Brief Communication

MOLECULAR METABOLISM

mTORC1-dependent increase in oxidative metabolism in POMC neurons regulates food intake and action of leptin

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ABSTRACT

Objective: Nutrient availability modulates reactive oxygen species (ROS) production in the hypothalamus. In turn, ROS regulate hypothalamic neuronal activity and feeding behavior. The mechanistic target of rapamycin complex 1 (mTORC1) pathway is an important cellular integrator of the action of nutrients and hormones. Here we tested the hypothesis that modulation of mTORC1 activity, particularly in Proopiomelanocortin (POMC)-expressing neurons, mediates the cellular and behavioral effects of ROS.

Methods: C57BL/6J mice or controls and their knockout (KO) littermates deficient either for the mTORC1 downstream target 70-kDa ribosomal protein S6 kinase 1 (S6K1) or for the mTORC1 component Rptor specifically in POMC neurons (POMC-*rptor*-KO) were treated with an intracerebroventricular (icv) injection of the ROS hydrogen peroxide (H_2O_2) or the ROS scavenger honokiol, alone or, respectively, in combination with the mTORC1 inhibitor rapamycin or the mTORC1 activator leptin. Oxidant-related signal in POMC neurons was assessed using dihydroethidium (DHE) fluorescence.

Results: Icv administration of H_2O_2 decreased food intake, while co-administration of rapamycin, whole-body deletion of *S6K1*, or deletion of *rptor* in POMC neurons impeded the anorectic action of H_2O_2. H_2O_2 also increased oxidant levels in POMC neurons, an effect that hinged on functional mTORC1 in these neurons. Finally, scavenging ROS prevented the hypophagic action of leptin, which in turn required mTORC1 to increase oxidant levels in POMC neurons and to inhibit food intake.

Conclusions: Our results demonstrate that ROS and leptin require mTORC1 pathway activity in POMC neurons to increase oxidant levels in POMC neurons and consequently decrease food intake.

Keywords Hypothalamus; Reactive oxygen species; mTORC1; Leptin; Food intake; POMC

1. INTRODUCTION

Cells use different type of signals to convey information about energy availability and coordinate cellular functions. In mammalian cells, adenosine-triphosphate (ATP) production encompasses mitochondrial oxygen consumption, which leads to the production of reactive oxygen species (ROS). ROS, including hydrogen peroxide (H_2O_2) and the superoxide anion (O_2), are oxidant radical species derived from oxygen acting as signal molecules able to modulate numerous intracellular pathways [1,2].

Hypothalamic neurons can sense, transmit, and convert ROS signals into appropriate intracellular responses [2,3]. Among these, increased ROS production has been involved in determining hypothalamic glucose and lipid sensing [4–6] and in mediating the central appetite-suppressant action of insulin [7]. Remarkably, nutrient availability directly impacts ROS levels in Neuropeptide Y/Agouti-related protein (NPY/AgRP) and proopiomelanocortin (POMC) expressing neurons of the hypothalamic arcuate nucleus (ARC) [8,9]. In turn, ROS modify the activity of these two neuronal populations [8,9], which control feeding behavior and peripheral metabolism by integrating information related to energy availability through neuronal inputs [10] and circulating metabolic cues [11–13]. Specifically, during fasting, ROS levels are not increased in NPY/AgRP neurons despite increased neuronal firing, thanks to a feed-forward ROS buffering mechanism involving mitochondrial uncoupling protein 2 (UCP2) [8]. However, if ROS generation is uncontrolled, firing of NPY/AgRP cells is impaired [8]. In contrast, while in fasting, ROS levels are low in hypothalamic POMC neurons, they are high upon refeeding in the same neuronal population [8,9]. Moreover, POMC neurons are depolarized and fire more in response to H_2O_2, while application of the H_2O_2 scavenging enzyme catalase during patch-clamp recordings hyperpolarizes and inhibits them [9,14]. Thus, a transient increase in ROS levels in POMC neurons

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favors satiety whereas suppression of ROS through intracerebroventricular (icv) administration of the ROS scavenger honokiol diminishes POMC neurons activity, as shown by the reduced expression of the neuronal activity marker c-fos, and promotes food intake [9]. Remarkably, ROS levels in POMC neurons of lean, chow-fed mice correlate with circulating leptin levels [9] and leptin induces ROS formation in hypothalamic cells *in vitro* [15]. However, whether this happens *in vivo* and if it is relevant for the effect of the hormone on food intake remains to be established.

Similarly to ROS, the mammalian or mechanistic target of rapamycin (mTOR) pathway is an important cellular integrator of the actions of nutrients and hormones on food intake [16]. mTOR is an evolutionary conserved serine/threonine kinase that forms two distinct complexes in cells (mTOR complex 1 or mTORC1, and mTOR complex 2 or mTORC2), controlling cellular proliferation and metabolism in response to nutrients, growth factors, mitogens, hormones, and cellular stress [16,17]. We and others have shown that mTORC1 signaling localizes in NPY/AgRP and POMC neurons of the ARC and that the hypothalamic mTORC1 pathway, which includes the 70-kDa ribosomal protein S6 kinase 1 (S6K1) as one of the downstream effectors, participates in the regulation of energy balance [18–23]. Increased hypothalamic mTORC1 activity is required for leptin-induced anorexia [18–21]. However, the specific neuronal substrates in which leptin-induced mTORC1 activity translates into decreased food intake are currently unknown.

Taking into account that there is evidence linking ROS and mTORC1 signaling, mostly in aging and cancer-related studies [24,25], here we have hypothesized that the mTORC1 pathway mediates ROS-dependent responses, specifically in POMC neurons, leading to consequent changes in food intake, and that ROS and mTORC1 signaling are intertwined and determine the effect of leptin on food intake.

By using genetic models and pharmacology, our study reveals that ROS require a functional mTORC1 pathway in POMC neurons to decrease food intake and that increased ROS and mTORC1 activity in POMC neurons are needed in order to observe the appetite-suppressant action of leptin.

2. MATERIAL AND METHODS

2.1. Animals

The experiments were conducted in strict compliance with European Union Directives (2010/63/EU) and were approved by the ethical committee of the University of Bordeaux (authorization number DIR1325). All procedures involving animals were performed in accordance with the ARRIVE guidelines [26]. Animal welfare was monitored daily for the length of the study.

Two-to 3- month-old male C57BL/6J mice (Janvier, France), *S6K1*-KO, and their wild-type (WT) littermates were used. The S6K1 mouse strain [27], kind gift of G. Thomas and S. Kozma (IDIBELL, Barcelona, Spain) was out-crossed at least twice to C57BL/6J mice upon arrival at the Neurocentre Magendie and was maintained on heterozygous breeding. $POMC^{Cre+/+}$::$Rptor^{flox/flox}$ mice (lacking the expression of Rptor, Regulatory-associated protein of mTOR, in POMC cells and thereafter called POMC-*rptor*-KO) and their $POMC^{Cre-/-}$::$Rptor^{flox/flox}$ control littermates were generated by crossing POMC-Cre mice [Tg(Pomc1-cre)16Lowl/J, JAX Stock #005965, The Jackson Laboratory, USA] with Rptor-Flox mice (B6.Cg-Rptortm1.1Dmsa/J, JAX Stock #013188, The Jackson Laboratory). This conditional mouse line was generated following a 3-step backcrossing method [28].

Mice were genotyped by PCR [28] using specific primers (see Supplementary Table 1). Effective Cre-mediated deletion of Rptor in POMC neurons was further assessed by immunohistochemistry.

Mice were housed individually with chow (Standard Rodent Diet A03, 3.2 kcal/g; SAFE, France) and water *ad libitum*, unless otherwise specified, under a 12h light/dark cycle (lights on at 01:00 am), at 22 ± 2 °C. At the end of the behavioral experiments, mice were killed by cervical dislocation or deeply anesthetized to undergo intracardiac perfusion for tissue collection and further analysis. The number of animals used for each experiment is detailed in the figure legends.

2.2. Surgery

Using a stereotaxic apparatus (David Kopf Instruments, USA), anesthetized mice were implanted with a cannula into the lateral cerebral ventricle [28]. Correct cannula placement was confirmed by administering 5 μg of the orexigenic peptide NPY (Phoenix Pharmaceuticals Inc, France) in 1 μl of 0.9% physiological saline. The cannula was judged correctly placed if the mice ate a minimum of 0.5 g of chow within 2h after the administration of NPY, during the light phase [29]. Mice that failed to respond correctly to the NPY test were removed from the study.

2.3. Drug administration and food intake studies

Drugs were administered through an intracerebroventricular (icv) cannula or by intraperitoneal (ip) injection. H_2O_2 (5 μM in 2 μL saline, icv, as in [9]) or its vehicle was administered just before the onset of the dark phase, after a 24h fast. To study the effect of a central mTORC1 inhibition on the appetite suppressant action of H_2O_2, mice received an acute icv injection of rapamycin [VWR International, France; 18 μg in 1 μL dimethyl sulfoxide (DMSO)], an inhibitor of mTORC1 [17], or its vehicle just before the dark phase, 40 min after an acute icv injection of H_2O_2 in 24h fasted C57BL/6J mice. In preliminary experiments (see Supplementary Fig. 1**)**, we tested the dose of rapamycin (18 or 25 μg in 1 μL DMSO, icv) to combine with H_2O_2. Mouse recombinant leptin (obtained from Dr A. F. Parlow, National Hormone and Pituitary Program, Torrance, CA) [2.5 μg/μL in 2 μL of phosphate buffered saline (PBS), icv] or its vehicle was administered in free-fed animals 4h before the onset of the dark phase [30]. When combined with the ROS scavenger honokiol, leptin (2.5 mg/kg) was given to free-fed animals by ip injection, while honokiol was administered icv (376 μM in in 2 μL DMSO).

2.4. Immunohistochemistry (IHC)

Mice were deeply anesthetized using pentobarbital (ip) and then perfused transcardially with ice-cold PBS, pH 7.4, followed by 4% paraformaldehyde (PFA, Sigma–Aldrich, France) in PBS with 0.2% picric acid. Brains were postfixed in 4% PFA overnight at 4 °C, then cryoprotected with 30% sucrose in PBS at 4 °C. Coronal sections (30 μm) were cut with a cryostat (CM1950, Leica, Germany), collected in PBS and stored in antifreeze solution (30% ethylene glycol, 30% glycerol in KPBS) at −20 °C until further used. Brain sections from $POMC^{Cre-/-}$::$Rptor^{flox/flox}$ control and their POMC-*rptor*-KO littermates were processed for the co-localization of Rptor and POMC. Sections were incubated with 10% normal goat serum (Dako) and then with rabbit anti-Rptor antibody (1:500, Abcam, UK) overnight at 4 °C. The next day, sections were washed in PBS and incubated for 1h with A647-conjugated secondary goat anti-rabbit antibody (1:500, Cell Signaling). Sections were washed in PBS and incubated with Goat Fab Fragment Anti-Rabbit IgG (1:100, Jackson ImmunoResearch Laboratories, USA) to avoid cross-reactivity. Sections were washed in PBS and blocked with 10% normal goat serum (Dako) and then incubated

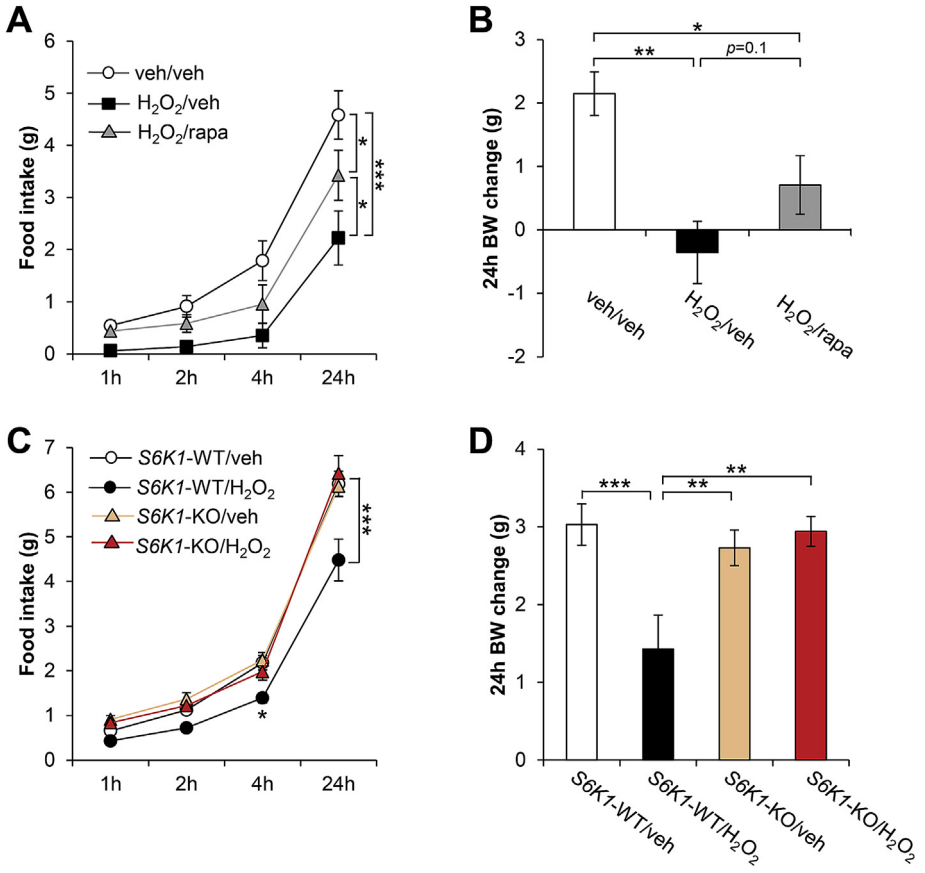

Figure 1: Central administration of the ROS H_2O_2 engages mTORC1 signaling to decrease food intake. (**A**) Acute icv co-administration of the mTORC1 inhibitor rapamycin with H_2O_2 blunts the effect of H_2O_2 on food intake (Repeated measures ANOVA: treatment effect $F_{(2,15)} = 6.39$, $P < 0.01$; time effect $F_{(3,45)} = 141.33$, $P < 0.0001$; treatment × time interaction $F_{(6,45)} = 4.59$, $P < 0.01$, n = 5–7 mice per group) and (**B**) 24h body weight (BW) change (One-way ANOVA: treatment effect $F_{(2,15)} = 9.37$, $P < 0.005$, n = 5–7 mice per group) in C57BL/6J mice. (**C**) Effect of an acute icv administration of H_2O_2 on food intake (Repeated measures ANOVA: treatment effect $F_{(1,40)} = 7.94$, $P < 0.01$; genotype effect $F_{(1,40)} = 11.24$, $P < 0.005$; treatment × genotype interaction $F_{(1,40)} = 6.18$, $P < 0.05$; time effect $F_{(3,120)} = 892.17$, $P < 0.0001$; treatment x genotype × time interaction $F_{(3,120)} = 7.58$, $P < 0.0005$, n = 10–12 mice per group) and (**D**) 24h BW change (two-way ANOVA: treatment effect $F_{(1,40)} = 5.71$, $P < 0.05$; genotype effect $F_{(1,40)} = 4.37$, $P < 0.05$; treatment × genotype interaction $F_{(1,40)} = 9.81$, $P < 0.005$, n = 10–12 mice per group) in WT and *S6K1*-KO littermates. Data are mean ± SEM. *P < 0.05, **P < 0.005, ***P < 0.0005.

with rabbit anti-POMC antibody (1:2000; Phoenix Pharmaceuticals, France) overnight at 4 °C. Sections were washed in PBS and incubated for 1h with A488-conjugated secondary goat anti-rabbit antibody (1:500, Cell Signaling). Fluorescent images were acquired with a confocal microscope (SP8-STED, Leica, Germany), and all sections containing the ARC (from Bregma −0.94 mm to Bregma −2.70 mm [31]) were rostro-caudally analyzed using ImageJ (https://imagej.nih.gov/ij/).

2.5. Analysis of oxidant levels in POMC neurons

Dihydroethidium (DHE, Sigma–Aldrich, France) was used, as in [8,9,32], to assess oxidant levels [33] in POMC neurons. Mice received first DHE (1 mg/mL in anhydrous DMSO, ip) and after 30 min, icv H_2O_2, leptin, or vehicle. Twenty min later, mice were deeply anesthetized with pentobarbital and perfused intracardially with 4% PFA in PBS. After post-fixation in 4% PFA overnight and cryoprotection with 30% sucrose in PBS, brains were processed and coronal brain sections (30 μm) containing the hypothalamus were obtained using a cryostat (CM1950, Leica). Sections were always protected from the light. After 30 min in PBS, they were incubated in blocking solution (10% normal goat serum in PBS containing 0.3% Triton X-100) for 1h. Primary anti-rabbit POMC antibody (1:2000, Phoenix Pharmaceuticals) was then applied overnight at 4 °C. The next day, sections were washed in PBS and incubated with the secondary antibody (goat anti-rabbit IgG alexa fluor 488, 1:1000, Cell Signaling) for 1h. Finally, the sections were mounted and confocal images were taken using a Spinning Disk microscope (Leica DMI 6000, Leica) equipped with a HQ2 camera (Photometrics, Tucson, USA). The diode lasers used were 491 and 561 mm. The objective used was a HCX PL APO CS 63× oil 1.32 NA. The z stacks were done with a piezo P721.LLQ (Physik Instrumente, Germany). Semi-quantitative analysis of DHE-associated fluorescence within POMC neurons was performed in ImageJ using a macro in which number and volume of the fluorescent spots within the cell were taken into account to determine the quantification of the fluorescent signal. The same area, designed within the ARC, was used to quantify the fluorescence within several POMC cells contained in the area of interest across different sections and animals. At least 20 POMC cells per animal were analyzed in the different experimental conditions.

2.6. Statistics

Values are expressed as mean ± SEM. Data were analyzed using unpaired t-test, two-way ANOVA, one and two-way repeated-measures ANOVA, using genotype and treatment as independent variables, followed by Fisher-LSD post-hoc analysis, with Statistica Version 9

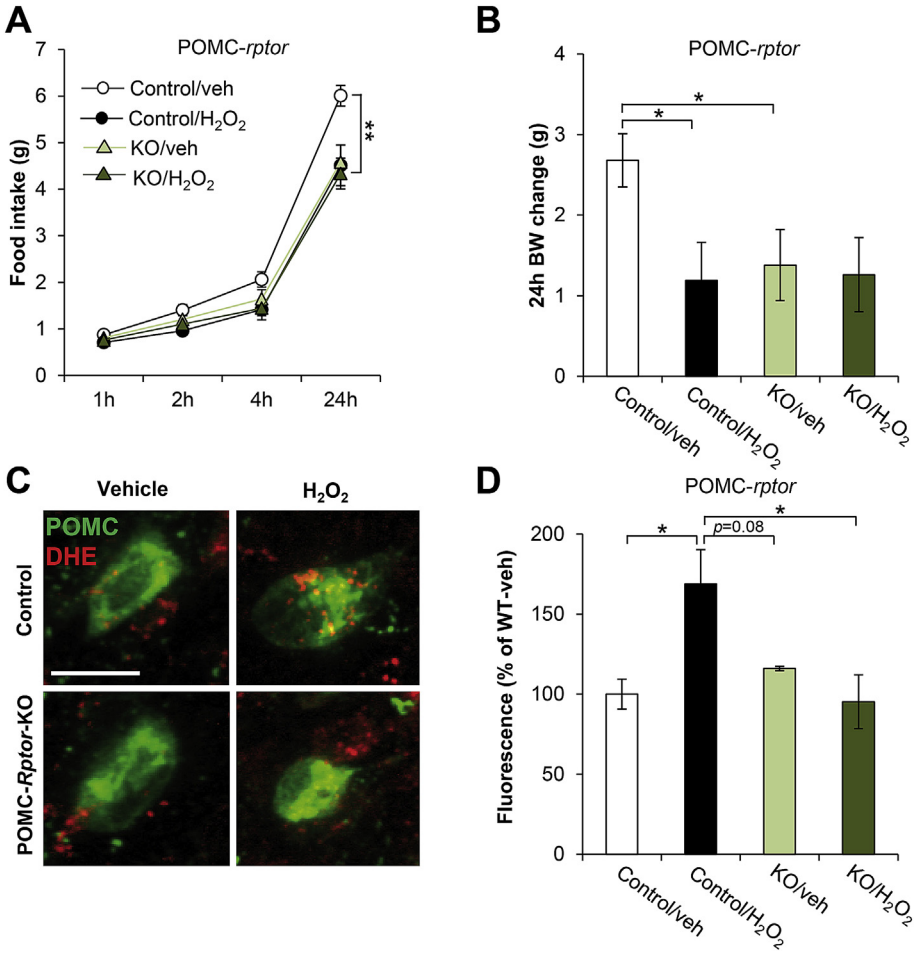

Figure 2: H_2O_2 requires activity of mTORC1 in POMC neurons to modulate food intake and intracellular oxidant levels. (**A**) Effect of an acute icv administration of H_2O_2 on food intake (Repeated measures ANOVA: treatment effect $F_{(1,25)} = 3.99$, $P = 0.05$; genotype effect $F_{(1,25)} = 1.25$, $P = 0.31$; treatment × genotype interaction $F_{(1,25)} = 4.73$, $P < 0.05$; time effect $F_{(3,75)} = 624.43$, $P < 0.0001$; treatment x genotype × time interaction $F_{(3,75)} = 6.63$, $P < 0.0005$, n = 7–8 mice per group) and (**B**) 24h BW change (Two-way ANOVA: treatment effect $F_{(1,25)} = 1.04$, $P = 0.31$; genotype effect $F_{(1,25)} = 0.33$, $P = 0.56$; treatment × genotype interaction $F_{(1,25)} = 6.07$, $P < 0.05$, n = 7–8 mice per group) in $POMC^{Cre-/-}$::$Rptor^{flox/flox}$ control and POMC-*rptor*-KO littermates. (**C**) Representative images of DHE-associated fluorescence in POMC neurons of control and POMC-*rptor*-KO littermates after icv administration of H_2O_2 and (**D**) related quantification (Two-way ANOVA: treatment effect $F_{(1,8)} = 1.70$, $P = 0.22$; genotype effect $F_{(1,8)} = 2.44$, $P = 0.1$; treatment × genotype interaction $F_{(1,8)} = 5.9$, $P < 0.05$, n = 3 mice per group). Data are mean ± SEM. *$P < 0.05$, **$P < 0.005$. Scale bar: 10 μm.

(Statsoft, Maisons-Alfort, France). $P < 0.05$ denoted statistical significance.

3. RESULTS

3.1. Central administration of H_2O_2 requires mTORC1 signaling to decrease food intake

To investigate the possible interaction between ROS and mTORC1 signaling in the regulation of food intake, we administered icv H_2O_2 to C57BL/6J mice, in combination with icv delivery of the mTORC1 inhibitor rapamycin at a dose that did not alter food intake (Supplementary Fig. 1). In agreement with previous studies [9], H_2O_2 significantly decreased 24h food intake and body weight (Figure 1 A, B), while co-administration of rapamycin blunted these effects (Figure 1 A, B). To further assess whether mTORC1 signaling was necessary to observe the behavioral changes induced by H_2O_2, we then tested the effects of this ROS in WT and *S6K1*-KO littermates. H_2O_2 given icv caused hypophagia and weight loss in WT, but not in *S6K1*-KO mice (Figure 1 C, D). These studies therefore suggest that H_2O_2 engages mTORC1 signaling to modulate food intake. However, they do not allow pinpointing the exact site(s) where this action is exerted.

3.2. mTORC1 activity in POMC neurons determines the behavioral and molecular effects of H_2O_2

ROS may inhibit food intake by activating hypothalamic POMC neurons, which mostly rely on oxidative metabolism [9]. Hence, we evaluated whether the ability of H_2O_2 to modify food intake and body weight required mTORC1 in POMC neurons. In order to do so, we generated mice lacking Rptor (a protein necessary for the assembling of mTORC1 [34,35]) and therefore characterized by defective mTORC1 activity. Rptor was expressed in 83.87% of POMC neurons of $POMC^{Cre-/-}$::$Rptor^{flox/flox}$ controls, while the protein was present in only 20.98% of POMC cells of POMC-*rptor*-KO littermates (Supplementary Fig. 2A). POMC-*rptor*-KO mice did not show any evident alteration in food intake or body weight under normal housing conditions as compared to controls (Supplementary Fig. 2 B, C). However, similarly to what previously observed using *S6K1* mice, while

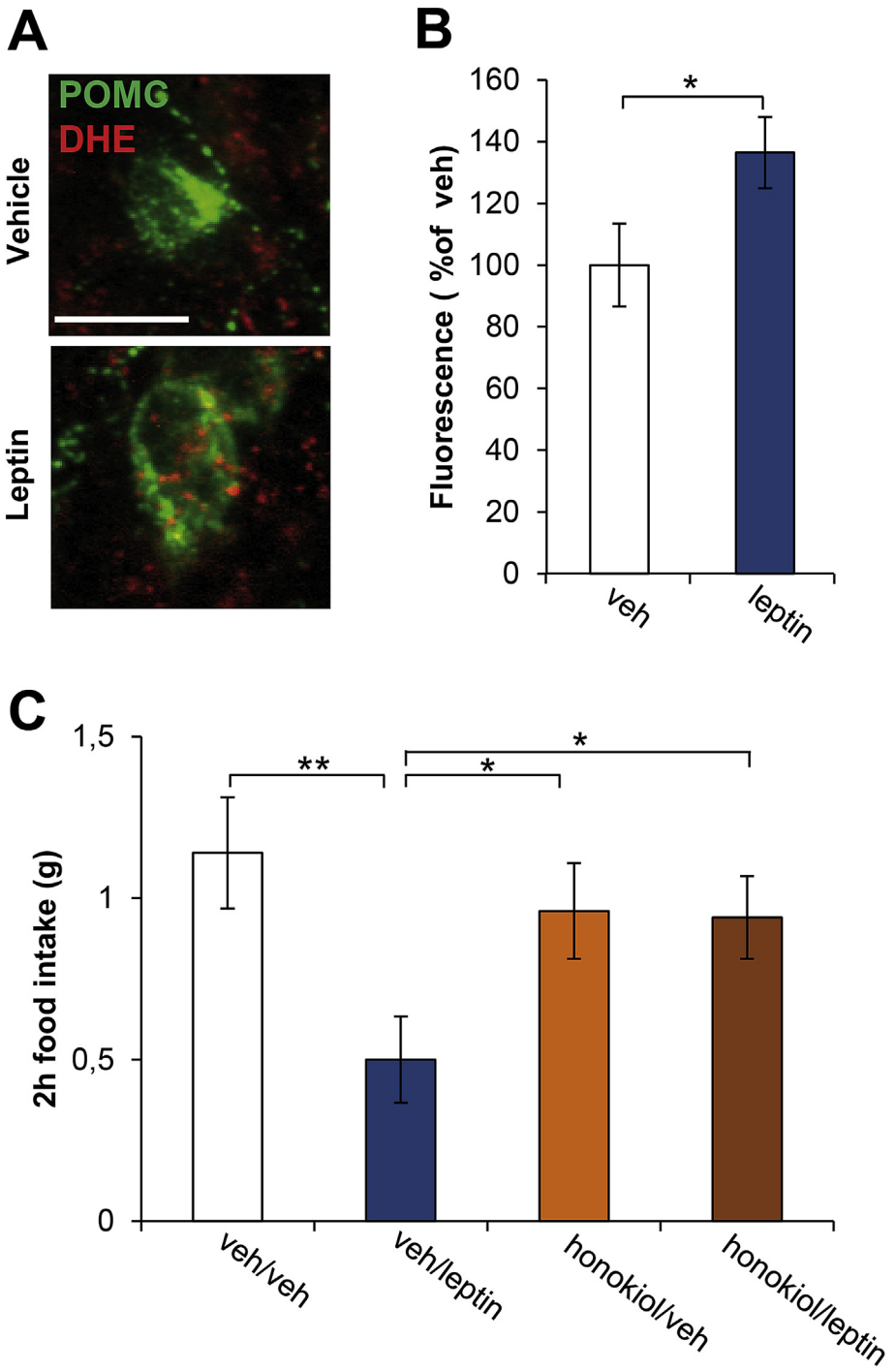

Figure 3: Leptin needs ROS formation to modulate food intake. (**A**) Representative images of DHE-related fluorescence in POMC neurons after icv administration of leptin in C57BL/6J mice and (**B**) related quantification (Unpaired t-test, one-tailed: $t_{(9)} = 1.94$, $P < 0.05$, $n = 5-6$ mice per group). (**C**) Effect of an acute ip administration of leptin combined with icv administration of the ROS scavenger honokiol on 2h food intake in C57BL/6J mice (Two-way ANOVA: honokiol effect $F_{(1,27)} = 0.80$, $P = 0.38$; leptin effect $F_{(1,27)} = 5.33$, $P < 0.05$; honokiol $\times$ leptin interaction $F_{(1,27)} = 4.53$, $P < 0.05$, $n = 7-9$ mice per group). Data are mean $\pm$ SEM. *$P < 0.05$, **$P < 0.005$. Scale bar: 10 μm.

$POMC^{Cre-/-}$::$Rptor^{flox/flox}$ controls decreased their food intake and body weight in response to icv delivery of H_2O_2, POMC-*rptor*-KO mice did not (Figure 2 A, B). Under these experimental conditions, in which animals were fasted for 24h before the administration of H_2O_2, we observed that POMC-*rptor*-KO, independently of the pharmacological treatment, ate less than control littermates (Figure 2 A, B). Accumulation of oxidants, as assessed by DHE-associated fluorescence, was evident in hypothalamic POMC neurons from controls receiving H_2O_2, but it was lacking in POMC-*rptor*-KO littermates (Figure 2 C, D). A trend ($P = 0.1$) toward a genotype effect was also observed, with decreased DHE-associated fluorescence in POMC-*rptor*-KO mice as compared to $POMC^{Cre-/-}$::$Rptor^{flox/flox}$ controls (Figure 2 C, D).

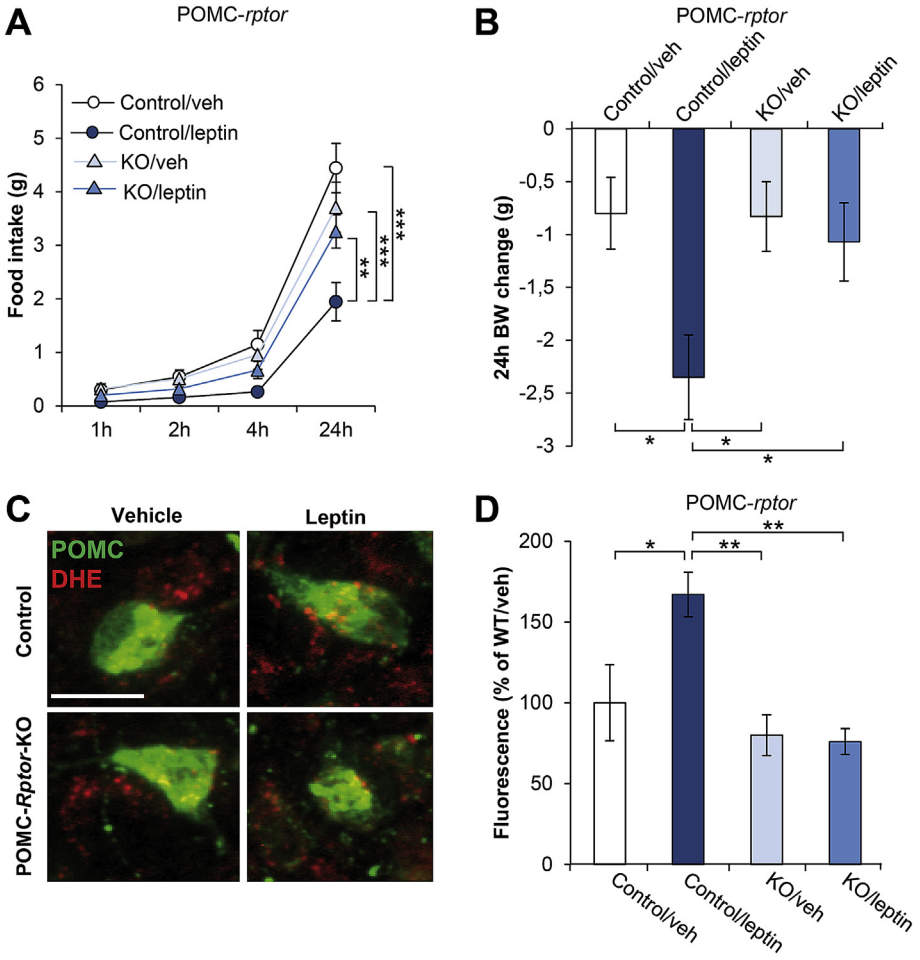

Figure 4: Leptin requires activity of mTORC1 in POMC neurons to modulate food intake and intracellular oxidant levels. (**A**) Effect of an acute icv administration of leptin on food intake (Repeated measures ANOVA: treatment effect $F_{(1,27)} = 11.1$, $P < 0.005$; genotype effect $F_{(1,27)} = 0.50$, $P = 0.49$; treatment × genotype interaction $F_{(1,27)} = 3.79$, $P = 0.06$; time effect $F_{(3,81)} = 314.54$, $P < 0.0001$; treatment x genotype × time interaction $F_{(3,81)} = 7.44$, $P < 0.0005$, n = 7–9 mice per group) and (**B**) 24h BW change (Two-way ANOVA: treatment effect $F_{(1,27)} = 6.01$, $P < 0.05$; genotype effect $F_{(1,27)} = 2.91$, $P = 0.10$; treatment × genotype interaction $F_{(1,27)} = 3.2$, $P = 0.08$, n = 7–9 mice per group) in POMC$^{Cre-/-}$::Rptor$^{flox/flox}$ control and POMC-*rptor*-KO littermates. (**C**) Representative images of DHE-related fluorescence in POMC neurons of control and POMC-*rptor*-KO littermates after icv administration of leptin and (**D**) related quantification (Two-way ANOVA: treatment effect $F_{(1,12)} = 5.04$, $P < 0.05$; genotype effect $F_{(1,12)} = 15.79$, $P < 0.005$; treatment × genotype interaction $F_{(1,12)} = 6.51$, $P < 0.05$, n = 3–5 mice per group). Data are mean ± SEM. *P < 0.05, **P < 0.005, ***P < 0.0005. Scale bar: 10 μm.

Thus, these data imply that mTORC1 activity in POMC neurons is required to observe both behavioral and molecular changes caused by exogenous ROS increase.

The hypothalamic mTORC1 pathway is involved in determining the actions of leptin on food intake [18,21]. In turn, leptin increases formation of ROS *in vitro* [15] and acts onto POMC neurons to modulate food intake [36,37]. Therefore, we finally tested whether the ability of leptin to modulate food intake requires ROS and mTORC1 in POMC neurons.

3.3. Leptin requires ROS formation to modulate food intake

First, we demonstrated that an acute icv administration of leptin rapidly increased oxidant levels in POMC neurons (Figure 3 A, B). Consequently, we assessed whether ROS scavenging could blunt the hypophagic action of leptin. To this purpose, an icv administration of the ROS scavenger honokiol at a dose that did not alter food intake (Figure 3C) was combined with a peripheral injection of leptin. As expected, leptin significantly reduced food intake, while co-administration of honokiol completely prevented leptin-induced hypophagia (Figure 3C).

3.4. mTORC1 in POMC neurons determines the ability of leptin to modulate food intake and intracellular oxidant levels

Lastly, to verify whether leptin requires mTORC1 in POMC neurons to regulate food intake and ROS formation, we investigated the central actions of the hormone in POMC-*rptor* mice. Icv administration of leptin significantly decreased food intake and body weight in POMC-$^{Cre-/-}$::Rptor$^{flox/flox}$, but not in POMC-*rptor*-KO littermates (Figure 4 A, B). The appetite suppressant action of the hormone was associated with an accumulation of oxidants in POMC neurons, which was lacking in cells of POMC-*rptor*-KO mice (Figure 4 C, D). A significant genotype effect was observed, with POMC-*rptor*-KO mice having less DHE-associated fluorescence than controls (Figure 4 C, D).

Overall, these findings imply that mTORC1 activity in POMC neurons is required for the ability of leptin to induce ROS production in POMC neurons and consequently regulate food intake.

4. DISCUSSION

In recent years, a series of studies have established that a transient increase in hypothalamic ROS is part of a second messenger

intracellular response to an increase in energy availability, which participates to the inhibition of feeding behavior. Indeed, increased hypothalamic ROS levels contribute to the decreased feeding observed in association with experimental hypertrygliceridemia [5] and to central delivery of insulin [7], implying that ROS participate in the intracellular decoding of the information provided by both nutrients and hormones. Accordingly, exogenous ROS and ROS scavengers can directly impact food intake by modulating the neuronal activity of NPY/AgRP and POMC neurons [8,9,14].

Here we demonstrated that an acute icv administration of H_2O_2 decreased food intake and body weight in lean chow-fed C57BL/6J mice. These data agree with previous investigations showing same behavioral changes in response to central administration of H_2O_2 [9]. Work by Andrews et al. and Diano et al. also demonstrated that hypothalamic POMC neurons appear to have high oxidants levels, as indicated by intracellular DHE-associated signal, when these cells are activated to promote satiety [8,9]. Increase in ROS levels would therefore result from mitochondrial activation due to full oxidation of glucose in POMC neurons during positive energy balance [8,9].

Our work now establishes that ROS signaling requires mTORC1 activity specifically in POMC neurons to regulate food intake. This is relevant not only for the action of ROS (and specifically H_2O_2) on feeding, but also for the ability of leptin to increase oxidant levels in POMC neurons and in turn inhibit food intake.

Our findings in particular demonstrate that both acute pharmacological inhibition of the mTORC1 pathway with central delivery of rapamycin as well as whole-body deletion of *S6K1* prevented the ability of H_2O_2 to decrease food intake. Finally, defective mTORC1 activity by deletion of *Rptor* in POMC neurons led to the inability of H_2O_2 to both induce accumulation of oxidants in these cells and inhibit food intake. Thus, these data suggest that mTORC1 activity is required in order for POMC cells to respond to ROS, and consequently decrease food intake. However, the exact molecular events connecting mTORC1 with ROS in POMC or other neuronal populations still need to be elucidated.

Several studies conducted in the fields of aging and cancer show that mTORC1 can affect both production and buffering of ROS [24]. mTORC1 can be purified in the mitochondrial fraction and increased mTORC1 activity increases mitochondrial oxygen consumption and oxidative metabolism, while its inhibition with rapamycin lowers mitochondrial ATP synthetic capacity while lowering mitochondrial ROS production [38,39]. Accordingly, mTORC1 was shown to control mitochondrial oxidative function through a YY1 (yin-yang 1) - PGC-1α (peroxisome proliferator-activated receptor-γ coactivator-1α) transcriptional complex [40] and phosphorylation of the signal transducer and activator of transcription 3 (STAT3) [41]. Thus, mTORC1 activity may determine the relative balance between mitochondrial and non-mitochondrial sources of ATP [38]. Besides, our findings show that exogenous H_2O_2 was unable to increase oxidant levels in POMC neurons of POMC-*rptor*-KO mice. This piece of evidence suggests that deletion of *rptor* increases ROS scavenging in these cells. In support of this interpretation, several studies have shown that rapamycin decreases ROS levels in different cell types by increasing anti-ROS enzymes superoxide dismutase and catalase through stimulation of autophagy [39,42,43]. The latter is due to rapamycin-dependent inhibition of mTORC1 activity [42] and reduction of rptor protein [44]. Accordingly, *in vivo* deletion of *rptor* in endothelial and beta cells induces autophagy and decreases oxidative stress [45,46].

Diano and colleagues demonstrated that ROS levels in hypothalamic POMC neurons of lean animals positively correlated with circulating levels of leptin [9], indicating that leptin may induce ROS formation in this neuronal population. We now demonstrate that exogenous leptin i) increases oxidant levels in POMC neurons, ii) exerts its appetite-suppressant action through ROS, as the ROS scavenger honokiol prevents the anorectic effect of leptin, and iii) engages mTORC1 in POMC neurons to modulate both oxidants production in POMC cells and food intake. Therefore, similarly to other signals of positive energy balance [4,5,7], leptin requires ROS production, a molecular response that necessitates activation of mTORC1 in POMC neurons to modulate food intake and body weight. The critical role of mTORC1 in POMC neurons in regulating leptin-dependent effects on food intake and body weight is in agreement with previous investigations showing that phosphatidylinositide 3-kinase (PI3K), which is upstream of mTORC1, is equally required in POMC neurons for the effects of leptin on energy balance [47,48]. Of note, mice that lack *S6K1* in POMC neurons are still responsive to leptin [22]. Thus, leptin would specifically need mTORC1 rather than S6K1 in POMC neurons to decrease food intake.

We have previously mentioned studies demonstrating how ROS affect both NPY/AgRP and POMC neuronal firing [8,9,14]. Similarly to what has been observed concerning the action of leptin on food intake, PI3K is required for the ability of leptin to increase firing of POMC neurons, while S6K1 is dispensable [22,47]. However, POMC neurons lacking S6K1 have lower resting membrane potential and spike firing frequency than control POMC cells, implying a reduction in basal transmitter release [22]. Similarly, sustained overactivation of mTORC1 in POMC neurons, induced by genetic manipulation or old age, impairs cellular activity, leading to hyperpolarization and silencing of POMC neurons through increase in the activity of ATP-sensitive potassium channels [49]. Thus, mTORC1 signaling plays an important role in the regulation of POMC neuronal activity; however, further investigations are needed in order to understand the exact link with ROS in this context.

In conclusion, our study has identified mTORC1 as a key intracellular pathway involved in the central regulation of food intake by ROS. Considering the known interactions existing between mitochondrial function, ROS and the mTORC1 signaling in aging and cancer, it will be particularly relevant to further investigate this molecular intertwining and its role on the function of neuronal circuits regulating energy balance in obesity and type 2 diabetes.

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CONTRIBUTION STATEMENT

MH, AF, VS, NS, ND, CA, SC, and OGQ performed the experiments and collected the data; MH, AF, VS, AT, and DC analyzed the data; DC conceptualized all studies and supervised the work; MH, AT, and DC wrote the manuscript. All authors have read and approved the final version of the manuscript.

DUALITY OF INTEREST

The authors declare no competing financial interest in relation to the work described.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1016/j.molmet.2018.04.002.

REFERENCES

[1] Droge, W., 2002. Free radicals in the physiological control of cell function. Physiological Reviews 82:47–95.

[2] Drougard, A., Fournel, A., Valet, P., Knauf, C., 2015. Impact of hypothalamic reactive oxygen species in the regulation of energy metabolism and food intake. Frontiers in Neuroscience 9:56.

[3] Shadel, G.S., Horvath, T.L., 2015. Mitochondrial ROS signaling in organismal homeostasis. Cell 163:560–569.

[4] Leloup, C., Magnan, C., Benani, A., Bonnet, E., Alquier, T., Offer, G., et al., 2006. Mitochondrial reactive oxygen species are required for hypothalamic glucose sensing. Diabetes 55:2084–2090.

[5] Benani, A., Troy, S., Carmona, M.C., Fioramonti, X., Lorsignol, A., Leloup, C., et al., 2007. Role for mitochondrial reactive oxygen species in brain lipid sensing: redox regulation of food intake. Diabetes 56:152–160.

[6] Fioramonti, X., Chretien, C., Leloup, C., Penicaud, L., 2017. Recent advances in the cellular and molecular mechanisms of hypothalamic neuronal glucose detection. Frontiers in Physiology 8:875.

[7] Jaillard, T., Roger, M., Galinier, A., Guillou, P., Benani, A., Leloup, C., et al., 2009. Hypothalamic reactive oxygen species are required for insulin-induced food intake inhibition: an NADPH oxidase-dependent mechanism. Diabetes 58:1544–1549.

[8] Andrews, Z.B., Liu, Z.W., Walllingford, N., Erion, D.M., Borok, E., Friedman, J.M., et al., 2008. UCP2 mediates ghrelin's action on NPY/AgRP neurons by lowering free radicals. Nature 454:846–851.

[9] Diano, S., Liu, Z.W., Jeong, J.K., Dietrich, M.O., Ruan, H.B., Kim, E., et al., 2011. Peroxisome proliferation-associated control of reactive oxygen species sets melanocortin tone and feeding in diet-induced obesity. Nature Medicine 17:1121–1127.

[10] Chen, Y., Lin, Y.C., Kuo, T.W., Knight, Z.A., 2015. Sensory detection of food rapidly modulates arcuate feeding circuits. Cell 160:829–841.

[11] Cota, D., Proulx, K., Seeley, R.J., 2007. The role of CNS fuel sensing in energy and glucose regulation. Gastroenterology 132:2158–2168.

[12] Zeltser, L.M., Seeley, R.J., Tschop, M.H., 2012. Synaptic plasticity in neuronal circuits regulating energy balance. Nature Neuroscience 15:1336–1342.

[13] Dietrich, M.O., Horvath, T.L., 2013. Hypothalamic control of energy balance: insights into the role of synaptic plasticity. Trends in Neurosciences 36:65–73.

[14] Pauliina Markkula, S., Lyons, D., Yueh, C.Y., Riches, C., Hurst, P., Fielding, B., et al., 2016. Intracerebroventricular catalase reduces hepatic insulin sensitivity and increases responses to hypoglycemia in rats. Endocrinology 157:4669–4676.

[15] Palomba, L., Silvestri, C., Imperatore, R., Morello, G., Piscitelli, F., Martella, A., et al., 2015. Negative regulation of leptin-induced reactive oxygen species (ROS) formation by cannabinoid CB1 receptor activation in hypothalamic neurons. Journal of Biological Chemistry 290:13669–13677.

[16] Haissaguerre, M., Saucisse, N., Cota, D., 2014. Influence of mTOR in energy and metabolic homeostasis. Molecular and Cellular Endocrinology 397:67–77.

[17] Saxton, R.A., Sabatini, D.M., 2017. mTOR signaling in growth, metabolism, and disease. Cell 168:960–976.

[18] Cota, D., Proulx, K., Smith, K.A., Kozma, S.C., Thomas, G., Woods, S.C., et al., 2006. Hypothalamic mTOR signaling regulates food intake. Science 312:927–930.

[19] Cota, D., Matter, E.K., Woods, S.C., Seeley, R.J., 2008. The role of hypothalamic mammalian target of rapamycin complex 1 signaling in diet-induced obesity. Journal of Neuroscience 28:7202–7208.

[20] Blouet, C., Ono, H., Schwartz, G.J., 2008. Mediobasal hypothalamic p70 S6 kinase 1 modulates the control of energy homeostasis. Cell Metabolism 8:459–467.

[21] Dagon, Y., Hur, E., Zheng, B., Wellenstein, K., Cantley, L.C., Kahn, B.B., 2012. p70S6 kinase phosphorylates AMPK on serine 491 to mediate leptin's effect on food intake. Cell Metabolism 16:104–112.

[22] Smith, M.A., Katsouri, L., Irvine, E.E., Hankir, M.K., Pedroni, S.M., Voshol, P.J., et al., 2015. Ribosomal S6K1 in POMC and AgRP neurons regulates glucose homeostasis but not feeding behavior in mice. Cell Reports 11:335–343.

[23] Burke, L.K., Darwish, T., Cavanaugh, A.R., Virtue, S., Roth, E., Morro, J., et al., 2017. mTORC1 in AGRP neurons integrates exteroceptive and interoceptive food-related cues in the modulation of adaptive energy expenditure in mice. Elife 6.

[24] Blagosklonny, M.V., 2008. Aging: ROS or TOR. Cell Cycle 7:3344–3354.

[25] Wellen, K.E., Thompson, C.B., 2010. Cellular metabolic stress: considering how cells respond to nutrient excess. Molecular Cell 40:323–332.

[26] Kilkenny, C., Browne, W., Cuthill, I.C., Emerson, M., Altman, D.G., 2010. Animal research: reporting in vivo experiments: the ARRIVE guidelines. British Journal of Pharmacology 160:1577–1579.

[27] Shima, H., Pende, M., Chen, Y., Fumagalli, S., Thomas, G., Kozma, S.C., 1998. Disruption of the p70(s6k)/p85(s6k) gene reveals a small mouse phenotype and a new functional S6 kinase. The EMBO Journal 17:6649–6659.

[28] Bellocchio, L., Soria-Gomez, E., Quarta, C., Metna-Laurent, M., Cardinal, P., Binder, E., et al., 2013. Activation of the sympathetic nervous system mediates hypophagic and anxiety-like effects of CB(1) receptor blockade. Proceedings of the National Academy of Sciences of the U S A 110:4786–4791.

[29] Brown, L.M., Clegg, D.J., Benoit, S.C., Woods, S.C., 2006. Intraventricular insulin and leptin reduce food intake and body weight in C57BL/6J mice. Physiology & Behavior 89:687–691.

[30] Cardinal, P., Bellocchio, L., Clark, S., Cannich, A., Klugmann, M., Lutz, B., et al., 2012. Hypothalamic CB1 cannabinoid receptors regulate energy balance in mice. Endocrinology 153:4136–4143.

[31] Franklin, K.B.J., Paxinos, G., 2008. The mouse brain in stereotaxic coordinates, Compact 3rd ed. Academic Press.

[32] Koch, M., Varela, L., Kim, J.G., Kim, J.D., Hernandez-Nuno, F., Simonds, S.E., et al., 2015. Hypothalamic POMC neurons promote cannabinoid-induced feeding. Nature 519:45–50.

[33] Zielonka, J., Kalyanaraman, B., 2010. Hydroethidine- and MitoSOX-derived red fluorescence is not a reliable indicator of intracellular superoxide formation: another inconvenient truth. Free Radical Biology and Medicine 48:983–1001.

[34] Kim, D.H., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., et al., 2002. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell 110:163–175.

[35] Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidayat, S., et al., 2002. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell 110:177–189.

[36] Cowley, M.A., Smart, J.L., Rubinstein, M., Cerdan, M.G., Diano, S., Horvath, T.L., et al., 2001. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature 411:480–484.

[37] Seeley, R.J., Yagaloff, K.A., Fisher, S.L., Burn, P., Thiele, T.E., van Dijk, G., et al., 1997. Melanocortin receptors in leptin effects. Nature 390:349.

[38] Schieke, S.M., Phillips, D., McCoy Jr., J.P., Aponte, A.M., Shen, R.F., Balaban, R.S., et al., 2006. The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity. Journal of Biological Chemistry 281:27643–27652.

[39] Lu, Q., Zhou, Y., Hao, M., Li, C., Wang, J., Shu, F., et al., 2017. The mTOR promotes oxidative stress-induced apoptosis of mesangial cells in diabetic nephropathy. Molecular and Cellular Endocrinology. https://doi.org/10.1016/j.mce.2017.12.012.

[40] Cunningham, J.T., Rodgers, J.T., Arlow, D.H., Vazquez, F., Mootha, V.K., Puigserver, P., 2007. mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. Nature 450:736–740.

[41] Wegrzyn, J., Potla, R., Chwae, Y.J., Sepuri, N.B., Zhang, Q., Koeck, T., et al., 2009. Function of mitochondrial Stat3 in cellular respiration. Science 323:793–797.

[42] Singh, A.K., Kashyap, M.P., Tripathi, V.K., Singh, S., Garg, G., Rizvi, S.I., 2017. Neuroprotection through rapamycin-induced activation of autophagy and PI3K/Akt1/mTOR/CREB signaling against amyloid-beta-induced oxidative stress, synaptic/neurotransmission dysfunction, and neurodegeneration in adult rats. Molecular Neurobiology 54:5815–5828.

[43] Dai, C., Ciccotosto, G.D., Cappai, R., Wang, Y., Tang, S., Hoyer, D., et al., 2018. Rapamycin confers neuroprotection against colistin-induced oxidative stress, mitochondria dysfunction, and apoptosis through the activation of autophagy and mTOR/akt/CREB signaling pathways. ACS Chemical Neuroscience. https://doi.org/10.1021/acschemneuro.7b00323.

[44] Martinez-Cisuelo, V., Gomez, J., Garcia-Junceda, I., Naudi, A., Cabre, R., Mota-Martorell, N., et al., 2016. Rapamycin reverses age-related increases in mitochondrial ROS production at complex I, oxidative stress, accumulation of mtDNA fragments inside nuclear DNA, and lipofuscin level, and increases autophagy, in the liver of middle-aged mice. Experimental Gerontology 83:130–138.

[45] Fan, W., Han, D., Sun, Z., Ma, S., Gao, L., Chen, J., et al., 2017. Endothelial deletion of mTORC1 protects against hindlimb ischemia in diabetic mice via activation of autophagy, attenuation of oxidative stress and alleviation of inflammation. Free Radical Biology and Medicine 108:725–740.

[46] Blandino-Rosano, M., Barbaresso, R., Jimenez-Palomares, M., Bozadjieva, N., Werneck-de-Castro, J.P., Hatanaka, M., et al., 2017. Loss of mTORC1 signalling impairs beta-cell homeostasis and insulin processing. Nature Communications 8:16014.

[47] Hill, J.W., Williams, K.W., Ye, C., Luo, J., Balthasar, N., Coppari, R., et al., 2008. Acute effects of leptin require PI3K signaling in hypothalamic proopiomelanocortin neurons in mice. Journal of Clinical Investigation 118:1796–1805.

[48] Al-Qassab, H., Smith, M.A., Irvine, E.E., Guillermet-Guibert, J., Claret, M., Choudhury, A.I., et al., 2009. Dominant role of the p110beta isoform of PI3K over p110alpha in energy homeostasis regulation by POMC and AgRP neurons. Cell Metabolism 10:343–354.

[49] Yang, S.B., Tien, A.C., Boddupalli, G., Xu, A.W., Jan, Y.N., Jan, L.Y., 2012. Rapamycin ameliorates age-dependent obesity associated with increased mTOR signaling in hypothalamic POMC neurons. Neuron 75:425–436.

