fatty acids (3), and amino acids (4,5).

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Recent evidence has highlighted the role of intracellular fuel sensing in the regulation of energy balance (6). In particular, the biochemical pathway underlying fatty acid metabolism has been involved in the regulation of both feeding and glucose homeostasis (6–10). Fatty acid synthase (FAS) catalyzes the condensation of malonyl-CoA and acetyl-CoA to generate long-chain fatty acids (LCFAs). Acetyl-CoA carboxylase (ACC) and FAS are expressed in the hypothalamus (7), where malonyl-CoA (11) and LCFA-CoA levels (8) decrease during fasting and increase after refeeding. Studies using FAS inhibitors and other pharmacological or genetic tools that modify the activity of different enzymes regulating fatty acid metabolism support a role for this pathway in the regulation of feeding (9,12–15).

Peripheral administration of the natural FAS inhibitor cerulenin (2,3-epoxy-4-oxo-6-dodecadienoylamide) or the synthetic inhibitor C75 (trans-4-carboxy-5-octyl-3-methylenebutyrolactone) causes profound dose-dependent anorexia and weight loss in several rodent models (12,13,15,16). Reduced food intake is also observed with central administration of much lower doses of C75, suggesting that the brain is the key site of action (12). Increased hypothalamic malonyl-CoA is necessary for the anorexic and weight-reducing effects of FAS inhibitors (9,15,17). Interestingly, the ability of leptin to reduce food intake depends on increased hypothalamic malonyl-CoA (8) and possibly palmitoyl-CoA (18), which are achieved through the concomitant inhibition of AMP-activated protein kinase (AMPK) and activation of ACC (18). Therefore, the hypothalamic fatty acid synthesis pathway appears to process different fuel signals and convert them into efferent outputs that prevent further consumption of nutrients.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that controls critical aspects of cell growth (19). mTOR is a component of at least two multiprotein complexes: mTOR complex 1 (mTORC1), which includes raptor, and mTOR complex 2 (mTORC2), which includes rictor. Whereas mTORC2 regulates phosphorylation of Akt, mTORC1 modulates the activity of S6 kinase 1 (S6K1) and 4E binding protein 1 (20). Notably, the phosphorylation of S6K1 at Thr 389 is one of the markers commonly used to evaluate mTORC1 activity in vivo (21). Insulin, IGF, amino acids, and glucose all activate intracellular cascades that lead to activation of mTORC1 (22). We reported that the anorexic action of central leptin and leucine are both dependent on the activation of mTORC1 signaling in the hypothalamus (4). Given the ability of mTORC1 to sense and integrate fuel signals, and the role it plays in controlling food intake (4), we hypothesized that mTORC1 signaling is involved in monitoring the biochemical changes induced by the modulation of hypothalamic fatty acid metabolism.

RESEARCH DESIGN AND METHODS

Animals. Adult male Long-Evans rats, *S6K1*^{-/-} mice, and their wild-type littermates (23) were used. Mice were 8–11 weeks old in the cerulenin study and 7 months old in the C75 study. All animals were housed individually and maintained on a 12:12-h light-dark cycle, standard lab chow (Harlan-Teklad, Indianapolis, IN), and water. All animal procedures were approved by the institutional animal care and use committee of the University of Cincinnati. Rats were implanted with intracerebroventricular (icv) cannulas as previously described (24) and allowed to recover for a minimum of 7 days. Cannula placement was confirmed by consuming >5 ml water after 10 ng angiotensin II icv.

Drugs. C75 (Calbiochem; EMD Bioscience, La Jolla, CA) was dissolved in RPMI (Gibco, Carlsbad, CA). Cerulenin (Sigma-Aldrich, St. Louis, MO) was first dissolved in DMSO followed by RPMI, to a final concentration of 25% DMSO. Rapamycin (Calbiochem, EMD Bioscience) was dissolved in DMSO. All vehicles served as controls.

Ketogenic diet. Rats were placed on a ketogenic diet (80.7% lipid, 14.3% protein, and 5% carbohydrate calories; Dyet 100959; Dyets, Bethlehem, PA) for 4 weeks (13). Blood β -hydroxybutyrate concentration was measured using the KetoSite test (Stanbio Laboratory, Boerne, TX).

Leucine measurement. Plasma samples were deproteinized with sulfosalicylic acid and analyzed using an automated Hitachi L8800 Amino Acid Analyzer.

Western blot. A wedge of mediobasal hypothalamus was dissected using as landmarks the mammillary bodies caudally, the optic chiasm rostrally, the optic tract laterally, and the apex of the third ventricle. Tissues were homogenized in radioimmunoprecipitation assay buffer with addition of phosphatase and protease inhibitors (Santa Cruz Biotechnology). Samples (70 μ g) were loaded on either a 9–13% (experiment 1) or a 10% (experiments 3 and 4) SDS-polyacrylamide gel (Bio-Rad). Proteins were separated by electrophoresis and transferred to nitrocellulose membranes. Membranes were processed as previously described (4) before overnight incubation at 4°C with either phosphorylated S6K1 (pS6K1) at Thr 389 (1:500; Cell Signaling Technology, Beverly, MA) or phosphorylated S6 (pS6) at Ser 240/244 (1:500; Cell Signaling Technology). After washing in Tris-buffered saline with Tween (TBST), the membranes were incubated for 1 h at room temperature with secondary antibody conjugated with horseradish peroxidase (goat anti-rabbit; 1:2,000; Cell Signaling Technology). After washes in TBST, immunopositive bands were visualized by chemiluminescence (Lumiglo reagent and peroxide kit; Cell Signaling Technology) using exposure to radiographic films (Denville Scientific, South Plainfield, NJ). After protein detection, membranes were stripped for 15 min at 55°C with a solution containing 62.5 mmol/l Tris-HCl, 100 mmol/l 2-mercaptoethanol, and 2% SDS before 2-h blocking in TBST with 5% skim milk powder at room temperature and reblotted with either rabbit anti-p70-S6K1 (Cell Signaling Technology, 1:250) or rabbit anti-S6 (1:500; Cell Signaling Technology). Density was determined by National Institutes of Health program Scion image.

Experimental design

Experiment 1: effect of C75 on hypothalamic mTORC1 signaling. One hour before dark, rats were injected with C75 (30 μ g in 2 μ l icv) or vehicle and killed 30 min, 1 h, or 6 h later. Brains were removed, immediately frozen in isopentane, and stored at -80°C until Western blot analysis.

Experiment 2: effect of rapamycin on C75-induced anorexia in rats. Three hours before dark, rats were injected with either DMSO or rapamycin (25 μ g in 1 μ l icv) followed by RPMI or C75 (50 μ g in 3 μ l icv) 2 h later. Food was weighed at 1, 4, 8, and 24 h. Body weight was measured after 24 h. As previously reported (18), we found that some CNS active compounds, including leptin and C75, are less effective when animals are pretreated with DMSO. In these conditions, the dose of C75 had to be raised to 50 μ g to reach the same degree of anorexia as that observed with 30 μ g.

Experiment 3: anorexic actions of C75 and cerulenin in *S6K1*^{-/-} mice. One hour before dark, mice were injected intraperitoneally with C75 (20 mg/kg body wt), cerulenin (160 mg/kg body wt), or their respective vehicles; and food was weighed at 1, 4, 10, and 24 h. Body weight was measured at 24 h. The experiments used a within-subjects design with treatment order counter-balanced and 1 week between each treatment.

Experiment 4: effect of cerulenin on hypothalamic mTORC1 signaling. Rats were injected intracerebroventricularly with cerulenin (90 μ g in 2 μ l) and killed as in experiment 1.

Experiment 5: effect of the ketogenic diet on C75-induced anorexia. Rats were divided into two groups (saccharin or sucrose). All rats had ad libitum access to a ketogenic diet, water, and a sucrose or saccharin solution (13). Four weeks later, rats were fasted for 24 h, and ketosis was confirmed by a significant elevation of blood β -hydroxybutyrate in rats that had access to saccharin versus sucrose. Three days later, rats were fasted overnight and

given RPMI or C75 (30 μ g in 2 μ l icv) 1 h before lights off. Food and bottles of sucrose or saccharin were returned and weighed at 4 and 24 h.

Experiment 6: effect of the ketogenic diet on C75-induced changes in pS6K1 and pS6. The protocol was the same as for experiment 5, except that food, sucrose, and saccharin were not returned after the injection to avoid any confounding effect of caloric intake. Rats were killed 1 h after receiving either C75 or RPMI, and brains were collected for Western blot, as described above.

Statistical analyses. All values are expressed as means \pm SE. Data were analyzed by two-way ANOVA for multiple group designs and one-way repeated measures ANOVA for within-subjects design, followed by Fisher's least significant differences post hoc tests. Designs with only two groups were assessed with unpaired *t* tests. Experiment-wise significance was two-tailed and set at *P* < 0.05.

RESULTS

C75 increases hypothalamic mTORC1 signaling. C75 had no effect on the phosphorylation of either S6K1 (pS6K1/S6K1: RPMI, 100.00 \pm 8.36% vs. C75, 86.49 \pm 11.68% of RPMI; *P* = 0.365) or S6 (pS6/S6: RPMI, 100 \pm 18.36% vs. C75, 105.59 \pm 14.45% of RPMI; *P* = 0.816) 30 min after injection. At 1 h, C75 increased the phosphorylation of S6 (Fig. 1A and B), and at 6 h, this was accompanied by a significant elevation of pS6K (Fig. 1C and D).

mTORC1 signaling mediates the anorexic action of C75. We have found that refeeding activates hypothalamic mTORC1 signaling, whereas pharmacological inhibition of CNS mTOR increases food intake in rats (4). Given that C75 increased hypothalamic pS6K1 and pS6, we evaluated whether C75-induced anorexia depends on activation of the mTORC1 signaling by using the potent and selective mTOR inhibitor rapamycin (25). There was a main effect of the second drug treatment on food intake ($F_{(1, 46)} = 29.03$, *P* < 0.001). Within the 1st h after injection, C75 reduced food intake (*P* < 0.01), and this effect persisted for the following 24 h (Fig. 2A and B). There was a main effect of the first drug treatment on food intake ($F_{(1, 46)} = 5.53$, *P* < 0.05). The dose of rapamycin used decreased feeding in the 1st h (*P* < 0.01; Fig. 2A), which was surprising given that we had never observed an effect of this dose in any prior studies (Appendix 1, which is available in an online appendix at <http://dx.doi.org/10.2337/db07-1690>) (4). Nonetheless, pretreatment with rapamycin blocked C75-induced anorexia by 4 h (*P* < 0.05; Fig. 2A), and the inhibition persisted at 24 h (Fig. 2A and B). There was an interaction between the first and second drug treatments on food intake at 8 h ($F_{(1, 46)} = 4.16$, *P* < 0.05). There were main effects of the first ($F_{(1, 46)} = 7.63$, *P* < 0.01) and second drug treatments on body weight ($F_{(1, 46)} = 10.57$, *P* < 0.01). Rapamycin prevented the weight loss effect of C75 over 24 h (*P* < 0.05; Fig. 2C). The interaction between the two drug treatments approached significance ($F_{(1, 46)} = 4.03$, *P* = 0.05).

To assess the role of the mTORC1 pathway in mediating the potent anorexic effects of FAS inhibitors, we used *S6K1*^{-/-} and their wild-type littermates. As previously reported (26), *S6K1*^{-/-} mice had lower body weights than wild-type mice (wild type, 29.36 \pm 1.30 vs. *S6K1*^{-/-}, 25.60 \pm 0.78 g; *P* < 0.05). However, their cumulative 24-h food intake was similar to that of controls, whether expressed as total intake (Fig. 2E) or intake per kilogram body weight (wild type/RPMI, 139.68 \pm 26.99 vs. *S6K1*^{-/-}/RPMI, 156.38 \pm 15.52 g/kg body wt; *P* = 0.603). There was a main effect of drug on feeding ($F_{(1, 10)} = 30.07$, *P* < 0.001). C75 significantly decreased food intake in both genotypes in the 1st h (*P* < 0.01; Fig. 2D). The suppression lasted at least 24 h in wild-type mice (*P* < 0.01; Fig. 2D and E). However, the response of *S6K1*^{-/-} mice was signifi-

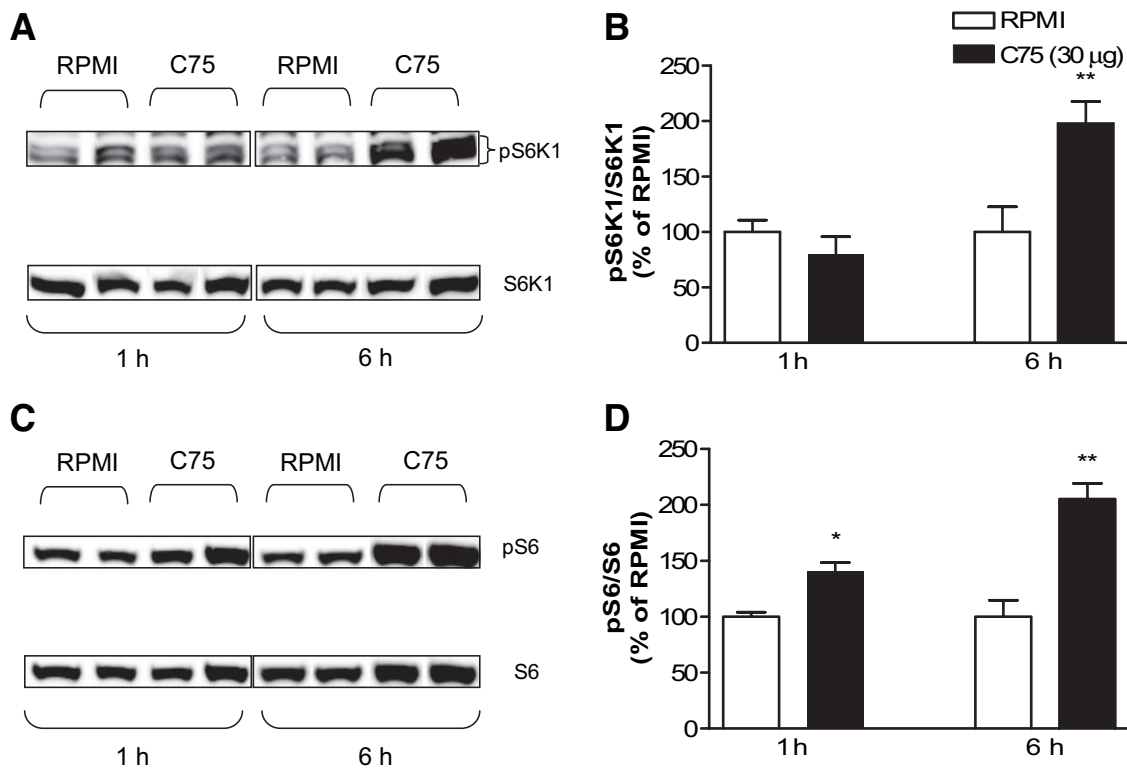


FIG. 1. C75 increases hypothalamic mTORC1 signaling. Representative Western blots from RPMI-treated or C75-treated (30 µg in 2 µl RPMI icv) rats (A and C) and quantification by image analysis of hypothalamic phosphorylation of S6K1 (B) and S6 (D). * $P < 0.05$; ** $P < 0.01$ vs. RPMI-treated rats. pS6K1: bands were quantified. Means \pm SE of seven rats in each condition.

cantly blunted at 4 h ($P < 0.05$; Fig. 2D), as revealed by a main effect of genotype ($F_{(1, 10)} = 5.69$, $P < 0.05$). There was a significant interaction between drug and genotype between 10 and 24 h ($F_{(1, 10)} = 7.06$, $P < 0.05$), even when expressed as grams per kilogram body weight (interval 10–24 h: wild type/C75, 8.95 ± 5.72 vs. $S6K1^{-/-}$ /C75, 29.34 ± 6.21 g/kg body weight; $P < 0.05$). We also found a main effect of drug on weight loss ($F_{(1, 10)} = 49.28$, $P < 0.001$), and there was a trend for C75 to be less potent in $S6K1^{-/-}$ ($P = 0.077$; Fig. 2F). Thus, the pharmacological and genetic data imply that intact mTORC1 signaling is necessary for the full anorexic effect of C75 in rats and mice at time points beyond 4 h.

Cerulein activates hypothalamic mTORC1 signaling, and this effect is required for its anorexic action. Because no pharmacological agent is specific to a single pathway, we further tested our hypothesis by using a second FAS inhibitor. Cerulein activated mTORC1 signaling in a pattern similar to that of C75 by phosphorylating both S6K1 and S6 (Fig. 3A and B). Moreover, cerulein also required intact mTORC1 signaling to cause anorexia. Consistent with previous reports (15,16), cerulein decreased food consumption, as revealed by a main effect of drug that started 1 h after injection in both genotypes ($F_{(1, 13)} = 33.61$, $P < 0.001$). This effect persisted in the following 4- to 24-h period in wild type ($P < 0.001$), but not in $S6K1^{-/-}$, whose food consumption was similar to that of VEH-injected $S6K1^{-/-}$ after 4 h (Fig. 3C). There was also a main effect of drug on 24-h weight loss ($F_{(1, 13)} = 22.00$, $P < 0.001$), but only in wild type (Fig. 3E). There was a trend for an interaction between genotype and drug on feeding at 4 h, which reached significance at 24 h ($F_{(1, 13)} = 15.84$, $P < 0.01$; Fig. 3D) and was also found for weight loss ($F_{(1, 13)} = 10.61$, $P < 0.01$; Fig. 3E). The observation that two different FAS inhibitors modulate

hypothalamic mTORC1 and require mTORC1 signaling for their anorexic action makes a strong case for a link between mTORC1 and FAS in the regulation of feeding.

The actions of C75 on hypothalamic mTORC1 signaling are blunted in ketotic rats. We had previously hypothesized that a key signal leading to C75-induced anorexia is derived, at least in part, from its ability to increase CNS glucose utilization, and we demonstrated that C75 does not reduce food intake under ketosis (13). Here, we explored the possibility that this is due to inability to modulate CNS mTORC1 signaling. Blood β -hydroxybutyrate was significantly elevated in rats given access to saccharin versus sucrose ($P < 0.001$; Fig. 4A). There was a main effect of drug on food intake at 4 h ($F_{(1, 28)} = 12.74$, $P < 0.01$) and at 24 h ($F_{(1, 28)} = 9.33$, $P < 0.01$). Consistent with its effect on chow, C75 reduced caloric intake in rats whose ketosis was prevented by access to sucrose ($P < 0.01$), and this effect lasted for 24 h. However, the caloric-reducing effect of C75 was blunted in ketotic rats receiving saccharin at 4 (data not shown) and 24 h (Fig. 4B) and was nearly statistically different from that of rats in the sucrose group ($P = 0.059$; Fig. 4B). These effects were accompanied by reduced hypothalamic mTORC1 signaling in ketotic rats compared with sucrose rats. There was a main effect of drug on pS6K1 ($F_{(1, 27)} = 5.56$, $P < 0.05$) and pS6 ($F_{(1, 26)} = 27.19$; $P < 0.001$). The effect of diet ($F_{(1, 26)} = 3.89$, $P = 0.059$) and the interaction between drug and diet ($F_{(1, 26)} = 3.92$, $P = 0.058$) nearly reached statistical significance for pS6. C75 was less efficient at increasing pS6 in ketotic rats versus sucrose rats ($P < 0.05$; Fig. 4D), and it increased pS6K1 only in sucrose rats ($P < 0.01$) (sucrose-C75 vs. saccharin-C75, $P < 0.05$; Fig. 4C). Thus, the anorexic action of C75 appears to depend on the rate of neuronal glucose uptake

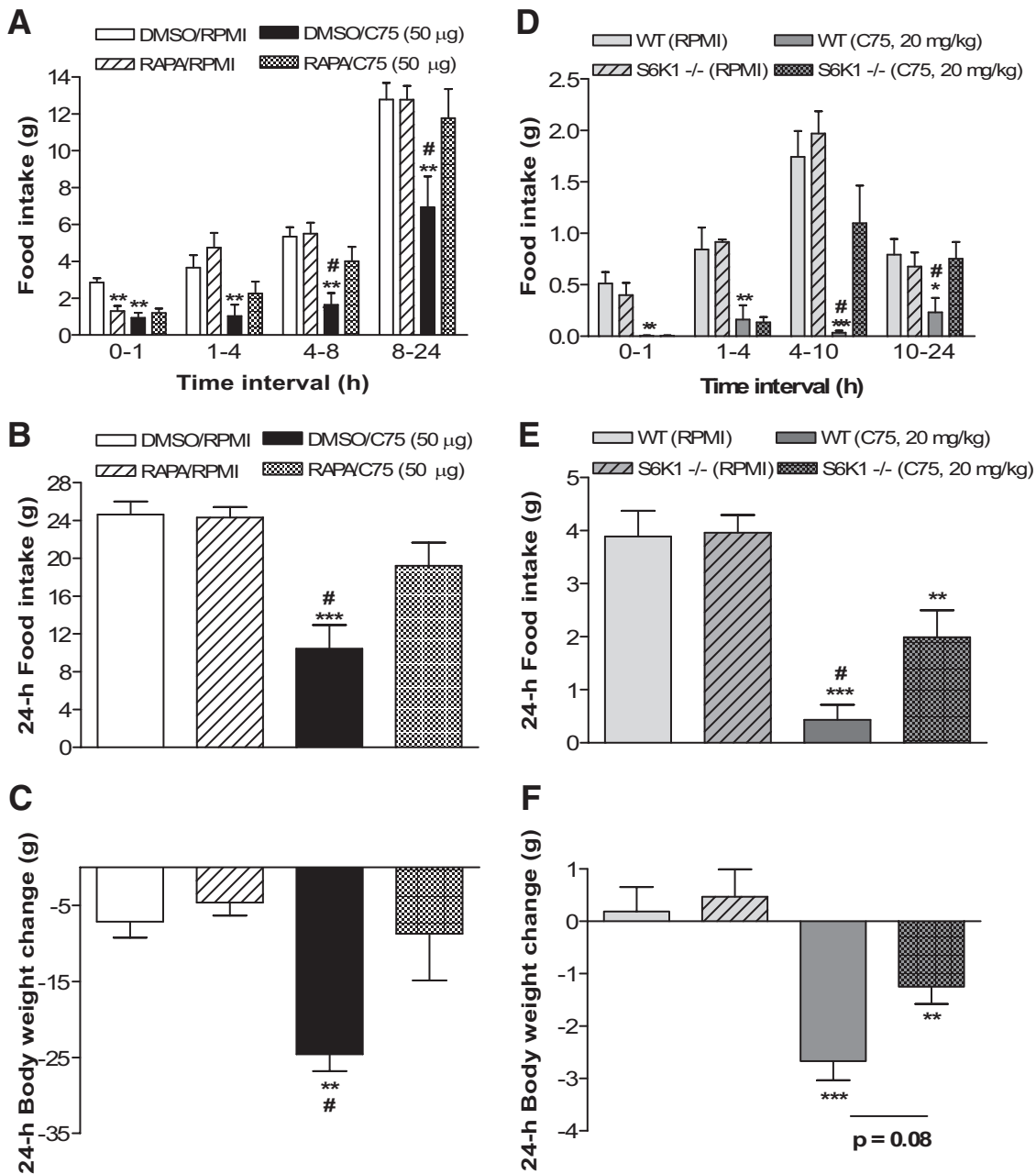


FIG. 2. mTORC1 signaling contributes to the anorexic effect of C75. Rapamycin (RAPA; 25 μ g in 1 μ l DMSO icv) prevents the effects of C75 (50 μ g in 3 μ l RPMI icv) on food intake (**A** and **B**) and on body weight change (**C**). Data are the mean of two separate experiments. Means \pm SE of 9–15 rats in each treatment group. ** P < 0.01; *** P < 0.001 vs. DMSO/RPMI-treated rats; # P < 0.05 vs. RAPA/C75-treated rats. **D** and **E**: The anorexic effect of C75 (20 mg/kg, in 1 ml/100 g body wt RPMI ip) is significantly reduced in *S6K1*^{-/-} mice. **F**: Body weight change over 24 h in mice injected with RPMI or C75. Means \pm SE of six mice in each treatment group. Food intake is expressed as the noncumulative amount consumed during different time intervals (**A** and **D**) and the cumulative amount eaten during 24 h (**B** and **E**). * P < 0.05; ** P < 0.01; *** P < 0.001 vs. wild-type (RPMI)-treated mice; # P < 0.05 vs. *S6K1*^{-/-} (C75)-treated mice.

and/or utilization. Moreover, this rate could also affect the ability of C75 to modulate mTORC1 signaling.

Ketosis is known to induce profound metabolic changes (27), so we compared sucrose- versus saccharin-treated rats on the same gel. Ketosis had no significant effect on the basal phosphorylation of S6K1 but caused a nonsignificant increase in S6 phosphorylation (Appendix 2, which is available in the online appendix). Rats fed a ketogenic diet have increased leucine levels in the cerebral cortex (28). Given the important role of leucine in regulating mTOR (29,30), we determined whether the ketogenic diet changes plasma leucine levels. However, we did not find

any difference in circulating leucine between the two groups (Fig. 4E).

DISCUSSION

Modulation of the FAS pathway represents a therapeutic strategy to produce weight loss and points to the important role of CNS metabolic pathways in regulating energy balance (6,31). Our previous work has demonstrated that activation of mTORC1 signaling in the hypothalamus reduces food intake (4). This led us to hypothesize that the ability of FAS inhibitors to modulate hypothalamic

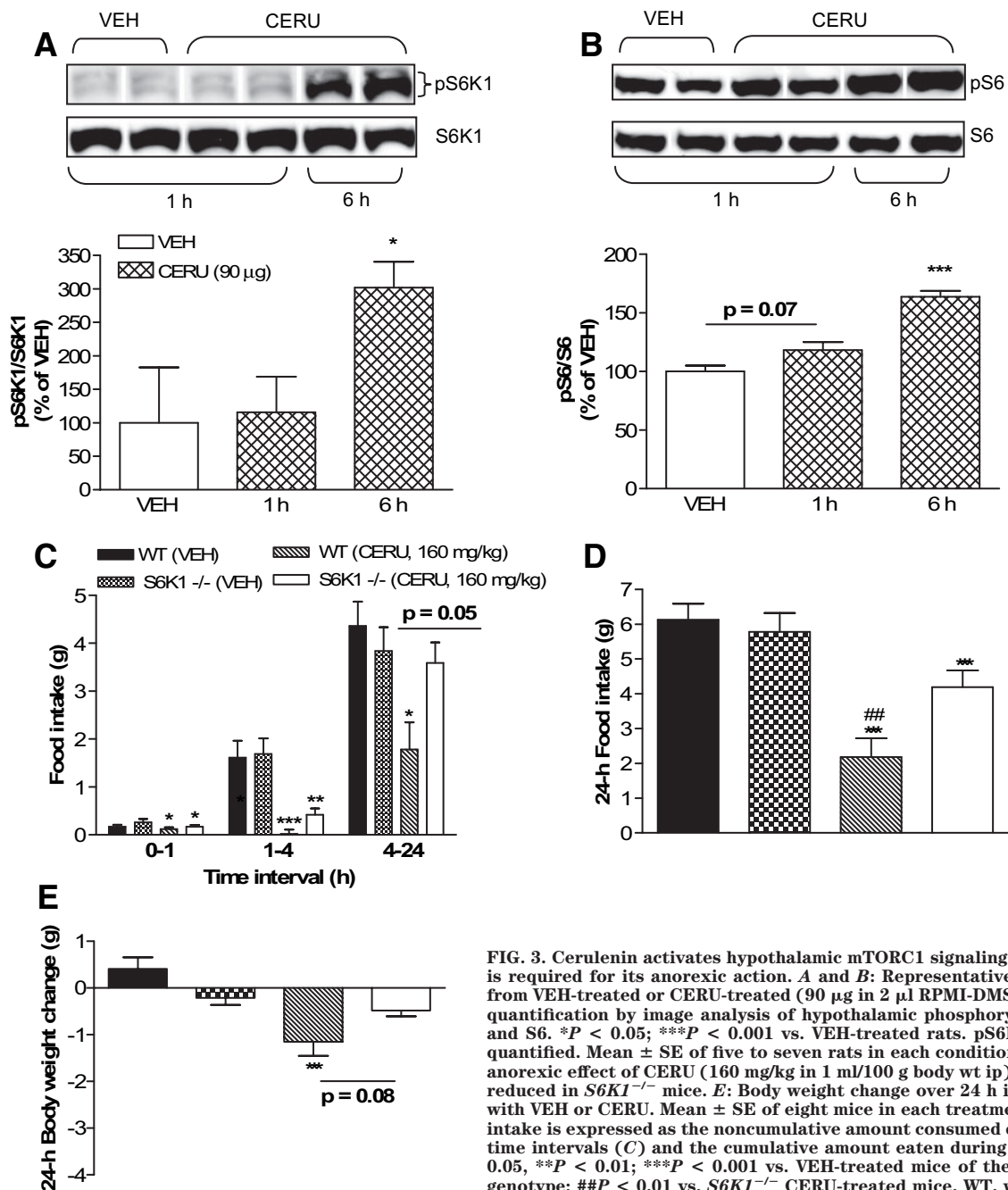


FIG. 3. Cerulenin activates hypothalamic mTORC1 signaling, and this effect is required for its anorexic action. **A** and **B**: Representative Western blots from VEH-treated or CERU-treated (90 µg in 2 µl RPMI-DMSO icv) rats and quantification by image analysis of hypothalamic phosphorylation of S6K1 and S6. * $P < 0.05$; *** $P < 0.001$ vs. VEH-treated rats. pS6K1: bands were quantified. Mean \pm SE of five to seven rats in each condition. **C** and **D**: The anorexic effect of CERU (160 mg/kg in 1 ml/100 g body wt ip) is significantly reduced in *S6K1*^{-/-} mice. **E**: Body weight change over 24 h in mice injected with VEH or CERU. Mean \pm SE of eight mice in each treatment group. Food intake is expressed as the noncumulative amount consumed during different time intervals (**C**) and the cumulative amount eaten during 24 h (**D**). * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$ vs. VEH-treated mice of the corresponding genotype; ## $P < 0.01$ vs. *S6K1*^{-/-} CERU-treated mice. WT, wildtype.

mTORC1 signaling is an important mechanism by which they cause anorexia.

Here, we report that central administration of C75 or cerulenin increases phosphorylation of hypothalamic S6K1 and S6. Furthermore, the ability of these compounds to modulate feeding depended on the activation of CNS mTORC1 signaling. In fact, intracerebroventricular rapamycin dampened the ability of C75 to reduce both feeding and body weight (4). We were surprised to observe an effect of rapamycin to reduce feeding in the 1st h after injection, given that we had not seen an effect of this dose in several prior studies (Appendix 1) (4). Nonetheless, it is important to point out that this effect on food intake is short-lived, with no effect observed beyond 1 h. Importantly, rapamycin actually resulted in an increase in intake in the presence of C75, which is incompatible with a major effect of rapamycin to reduce food intake.

Although rapamycin is quite specific in its effect on mTORC1 signaling (4), pharmacological inhibitors can have drawbacks that limit the interpretation of experimental results. Hence, we further tested our hypothesis in mice lacking *S6K1*, a key component of the mTORC1 pathway. C75 or cerulenin reduced both food intake and body weight in wild-type mice, as reported before (17,32). However, this effect was significantly reduced in *S6K1*^{-/-} mice. Interestingly, the data arising from both the pharmacological and genetic studies indicate that the activity of the mTORC1 pathway critically contributes to the anorexic effects of FAS inhibitors 4 h after drug injection. The time course observed in *S6K1*^{-/-} mice is a remarkable match for the observation made in rats that activation of mTORC1 remains elevated 6 h after either C75 or cerulenin. Such data underscore the importance of the mTORC1 signaling as one component

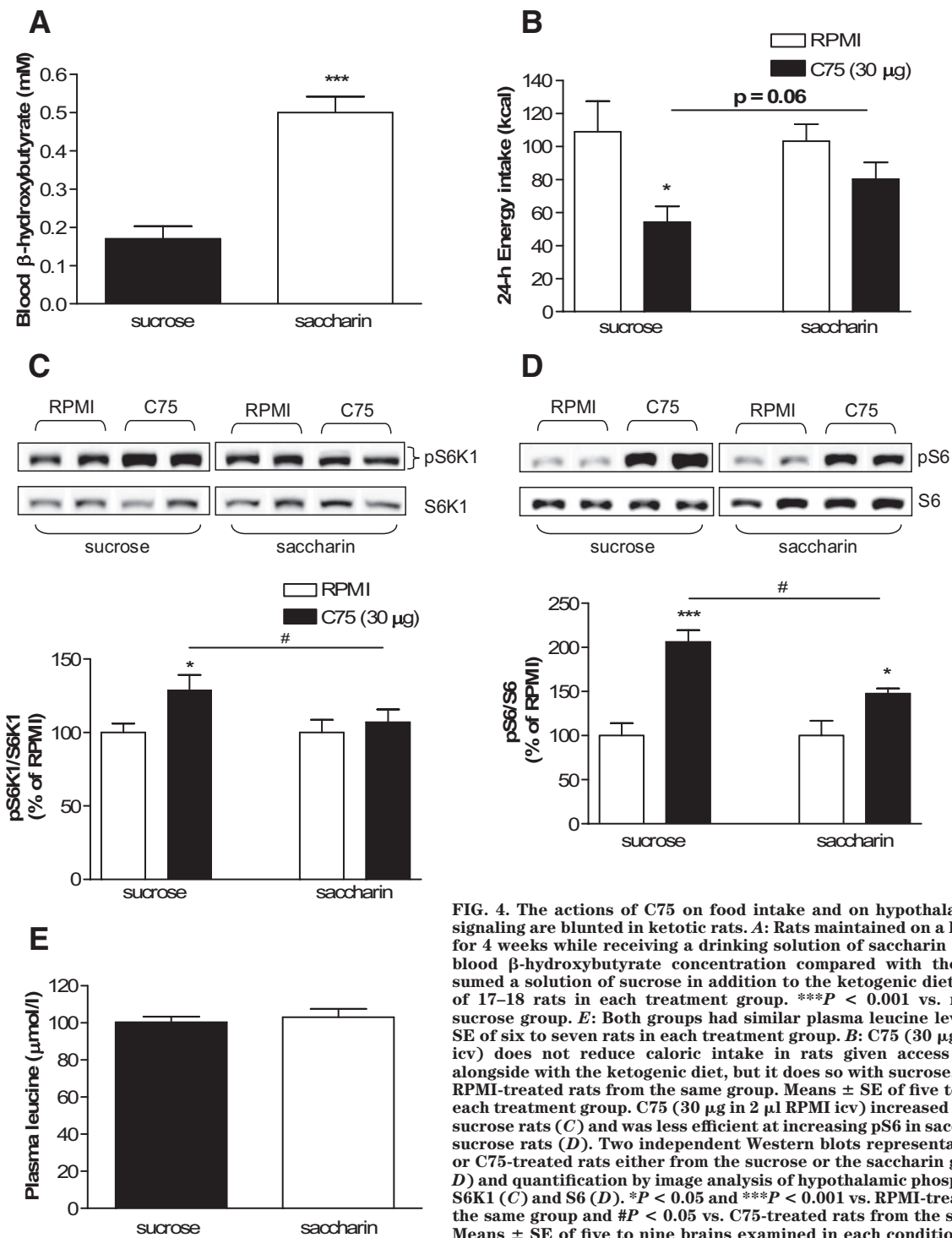


FIG. 4. The actions of C75 on food intake and on hypothalamic mTORC1 signaling are blunted in ketotic rats. **A:** Rats maintained on a ketogenic diet for 4 weeks while receiving a drinking solution of saccharin have elevated blood β -hydroxybutyrate concentration compared with those that consumed a solution of sucrose in addition to the ketogenic diet. Means \pm SE of 17–18 rats in each treatment group. *** P < 0.001 vs. rats from the sucrose group. **E:** Both groups had similar plasma leucine levels. Means \pm SE of six to seven rats in each treatment group. **B:** C75 (30 μ g in 2 μ l RPMI icv) does not reduce caloric intake in rats given access to saccharin alongside with the ketogenic diet, but it does so with sucrose. * P < 0.05 vs. RPMI-treated rats from the same group. Means \pm SE of five to eight rats in each treatment group. C75 (30 μ g in 2 μ l RPMI icv) increased pS6K1 only in sucrose rats (**C**) and was less efficient at increasing pS6 in saccharin rats vs. sucrose rats (**D**). Two independent Western blots representative of RPMI- or C75-treated rats either from the sucrose or the saccharin groups (**C** and **D**) and quantification by image analysis of hypothalamic phosphorylation of S6K1 (**C**) and S6 (**D**). * P < 0.05 and *** P < 0.001 vs. RPMI-treated rats from the same group and # P < 0.05 vs. C75-treated rats from the sucrose group. Means \pm SE of five to nine brains examined in each condition.

of a cascade that is essential to the anorexic effects of FAS inhibitors.

One proposed mechanism by which FAS inhibitors suppress food ingestion is by increasing CNS glucose utilization (13). Glucose metabolism is a biochemical index of nutrient status that plays a key role in the control of feeding. Quantitative and temporal changes in glucose concentration are closely monitored and integrated by specific subpopulations of “glucosensing” neurons in the CNS that are crucial to the regulation of feeding (6). A key finding to support the involvement of increased glucose use in mediating the effect of FAS inhibition is that rats on

a very low carbohydrate diet, which forces them to produce ketone bodies that neurons use in preference to glucose (33), do not reduce their food intake in response to C75 (13). In accordance with those previous results, we observed that rats maintained on a ketogenic diet but given sucrose to prevent ketosis responded to C75. In contrast, rats maintained on the ketogenic diet but given saccharin did not significantly reduce their food intake after C75. C75 increased S6 phosphorylation in rats on the ketogenic diet supplemented with sucrose, but this effect is blunted in those on the same diet supplemented with saccharin. This effect is consistent with what has been

observed under chow diet. It remains unknown how C75 increases the phosphorylation of S6K1 1 h after injection under a ketogenic diet supplemented with sucrose, whereas this phenomenon happens only at 6 h under a chow diet. This is an issue that we tested several times with multiple time points. In several studies, there was a strong trend for increased pS6K1 at 1 h, but this effect was not consistent across experiments, unlike the reliable effect to increase pS6. There could be a number of possibilities to explain why the time course of C75-induced phosphorylation of S6K1 differs between the two diets. The ketogenic diet induces a wide range of metabolic effects in addition to reducing glucose availability (27). These differences provide an important rationale for why we used the ketogenic diet supplemented with sucrose instead of a normal chow diet as the control for experiment 4. Nonetheless, these findings not only point to the permissive role played by neuronal glucose metabolism in the FAS inhibitors-induced anorexia, but they also highlight a mechanism through which the inhibition of FAS leads to the modulation of the mTORC1 signaling.

Kim et al. (17) ascribed the anorexic action of C75 to its ability to inhibit hypothalamic AMPK. Like mTOR, AMPK is a fuel sensor that plays a critical role in integrating signals of energy status in the hypothalamus and regulating food intake. However, in contrast to mTOR, hypothalamic AMPK is activated during negative energy balance, in which case it stimulates feeding (34). The relationship between these two fuel sensors is rather complex and appears to be bidirectional (35,36). Given the highly regulated crosstalk between mTOR and AMPK, the current experiments cannot rule out whether the effects of C75 on the mTORC1 pathway are directly mediated or occur as a result of inhibition of hypothalamic AMPK. Nonetheless, the data of Kim et al. (17) suggest that AMPK activity is involved primarily in the short-term actions of C75 because AMPK activators inhibit the effect of C75 specifically during the 1st h after drug administration, whereas our data suggest that mTORC1 signaling might be critical at later time points.

In conclusion, we provide evidence that links the FAS pathway to the mTORC1 signaling cascade in the CNS and, although the current experimental design does not allow us to conclude whether the ability of FAS inhibition to reduce food intake is due exclusively to actions in the hypothalamus, the literature would argue that it is the case. Several studies have attributed the hypophagic action of FAS inhibition to direct effects on hypothalamic neurons (31,37–40), and this model has been further supported in the recent study of Chakravarthy et al. (40). This group demonstrated that genetic inactivation of FAS in the hypothalamus and β -cells results in anorexia entirely due to inactivation of the enzyme in the hypothalamus. Moreover, nutritional regulation of mTORC1 signaling occurs specifically in the hypothalamus (4). Taking into account that leptin also activates mTORC1 in the hypothalamus (4) and reduces food intake through actions on CNS fatty acid metabolism (18), this suggests that mTORC1 is an important integrator of multiple signals in the CNS. mTORC1 activity can be found in both agouti-related protein- and proopiomelanocortin-producing neurons in the arcuate nucleus (4), and, thus, increased mTORC1 activity in the CNS may contribute to the similar effects of C75 and leptin on both food intake and hypothalamic gene expression (15,17,41). The current data connect two separate metabolic pathways that have been

independently implicated in the CNS regulation of energy balance, thus providing new knowledge on the relationship among key signaling pathways able to affect feeding behavior. Identifying the specific metabolic signals that are integrated by energy-sensing neurons is a crucial scientific question that could give important insights into potential etiologies and therapies for obesity and other metabolic disorders.

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