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FATTY ACID SYNTHASE (FAS) INHIBITORS MODULATE ENERGY BALANCE VIA MTOR COMPLEX 1 (MTORC1) SIGNALING IN THE CENTRAL NERVOUS SYSTEM (CNS)

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ABSTRACT

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 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), following 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 following 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 (CNS) to regulate energy balance, possibly via modulation of neuronal glucose utilization.

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 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).

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). FAS catalyzes the condensation of malonyl-CoA and acetyl-CoA to generate long-chain fatty acids (LCFA). 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 following refeeding. Studies using FAS inhibitors, as well as 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 multi-protein complexes: mTORC1 which includes raptor, and mTORC2 which includes rictor. While mTORC2 regulates phosphorylation of Akt, mTORC1 modulates the activity of 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 upon 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 (WT) littermates (23) were used. Mice were 8 to 11 weeks old in the cerulenin study, and 7 months in the C75 study. All animals were housed individually, maintained on a 12:12-h light-dark cycle, and standard lab chow (Harlan-Teklad, Indianapolis, IN) and water. All animal procedures were approved by the IACUC of the University of Cincinnati. Rats were implanted with intra-3rd-ventricular

(icv) cannulas as previously described (24) and allowed to recover for a minimum of 7 days. Cannula placement was confirmed by consuming more than 5 ml of water following icv angiotensin II (10 ng).

Drugs. C75 (Calbiochem, EMD Bioscience Inc., La Jolla, CA) was dissolved in RPMI (Gibco, Carlsbad, CA). Cerulenin (Sigma-Aldrich Inc, St. Louis, MO) was first dissolved in DMSO followed by RPMI, to a final concentration of 25% DMSO. Rapamycin (25) (Calbiochem, EMD Bioscience Inc., La Jolla, CA) 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 Inc., 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 3rd ventricle. Tissues were homogenized in RIPA with addition of phosphatase and protease inhibitors (Santa Cruz Biotechnology). Samples (70 μg) were loaded on either a 9-13% (Experiment 1) or a 10 % SDS-polyacrylamide gel (Experiments 3 and 4) (BioRad). 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 (Cell Signaling Technology, Beverly, MA, 1:500) or phosphorylated S6 (pS6) at Ser 240/244 (Cell Signaling Technology, 1:500). After washing in TBS-T, the membranes were incubated for 1h at RT with secondary antibody conjugated with horseradish peroxidase (goat anti-rabbit, 1:2000, Cell Signaling Technology). After washes in TBS-T, 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 mM Tris-HCl, 100 mM 2-mercaptoethanol, and 2 % SDS before 2-h blocking in TBS-T with 5 % skim milk powder at RT, and re-blotted with either rabbit anti p70 S6K1 (Cell Signaling Technology, 1:250) or rabbit anti S6 (Cell Signaling Technology, 1:500). Density was determined by NIH program Scion image.

Experimental design:

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

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

Experiment 3: Anorexic actions of C75 and cerulenin in S6K1$^{-/-}$ mice. One h before dark, mice were injected ip with either C75 (20 mg/kg BW), cerulenin (160 mg/kg BW) or their respective vehicles, food was weighed at

1, 4, 10 and 24h. Body weight was measured at 24h. The experiments utilized a within-subjects design with treatment order counterbalanced and one week between each treatment.

Experiment 4: Effect of cerulenin on hypothalamic mTORC1 signaling. Rats were injected icv with cerulenin (90 μg in 2 μl) and sacrificed as in Experiment 1.

Experiment 5: Effect of the ketogenic diet on C75-induced anorexia. Rats were divided into 2 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 24h and ketosis was confirmed by a significant elevation of blood β-hydroxybutyrate in rats that had access to saccharin *vs*. sucrose. Three days later, rats were fasted overnight and given RPMI or C75 (30 μg in 2 μl, icv) 1h before lights off. Food and bottles of sucrose or saccharin were returned and weighed at 4 and 24h.

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, in order to avoid any confounding effect of caloric intake. Rats were sacrificed 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 ± SEM. Data were analyzed by two-way ANOVA for multiple group designs and one-way repeated measures ANOVA for within-subjects design, followed by Fisher LSD post hoc tests. Designs with only two groups were assessed with unpaired *t* tests. Experiment-wise significance was set at $P < 0.05$, two-tailed.

RESULTS

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

mTORC1 signaling mediates the anorexic action of C75. We have found that refeeding activates hypothalamic mTORC1 signaling, while 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 RAPA (26). There was a main effect of the second drug treatment on food intake ($F_{(1, 46)}$= 29.03, P<0.001). Within the first hour after injection, C75 reduced food intake (P<0.01), and this effect persisted for the following 24h (Fig. 2A-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 RAPA used decreased feeding in the first hour (P<0.01; Fig. 2A), which was surprising given that we had never observed an effect of this dose in any prior studies (Online Appendix 1) (4). Nonetheless, pre-treatment with RAPA blocked C75-induced anorexia by 4h (P<0.05; Fig. 2A), and the inhibition persisted at 24h (Fig. 2A-B). There was an interaction between the first and second drug treatments on food intake at 8h ($F_{(1, 46)}$= 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). RAPA prevented the weight loss effect of C75 over 24h (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 WT littermates. As previously reported (27), S6K1$^{-/-}$ mice had lower body weights than WT (WT=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/kilogram BW (WT/RPMI=139.68 $\pm$26.99 *vs.* S6K1$^{-/-}$ /RPMI=156.38 $\pm$15.52 g/kg BW; P=0.603). There was a main effect of drug on feeding ($F_{(1, 10)}$= 30.07, P<0.001). C75 significantly decreased food intake in both genotypes in the first hour (P<0.01; Fig. 2D). The suppression lasted at least 24h in WT mice (P<0.01; Fig. 2D-E). However, the response of S6K1$^{-/-}$ mice was significantly blunted at 4h (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 24h ($F_{(1, 10)}$= 7.06, P<0.05), even when expressed as g/kg (interval 10-24 h: WT/C75=8.95 $\pm$ 5.72 *vs.* S6K1$^{-/-}$/C75=29.34 $\pm$ 6.21 g/kg BW; 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 4h.

Cerulenin 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. Cerulenin activated mTORC1 signaling in a pattern similar to that of C75 by phosphorylating both S6K1 and S6 (Fig. 3A-B). Moreover, cerulenin also required intact mTORC1 signaling in order to cause anorexia. Consistent with previous reports (15; 16), cerulenin decreased food consumption, as revealed by a main effect of drug that started 1h post-injection in both genotypes ($F_{(1, 13)}$= 33.61, P<0.001). This effect persisted in the following 4-24h period in WT (P<0.001), but not in S6K1$^{-/-}$, whose food consumption was similar to that of VEH-injected S6K1$^{-/-}$ after 4h (Fig. 3C). There was also a main effect of drug on 24h weight loss ($F_{(1, 13)}$= 22.00, P<0.001), but only in WT (Fig. 3E). There was a trend for an interaction between genotype and drug on feeding at 4h, which reached significance at 24h ($F_{(1, 13)}$= 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 *vs.* sucrose (P<0.001; Fig. 4A). There was a main effect of drug on food intake at 4h ($F_{(1,28)}$= 12.74, P<0.01) and at 24h ($F_{(1,28)}$= 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 24h. However the caloric-reducing effect of C75 was blunted in ketotic rats receiving saccharin at 4h and 24h (Fig. 4B) and was nearly statistically different from that of rats in the sucrose group (P=0.059; Fig. 4B). These effects were accompanied with reduced hypothalamic

mTORC1 signaling in ketotic rats as compared to 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 *vs.* 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 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 (28), so we compared sucrose *vs.* saccharin-treated rats on the same gel. Ketosis had no significant effect on the basal phosphorylation of S6K1, but caused a non-significant increase in S6 phosphorylation (Online Appendix 2). Rats fed a ketogenic diet have increased leucine levels in the cerebral cortex (29). Given the important role of leucine in regulating mTOR (30; 31), 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; 32). 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 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 upon the activation of CNS mTORC1 signaling. In fact, icv 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 first hour post-injection, given that we had not seen an effect of this dose in several prior studies (Online Appendix 1) (4). Nonetheless, it is important to point out that this effect on food intake is short-lived, with no effect observed beyond 1hour. 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.

While 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 WT mice, as reported before (17; 33). 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 following 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 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 (34), do not reduce their food intake in response to C75 (13). In accordance with those earlier 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 1h post-injection under a ketogenic diet supplemented with sucrose, while 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 of reducing glucose availability (28). 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 and colleagues ascribed the anorexic action of C75 to its ability to inhibit hypothalamic AMP-activated kinase (AMPK) (17). 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 (35). The relationship between these two fuel sensors is rather complex, and appears to be bidirectional (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.* suggest that AMPK activity is involved primarily in the short-term actions of C75, since AMPK activators inhibit the effect of C75 specifically during the first hour following drug administration (17), while 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 (32; 37-40), and this model has been further supported in the recent study of Chakravarthy *et al* (32; 37-

40). This group demonstrated that genetic inactivation of FAS in the hypothalamus and β-cells result 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. Indeed, mTORC1 activity can be found in both AgRP and POMC-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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Figure 1. C75 increases hypothalamic mTORC1 signaling.
Representative Western blots from RPMI- or C75 (30 µg in 2 µl RPMI, icv)-treated 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. } = bands that were quantified. Mean ± SEM of 7 rats in each condition.

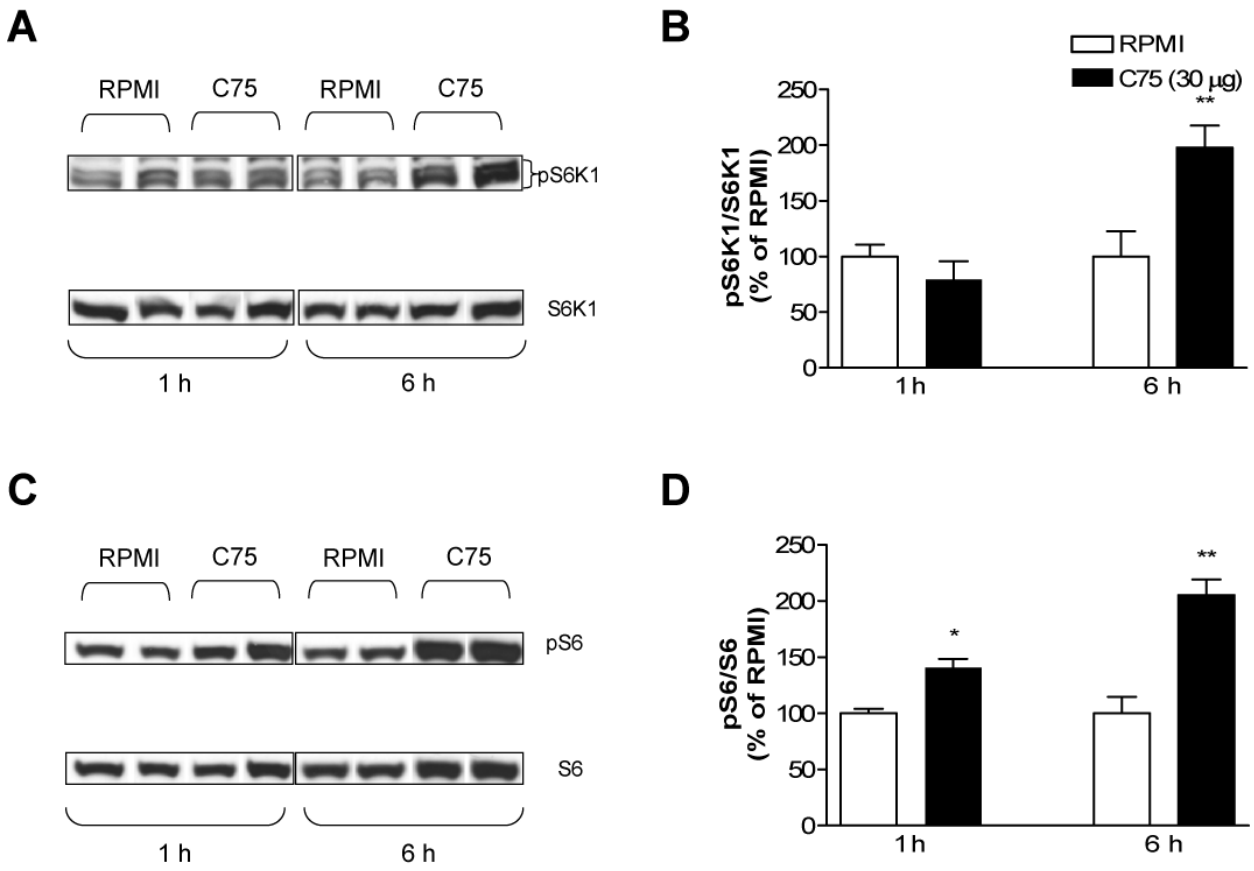


Figure 2. mTORC1 signaling contributes to the anorexic effect of C75.
Rapamycin (RAPA, 25 µg in 1 µl DMSO, icv) prevents the effects of C75 (50 µg in 3 µl RPMI, icv) on food intake (A and B) and on body weight change (C). Data are the mean of 2 separate experiments. Mean ± SEM of 9 to 15 rats in each treatment group. ** $P < 0.01$; *** $P < 0.001$ *vs*. DMSO/RPMI-treated rats; $^{\#}$ $P < 0.05$ *vs*. RAPA/C75 treated-rats. The anorexic effect of C75 (20 mg/kg, in 1ml/100 g BW RPMI, ip) is significantly reduced in S6K1$^{-/-}$ mice (D and E). Body weight change over 24 h in mice injected with RPMI or C75 (F). Mean ± SEM of 6 mice in each treatment group. Food intake is expressed as the non-cumulative amount consumed during different time intervals (A and D), as well as the cumulative amount eaten during 24 h (B and E). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ *vs.* WT (RPMI)-treated mice; $^{\#}$ $P < 0.05$ *vs.* S6K1$^{-/-}$ (C75)-treated mice.

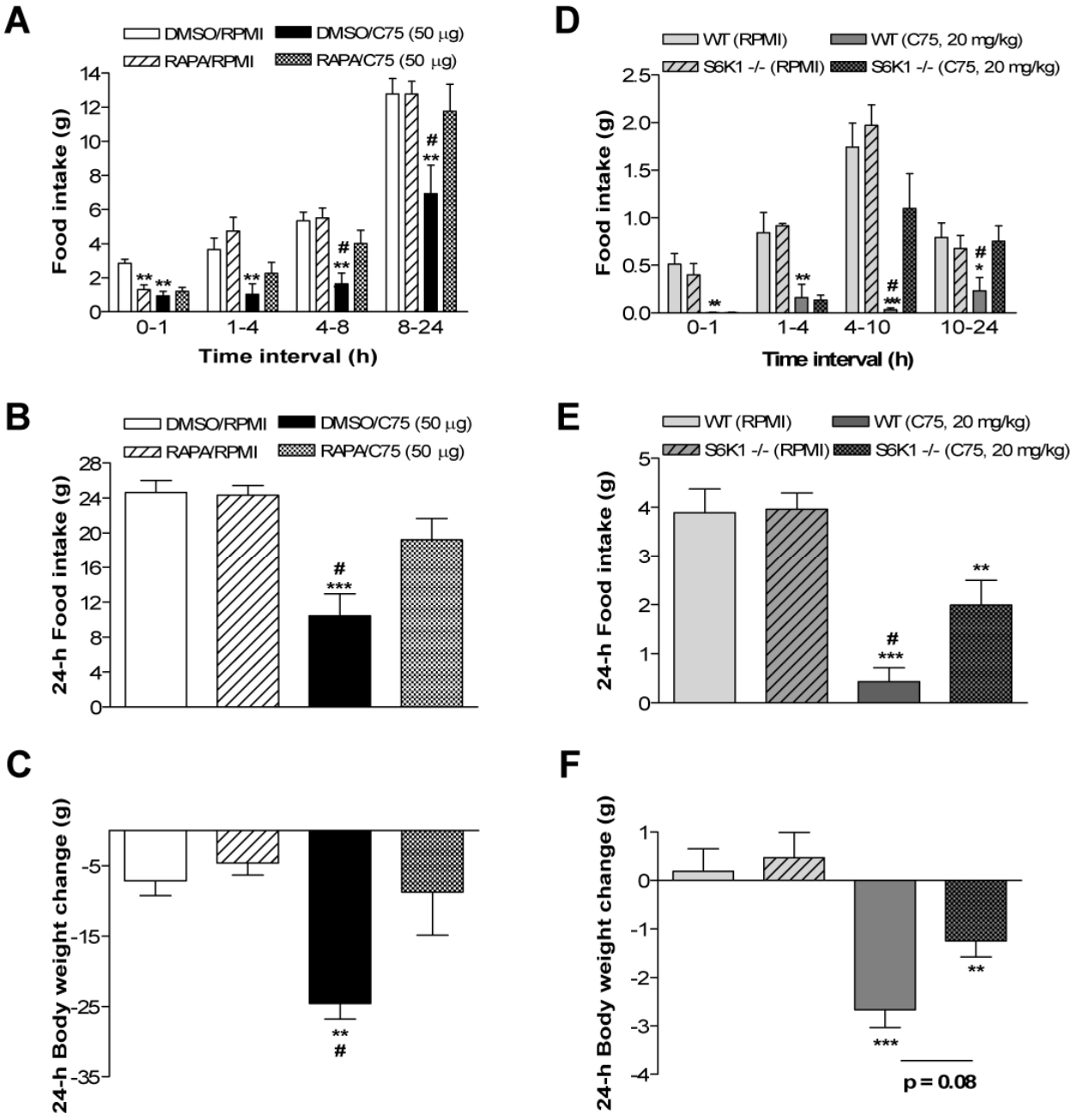

A
DMSO/RPMI
DMSO/C75 (50 μg)
RAPA/RPMI
RAPA/C75 (50 μg)
Food intake (g)
Time interval (h)
0-1
1-4
4-8
8-24
B
24-h Food intake (g)
C
24-h Body weight change (g)
D
WT (RPMI)
WT (C75, 20 mg/kg)
S6K1 -/- (RPMI)
S6K1 -/- (C75, 20 mg/kg)
Food intake (g)
Time interval (h)
0-1
1-4
4-10
10-24
E
24-h Food intake (g)
F
24-h Body weight change (g)
p = 0.08

Figure 3. Cerulenin activates hypothalamic mTORC1 signaling and this effect is required for its anorexic action.

Representative Western blots from VEH- or CERU (90 µg in 2 µl RPMI-DMSO, icv)-treated rats (A and B) and quantification by image analysis of hypothalamic phosphorylation of S6K1 and S6. * $P < 0.05$; *** $P < 0.001$ *vs.* VEH-treated rats. } = bands that were quantified. Mean ± SEM of 5-7 rats in each condition. The anorexic effect of CERU (160 mg/kg, in 1ml/100 g BW, ip) is significantly reduced in $S6K1^{-/-}$ mice (C and D). Body weight change over 24 h in mice injected with VEH or CERU (E). Mean ± SEM of 8 mice in each treatment group. Food intake is expressed as the non-cumulative amount consumed during different time intervals (C), as well as 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.

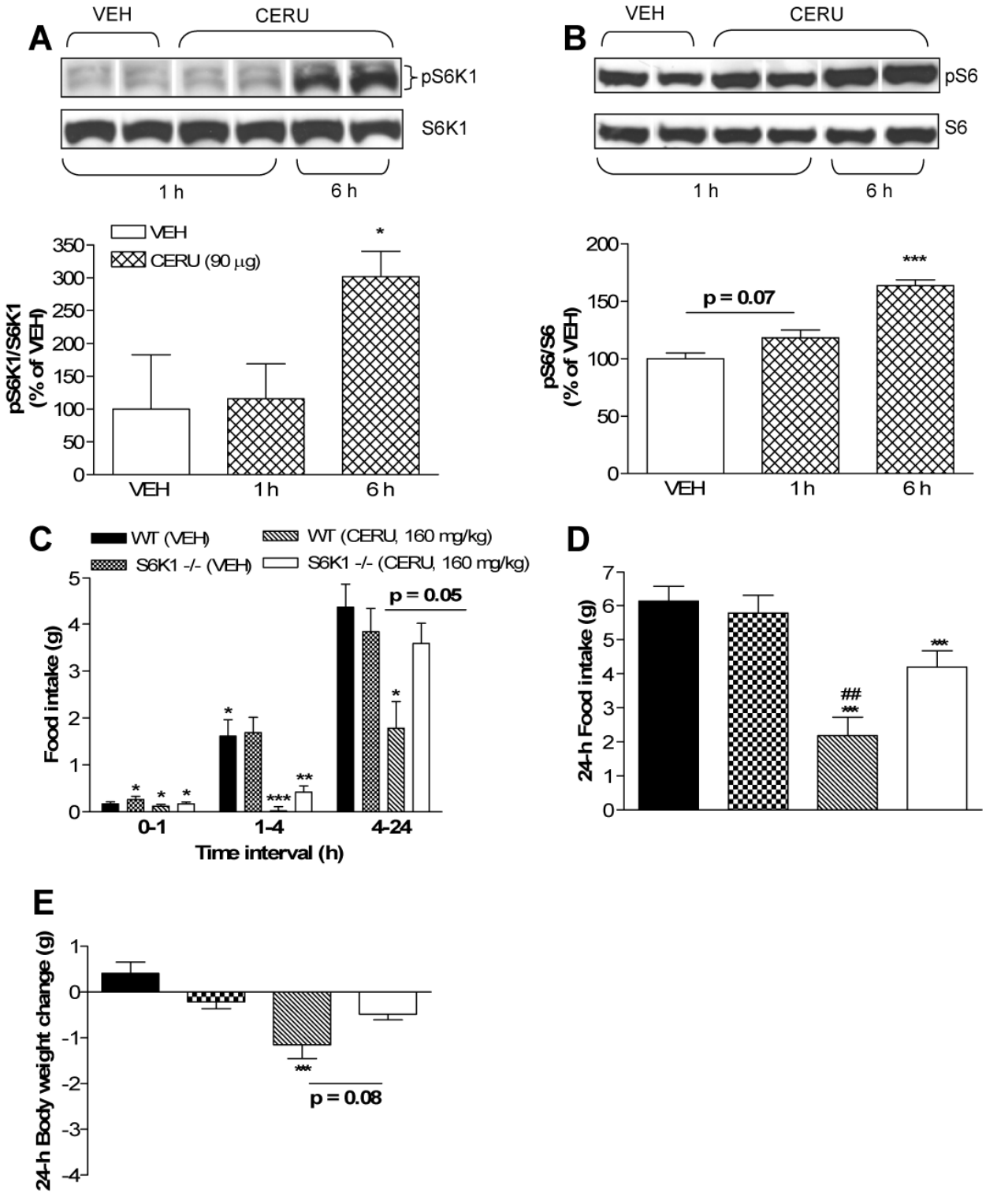
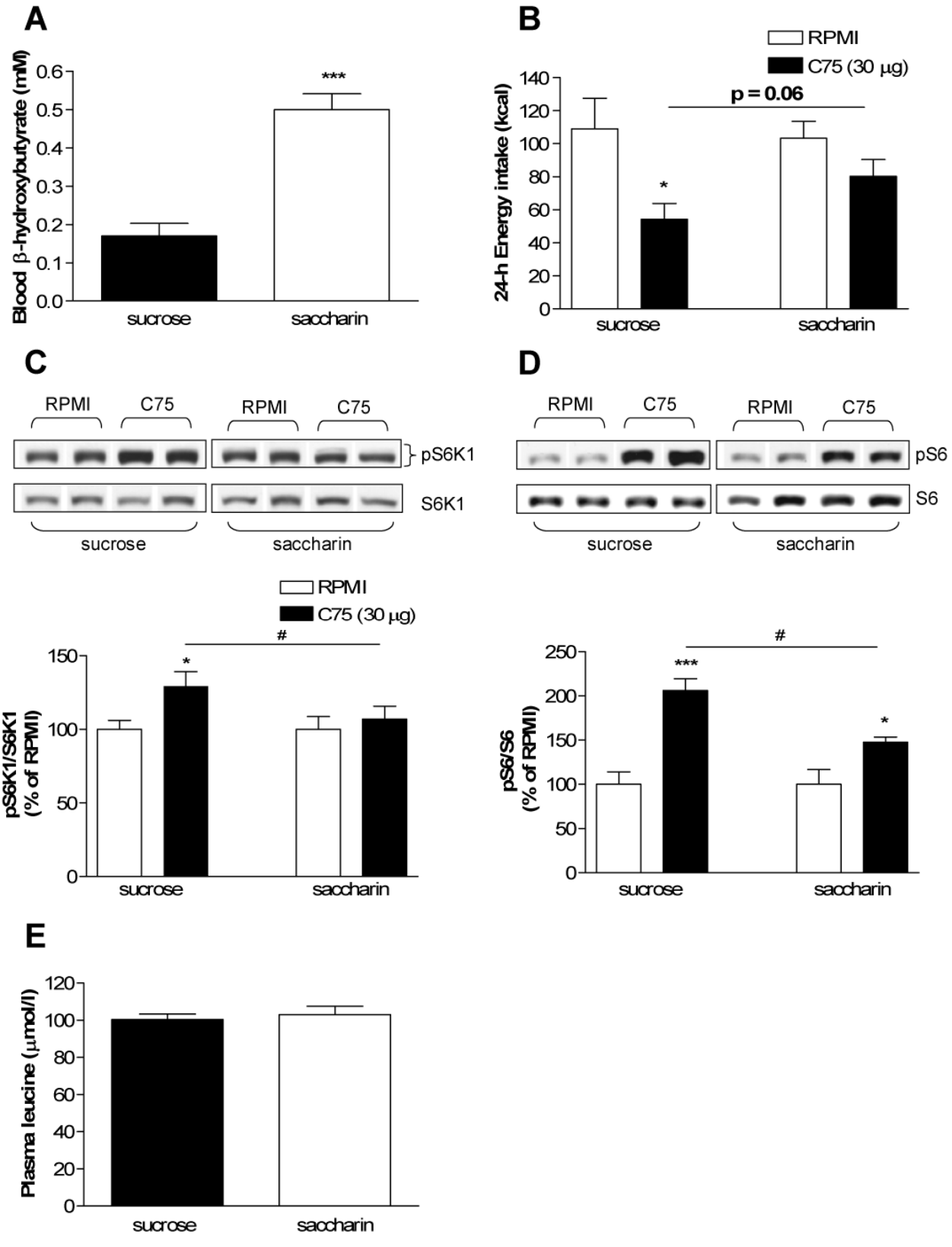

A
VEH
CERU
pS6K1
S6K1
1 h
6 h
VEH
CERU (90 μg)
pS6K1/S6K1 (% of VEH)
B
pS6
S6
pS6/S6 (% of VEH)
p = 0.07
C
WT (VEH)
S6K1 -/- (VEH)
WT (CERU, 160 mg/kg)
S6K1 -/- (CERU, 160 mg/kg)
Food intake (g)
p = 0.05
0-1
1-4
4-24
Time interval (h)
D
24-h Food intake (g)
E
24-h Body weight change (g)
p = 0.08

Figure 4. The actions of C75 on food intake and on hypothalamic mTORC1 signaling are blunted in ketotic rats.

Rats maintained on a ketogenic diet for 4 weeks while receiving a drinking solution of saccharin have elevated blood β-hydroxybutyrate concentration as compared to those that consumed a solution of sucrose in addition to the ketogenic diet (A). Mean ± SEM of 17-18 rats in each treatment group. *** $P < 0.001$ *vs.* rats from the sucrose group. Both groups had similar plasma leucine levels (E). Mean ± SEM of 6-7 rats in each treatment group. C75 (30 µg in 2 µl RPMI, icv) does not reduce caloric intake in rats given access to saccharin alongside with the ketogenic diet, but does so with sucrose (B). * $P < 0.05$ *vs.* RPMI-treated rats from the same group. Mean ± SEM of 5-8 rats in each treatment group. C75 (30 µg in 2 µl 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. Mean ± SEM of 5-9 brains examined in each condition.


A
Blood β-hydroxybutyrate (mM)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
sucrose
saccharin
B
RPMI
C75 (30 μg)
24-h Energy intake (kcal)
140
120
100
80
60
40
20
0
p = 0.06
*
sucrose
saccharin
C
RPMI
C75
RPMI
C75
pS6K1
S6K1
sucrose
saccharin
D
RPMI
C75
RPMI
C75
pS6
S6
sucrose
saccharin
RPMI
C75 (30 μg)
#
pS6K1/S6K1
(% of RPMI)
150
100
50
0
*
sucrose
saccharin
#
pS6/S6
(% of RPMI)
250
200
150
100
50
0
***
*
sucrose
saccharin
E
Plasma leucine (μmol/l)
120
100
80
60
40
20
0
sucrose
saccharin