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mTORC1 signaling in Agrp neurons mediates circadian expression of Agrp and NPY but is dispensable for regulation of feeding behavior

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ABSTRACT

Orexigenic agouti-related protein/neuropeptide Y (Agrp/NPY) neurons and an orexigenic pro-opiomelanocortin (POMC) neurons of the hypothalamus regulate feeding behavior and energy homeostasis. An understanding of the molecular signaling pathways that regulate Agrp/NPY and POMC function could lead to novel treatments for metabolic disorders. Target of Rapamycin Complex 1 (TORC1) is a nutrient-activated protein kinase and central controller of growth and metabolism. We therefore investigated the role of mammalian TORC1 (mTORC1) in Agrp neurons. We generated and characterized Agrp neuron-specific *raptor* knockout (Agrp-*raptor* KO) mice. Agrp-*raptor* KO mice displayed reduced, non-circadian expression of *Agrp* and *NPY* but normal feeding behavior and energy homeostasis on both normal and high fat diet. Thus, mTORC1 in Agrp neurons controls circadian expression of orexigenic neuropeptides but is dispensable for the regulation of feeding behavior and energy metabolism.

1. Introduction

The arcuate nucleus (ARC) of the hypothalamus mediates whole body energy homeostasis including feeding behavior. It contains orexigenic agouti-related protein/neuropeptide Y (Agrp/NPY) neurons that secrete the neuropeptides Agrp and NPY, and anorexigenic pro-opiomelanocortin (POMC) neurons that secrete the neuropeptide POMC. Agrp/NPY neurons induce feeding and decrease energy expenditure while POMC neurons inhibit feeding and increase energy expenditure [1–3]. The ARC receives input from peripheral organs via hormones, such as insulin from the pancreas, leptin from adipose tissue and ghrelin from the intestine [4]. Thus, dysfunction of Agrp/NPY and POMC neurons can contribute to the development of obesity [5]. For example, Agrp/NPY and POMC neurons of obese patients often develop resistance to leptin and insulin, leading to altered feeding behavior and disturbed energy metabolism [6]. It is therefore important to understand the signaling pathways that regulate Agrp/NPY and POMC function.

The mammalian Target of Rapamycin (mTOR) signaling pathway integrates energy and nutrient levels to regulate cellular and organismal growth and metabolism [7–10]. mTOR forms two structurally and functionally distinct complexes, mTOR complex 1 (mTORC1) and mTORC2 [11]. mTORC1 is activated by growth factors, amino acids and cellular energy. Activation of mTORC1 by growth factors is mediated via the PI3K-PDK1-Akt signaling pathway. mTORC1 signaling stimulates protein synthesis, nucleotide biosynthesis, and lipogenesis, while it inhibits autophagy [7,10]. Due to its central role in cell growth and metabolism, deregulation of mTORC1 signaling is often associated with the development of metabolic disorders, such as diabetes and obesity [12]. Several studies have implicated hypothalamic mTORC1 signaling in the regulation of energy homeostasis and food intake. For example, feeding status regulates mTORC1 activity in both Agrp/NPY and POMC neurons [13], and hyperactivation of mTORC1 in POMC neuron leads to hyperphagia-induced obesity [14].

To further examine the role of mTORC1 signaling in the ARC, we generated Agrp neuron-specific *raptor* knockout (Agrp-*raptor* KO) mice and assessed whole-body energy metabolism, feeding behavior and adaptation to metabolic stress. Ablation of mTORC1 signaling in Agrp neurons resulted in reduced, non-circadian expression of orexigenic neuropeptides. However, this did not affect *ad libitum* food intake or ghrelin- or fasting-induced feeding. Furthermore, Agrp-*raptor* KO mice did not display changes in whole-body metabolism compared to control mice. On both normal and high fat diet (HFD), Agrp-*raptor* KO mice displayed similar body weight, glucose homeostasis and whole-body metabolic rates compared to control mice. Hence, mTORC1 signaling in

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Agrp neurons is dispensable for regulation of whole-body metabolism and feeding behavior.

2. Material and methods

2.1. Animals

Agrp-*raptor* KO mice were generated by crossing Agrp-IRES-Cre mice [15] with *raptor*$^{LoxP/LoxP}$ mice [16]. For immunohistochemistry Agrp-IRES-Cre or Agrp-*raptor* KO mice were crossed with Rosa26-STOP$^{LoxP/LoxP}$-EGFP mice [17] to obtain mice with EGFP expression in Agrp neurons. Mice were housed at 22 °C in a conventional facility with a 12 h light/12 h dark cycle. The standard diet contained 15.8 kcal% of fat (Promivi Kliba). The high fat diet contained 60 kcal % of fat (Harlan). All experiments were performed in accordance with the federal guidelines for animal experimentation and were approved by the Kantonales Veterinäramt of Kanton Basel-Stadt.

2.2. Locomotor activity, metabolic rate, and food intake

Locomotor activity, metabolic rate, and food intake was measured for 48 h using a comprehensive laboratory animal monitoring system (CLAMS, Linton Instrumentation and Columbus Instruments) after 24 h of acclimatization.

2.3. Glucose tolerance test

Mice were starved for 6 h and subsequently injected I.P. with glucose (2 g/kg). Blood glucose was measured in tail vain blood using a glucose meter (Accu-Chek, Roche).

2.4. Microdissection of ARC

ARC was isolated from brains by micropunch [18]. Brains were sliced in 0.5 mm thick coronal sections with a rodent brain slicer matrix (Zivic Instruments). Sections containing the ARC were identified and ARC was excised with a hollow needle (1.25 mm diameter).

2.5. RNA isolation and RT-PCR

Total RNA from hypothalamus or ARC was isolated with SV total RNA isolation kit (Promega) followed by cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad). Semi-quantitative real-time PCR analysis was performed using fast SYBR green (Applied Biosystems) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Relative expression levels were determined by normalizing to *TBP* expression using the ΔΔCt method. Primers used: TBP fw: TGCTGTTGGTGATTGTTGGT; TBP rv: CTGGC TTGTGTGGGAAAGAT; Agrp fw: AACCTCTGTAGTCGCACCTAGC; Agrp rv: AAACCGTCCCATCCTTTATTCT; NPY fw: AGGCTTGAAGACCCTTC-CAT; NPY rv: GATGAGGGTGGAAACTTGGA; POMC fw: GAGCTGAT-GACCTCTAGCCTCT; POMC rv: ATCAGAGCCGACTGTGAAATCT.

2.6. Protein isolation and western blot

Hypothalamus or ARC tissue was homogenized in lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease inhibitors (Roche) and phosphatase inhibitors (Sigma–Aldrich)). Proteins were separated on SDS-PAGE and transferred onto nitrocellulose membranes (Whatman). Antibodies used: Akt (Santa Cruz), Akt-pS473, Akt-pT308, S6, S6-pS235/236, 4E-BP1, 4EBP1-pS37/48 (all Cell Signaling), actin (Millipore).

2.7. Recombination

Genomic DNA was isolated by incubating the tissues in PBND buffer containing 0.1 mg Proteinase K at 57 °C over night, followed by proteinase K inactivation at 95 °C for 10 min. PCR was performed for Cre or for the genomic region flanking the floxed sites in the *raptor* gene to determine *raptor* deletion. Primers used: Cre fw: TGTGGCTGATGATCCGAATA; Cre rv: GCTTGCATGATCTCCGGTAT; *raptor* deletion fw: ATGGTAGCAGGCACACTCTTCATG; *raptor* deletion rv: CTCAGAGAACTGCAGTGCTGAAGG.

2.8. Immunostaining

Mice were transcardially perfused with 4% paraformaldehyde, followed by over night fixation of the brain in 4% paraformaldehyde. Brains were dehydrated, embedded in paraffin and cut in 5um thick coronal sections. Antibodies used: GFP (Abcam) and pS6 S235/236 (Cell Signaling). DAPI was used to stain the nuclei.

2.9. Blood analysis

Blood glucose was measured in tail vain blood using a glucose meter (Accu-Chek, Roche).

2.10. Fasting refeeding

Mice were starved over night prior to the experiment. At ZT2 mice were refed and food was weighted at the indicated times after refeeding.

2.11. Ghrelin stimulation

Mice were fed *ad libitum* and injected I.P. with 1 mg/kg of ghrelin or vehicle. Food intake was measured 30 min after ghrelin administration.

3. Results

3.1. Deletion of raptor in Agrp neurons leads to inactivation of mTORC1 signaling

To investigate the role of mTORC1 signaling in Agrp neurons, we generated Agrp-*raptor* KO mice by crossing Agrp-IRES-Cre mice [15] with *raptor*$^{LoxP/LoxP}$ mice [16]. To assess the tissue specificity of the knockout, we performed PCR to monitor *raptor* ablation. Excision of the floxed *raptor* allele was detected in the ARC of Agrp-*raptor* KO mice while the hypothalamic region surrounding the ARC and all other organs tested revealed an intact *raptor* gene (Fig. 1A). Thus, *raptor* ablation was specific to the ARC. Next, we investigated PI3K-mTORC1 signaling in the ARC of control and Agrp-*raptor* KO mice in a fasting-refeeding paradigm. In the ARC of control mice, feeding induced phosphorylation of Akt at S473 and of the mTORC1 target S6 at S235/236 (Fig. 1B). These findings demonstrate that the PI3K-mTORC1 signaling pathway is activated in the ARC upon feeding, as shown previously [13]. In contrast, phosphorylation of S6 at S235/236 was absent in the ARC of Agrp-*raptor* KO mice, indicating that mTORC1 signaling was indeed inactive upon knockout of *raptor* (Fig. 1B). In line with previous studies using *raptor*-deficient mouse models [16,19], Agrp-*raptor* KO mice displayed hyper-phosphorylation of Akt-S473 and -T308 in the ARC (Fig. 1B), due to absence of the S6K-mediated negative feedback loop [20–23]. To assess mTORC1 signaling specifically in Agrp neurons, we immunostained phospho-S6 in coronal brain sections of Agrp-*raptor* KO and control mice expressing EGFP specifically in

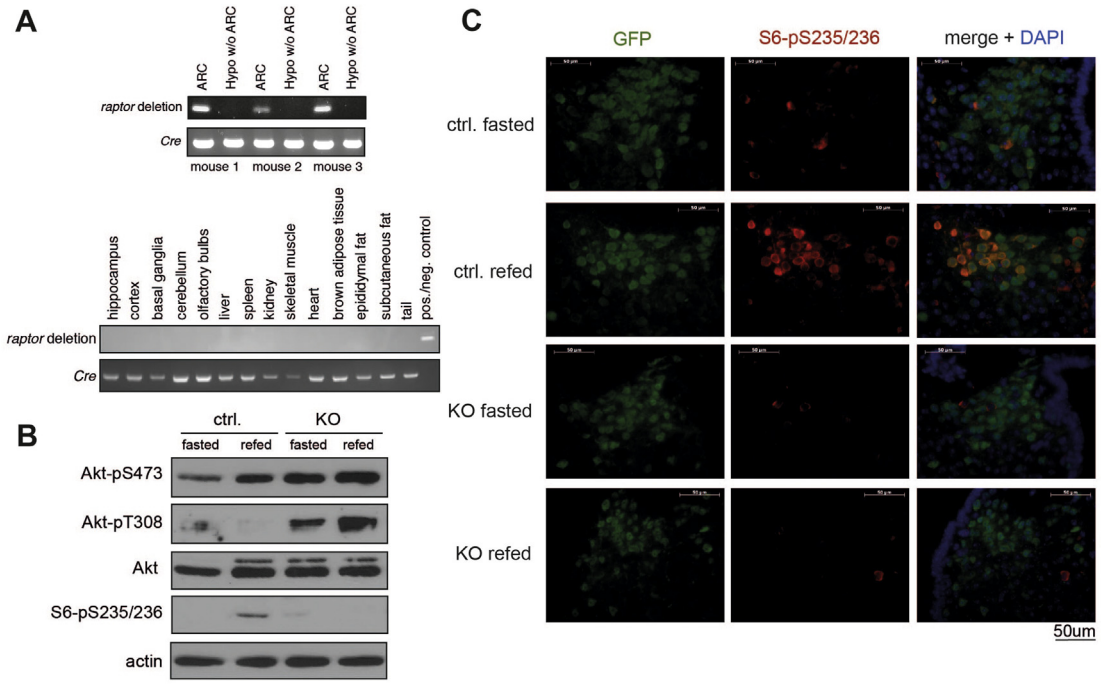

Fig. 1. Deletion of raptor in Agrp neurons leads to inactivation of mTORC1 signaling. (A) PCR for *Cre* and *raptor* deletion in the indicated organs from Agrp-*raptor* KO mice. (B) Immunoblot of protein lysates from the ARC of Agrp-*raptor* KO and control mice with the indicated antibodies. (C) Immunostaining of coronal brain sections from Agrp-*raptor* KO reporter mice and control reporter mice for EGFP to visualize Agrp neurons and S6-pS235/236 to visualize mTORC1 activity.

Agrp neurons. In line with our previous findings (Fig. 1B), S6 phosphorylation was induced in Agrp neurons upon refeeding in control mice (Fig. 1C), but was absent in Agrp neurons of Agrp-*raptor* KO mice. Thus, as expected, mTORC1 signaling was defective in Agrp neurons upon raptor deletion.

3.2. Inactivation of mTORC1 signaling in Agrp neurons does not affect whole-body metabolism

To investigate the role of mTORC1 signaling in Agrp neurons on whole-body metabolism, we measured body weight, body composition, glucose tolerance, metabolic rate and locomotor activity in young (3 months old) and old (1 year old) Agrp-*raptor* KO and control mice. Surprisingly, Agrp-*raptor* KO mice did not display any differences in body weight (Fig. 2A), body composition (Fig. 2B) or glucose tolerance (Fig. 2C) compared to control mice at either age. In the aged group, Agrp-*raptor* KO mice showed a trend toward higher body weight compared to control littermates (Fig. 2A). However, this difference did not reach statistical significance. Moreover, there was no difference in oxygen consumption (Fig. 2D), carbon dioxide production (Fig. 2E), respiratory exchange ratio (Fig. 2F) and locomotor activity (Fig. 2G) in Agrp-*raptor* KO mice compared to control mice. Taken together, these findings demonstrate that inactivation of mTORC1 signaling in Agrp neurons does not affect whole-body metabolism in either young or aged mice.

3.3. Agrp-raptor KO mice display impaired circadian rhythm of Agrp and NPY expression but not altered feeding behavior

Since Agrp neurons regulate feeding behavior in a circadian manner, we investigated food intake, mTORC1 signaling, and *Agrp* and *NPY* mRNA levels over a 24 h cycle in Agrp-*raptor* KO and control mice fed *ad libitum*. Mice were sacrificed at Zeitgeber 0 (ZT0), ZT12, ZT18 and ZT24. At ZT0, the lights turn on, ZT12 marks when the light turns off. Similar to other organs [24–26], phosphorylation of the mTORC1 downstream targets S6 (S235/236) and 4E-BP1 (T37/48) were regulated in a circadian manner in the ARC of control mice. mTORC1 signaling activity in the ARC peaked at ZT18, during the dark phase when mice are active and eat (Fig. 3A). In Agrp-*raptor* KO mice, phosphorylation of S6 and 4E-BP1 was undetectable in the ARC at all time points, confirming that mTORC1 signaling was ablated (Fig. 3A). Importantly, mRNA expression of both *Agrp* and *NPY* also oscillated in a circadian manner similar to mTORC1 signaling activity (Fig. 3B). Similar to S6 (S235/236) and 4E-BP1 (T37/48) phosphorylation, *Agrp* and *NPY* expression was highest at ZT18 (Fig. 3B). Interestingly, expression of both *Agrp* and *NPY* was reduced and non-oscillating in Agrp-*raptor* KO mice, leading to a significant reduction in *Agrp* and *NPY* levels at ZT18 (Fig. 3B). Hence, the circadian pattern of mTORC1 signaling in the ARC might regulate the circadian oscillation of *Agrp* and *NPY* expression. In contrast to this, *POMC* expression was not circadian and did not differ between the genotypes (Fig. 3B). Thus, inactivation of mTORC1 in Agrp neurons does not lead to compensatory alterations in *POMC* levels. Surprisingly, despite a significant decrease in *Agrp* and *NPY* mRNA levels, Agrp-*raptor* KO mice did not display a decrease in *ad libitum* food intake compared to controls (Fig. 3C).

Since *Agrp* and *NPY* expression was reduced in Agrp-*raptor* KO mice, we hypothesized that induction of *Agrp* and *NPY* expression might also be impaired in starved KO mice. To test this, we measured *Agrp* and *NPY* mRNA in Agrp-*raptor* KO and control mice that were starved overnight or fed *ad libitum*. Unlike the decreased circadian *Agrp* and *NPY* expression, Agrp-*raptor* KO mice were able to induce both *Agrp* and *Npy* mRNA expression upon fasting (Fig. 3D). Hence, induction of *Agrp* and *NPY* mRNA upon fasting is likely mediated by an mTORC1-independent mechanism. In line with the unchanged induction of *Agrp* and *NPY* expression upon starvation, Agrp-*raptor* KO mice displayed similar feeding in response to a fasting-refeeding challenge as compared to control mice. We fasted Agrp-*raptor* KO and control mice over night and

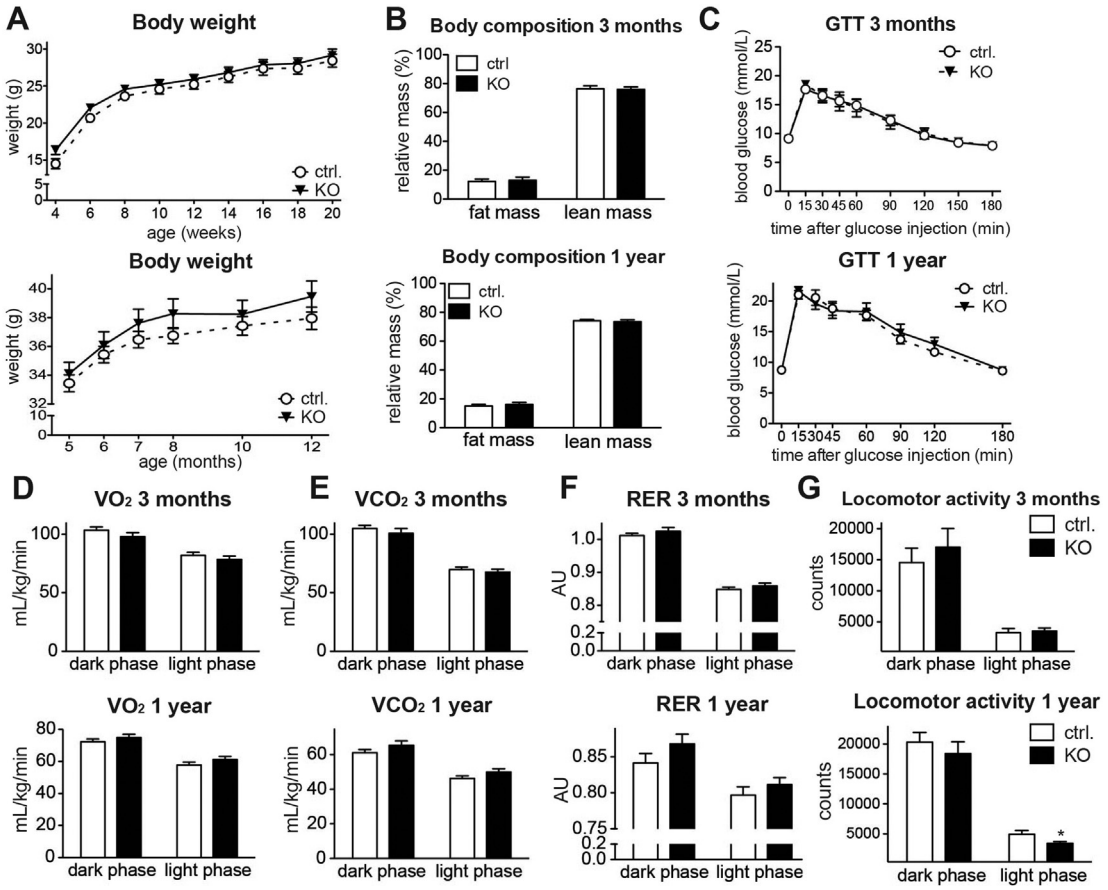

Fig. 2. Inactivation of mTORC1 signaling in Agrp neurons does not affect whole-body metabolism. (A) Body weight of young and old Agrp-*raptor* KO and control mice. (B) Body composition of young and old Agrp-*raptor* KO and control mice. (C) Glucose tolerance test (GTT) of young and old Agrp-*raptor* KO and control mice. (D) Oxygen consumption (VO_2) of young and old Agrp-*raptor* KO and control mice. (E) Carbon dioxide production (VCO_2) of young and old Agrp-*raptor* KO and control mice. (F) Respiratory exchange ratio (RER) of young and old Agrp-*raptor* KO and control mice. (G) Locomotor activity of young and old Agrp-*raptor* KO and control mice. Data represent mean ± SEM. Statistically significant differences between Agrp-*raptor* KO and control mice are indicated with asterisks (* = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$).

measured food intake after refeeding in the morning. Agrp-*raptor* KO mice consumed a similar amount of food compared to control mice at all time points after refeeding (Fig. 3E). Next, we investigated whether mTORC1 signaling is required for induction of feeding upon ghrelin stimulation. Ghrelin is an orexigenic peptide that is secreted upon starvation from ghrelin-producing cells in the gastrointestinal tract and is able to activate Agrp neurons [27,28]. We injected fed Agrp-*raptor* KO and control mice with ghrelin and measured food intake. Similar to our results above, Agrp-*raptor* KO mice again failed to display a defect in food intake (Fig. 3F). The above taken together indicates that feeding behavior is not impaired in Agrp-*raptor* KO mice.

3.4. *Agrp-raptor KO mice respond to metabolic stress*

Chronic, excessive consumption of nutrients, in particular fat, is a metabolic stress that induces compensatory metabolic alterations. Since Agrp-*raptor* KO mice failed to display metabolic alterations when fed a standard diet, we investigated the response of Agrp-*raptor* KO mice to metabolic stress. To induce metabolic stress, we fed Agrp-*raptor* KO and control mice a high fat diet (HFD) (60 kcal% from fat) for 10 weeks. Similar to the results obtained on a normal diet, Agrp *raptor*-KO mice displayed similar weight gain compared to controls (Fig. 4A). Moreover, body composition and food intake did not significantly differ between the genotypes (Fig. 4B and C). To assess glucose tolerance in HFD-fed Agrp-*raptor* KO mice we performed an intraperitoneal glucose tolerance test (GTT) and measured blood glucose in both fasted and fed animals. While HFD feeding led to impaired glucose tolerance in control and Agrp-*raptor* KO mice, no difference in glucose clearance rates could be detected between genotypes (Fig. 4D). Furthermore, control and Agrp-*raptor* KO mice displayed similar blood glucose levels upon HFD feeding (Fig. 4E). Finally, we measured oxygen consumption (Fig. 4F), carbon dioxide production (Fig. 4G), respiratory exchange ratio (Fig. 4H), and locomotor activity (Fig. 4I) in Agrp-*raptor* KO and control mice fed a HFD. In line with our results obtained with mice on a normal diet, HFD-fed Agrp-*raptor* KO mice failed to display any alteration in these parameters as compared to control mice. Taken together, these findings demonstrate that ablation of mTORC1 signaling in Agrp neurons does not alter the response to metabolic stress such as that caused by a HFD.

4. Discussion

mTOR is a nutrient sensor. Consequently, mTORC1 signaling in the hypothalamus has been suggested to be involved in the regulation of whole-body energy homeostasis and feeding behavior. Indeed, hyper-activation of mTORC1 signaling specifically in POMC neurons, through knockout of *TSC1,* results in hyperphagia-induced obesity [14]. These findings demonstrate a role for mTORC1 signaling in POMC neurons in the regulation of feeding behavior. However, the role of mTORC1 signaling in Agrp neurons has so far

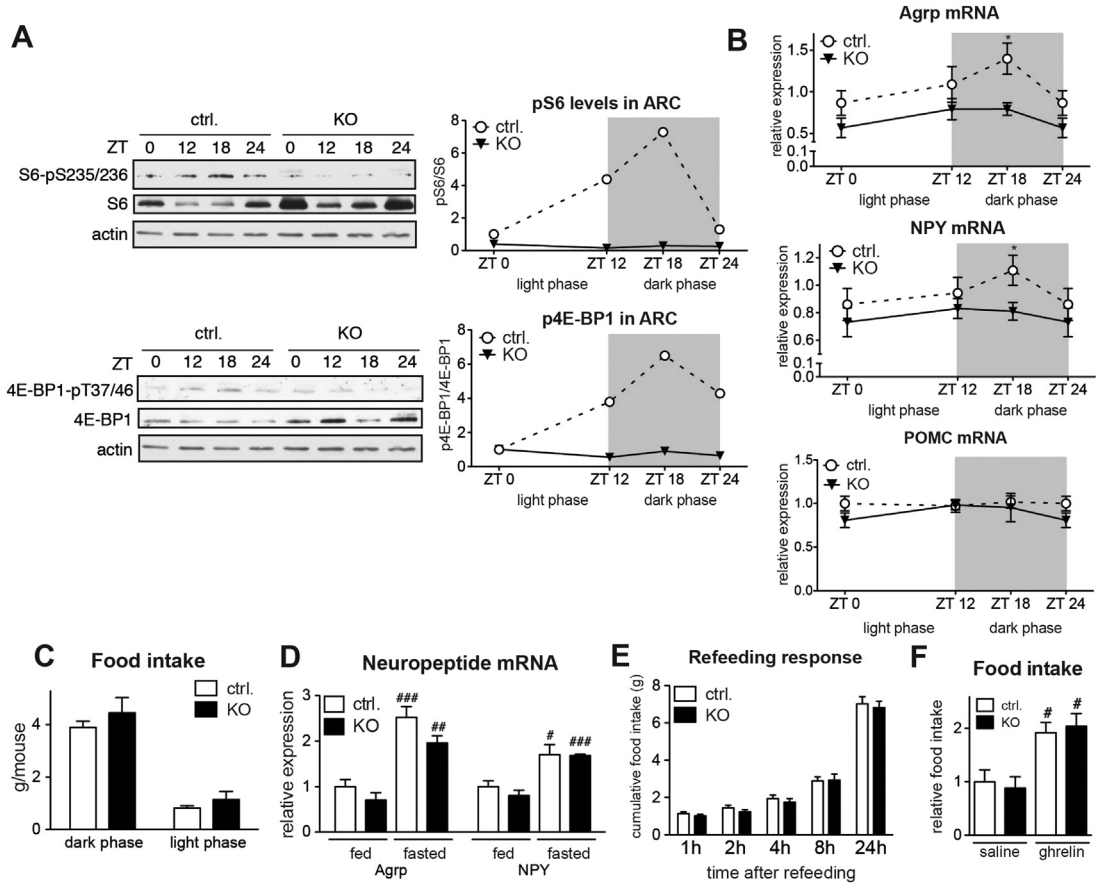

Fig. 3. Agrp-raptor KO mice display impaired circadian rhythm of *Agrp* and *NPY* expression but not altered feeding behavior. (A) Immunoblot analysis with the indicated antibodies of protein lysates from the ARC of Agrp-*raptor* KO and control mice sacrificed at the indicated time points. (B) qRT-PCR analysis of *Agrp*, *NPY* and *POMC* mRNA in the hypothalamus of Agrp-*raptor* KO and control mice sacrificed at the indicated time points. (C) *Ad libitum* food intake of Agrp-*raptor* KO and control mice. (D) qRT-PCR analysis of *Agrp* and *NPY* mRNA in the hypothalamus of Agrp-*raptor* KO and control mice fed *ad libitum* (fed) or starved over night (fasted). (E) Refeeding response of Agrp-*raptor* KO and control mice after over night starvation. (F) Feeding response of Agrp-*raptor* KO and control mice after ghrelin injection. Data represent mean ± SEM. Statistically significant differences between Agrp-*raptor* KO and control mice are indicated with asterisks (* = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$). Statistically significant differences between feeding conditions or ghrelin stimulation are indicated with a number sign (# = $p < 0.05$; ## = $p < 0.01$; ### = $p < 0.001$).

not been thoroughly examined. To elucidate the role of mTORC1 signaling in these neurons, we generated Agrp-*raptor* KO mice. Surprisingly, Agrp-*raptor* KO mice displayed no impairment in whole-body energy metabolism, glucose homeostasis and feeding behavior, both on normal and high fat diet. Hence, mTORC1 signaling in Agrp neurons is dispensable for the regulation of energy balance and feeding behavior. This is in agreement with a recent publication demonstrating that hyper-activation of mTORC1 in Agrp neurons, through deletion of *TSC1*, did not affect feeding behavior or energy homeostasis [29]. Thus, disruption of mTORC1 signaling in POMC neurons seemingly affects whole-body energy homeostasis, whereas perturbation of mTORC1 signaling in Agrp neurons does not. Interestingly, similar to mTORC1, Agrp-specific inactivation of mTORC2 signaling, through deletion of *rictor*, did not result in any alterations in feeding behavior and energy homeostasis, while POMC-specific deletion of *rictor* caused hyperphagia-induced obesity [30].

These findings are in line with the observation that perturbation of POMC neuronal function has a more profound effect on feeding behavior and energy metabolism as compared to perturbation of Agrp/NPY neuronal function. For example, inhibition of *Agrp* or *NPY* expression does not affect feeding behavior [31–34]. In contrast, impaired *POMC* expression or POMC neuronal function results in hyperphagia, obesity and disturbance in energy homeostasis [35,36]. While depletion of Agrp neurons in neonates only mildly affects feeding behavior, ablation of Agrp neurons in adult mice results in a strong decrease in food intake, leading to starvation [37]. These findings imply that the requirement for Agrp neurons for the regulation of feeding can be circumvented when their function is impaired early in life. A limitation of the Agrp-*raptor* KO mice used in this study is therefore that mTORC1 signaling is inactivated in Agrp neurons already in the neonate state. It could thus be that compensatory mechanisms have developed to circumvent the requirement for mTORC1 signaling for Agrp neuronal function in Agrp-*raptor* KO mice. Future studies should address whether disruption of mTORC1 signaling in Agrp neurons in adult mice impacts energy homeostasis and feeding behavior.

We found that Agrp-*raptor* KO mice display impaired circadian expression of *Agrp* and *NPY* but normal feeding behavior. Interestingly, mTORC1 activity in the ARC of control mice was highest during the dark phase, similar to the expression pattern of *Agrp* and *NPY* mRNA. Since Agrp-*raptor* KO mice were unable to induce mTORC1 signaling in Agrp neurons during the dark phase, it is likely that circadian mTORC1 signaling regulates circadian *Agrp* and *NPY* mRNA expression. In contrast to the defective *Agrp* and *NPY* expression observed in *ad libitum* fed mice, Agrp-*raptor* KO mice strongly induced *Agrp* and *NPY* mRNA expression in response to over night starvation as observed in control mice. Importantly, mTORC1 activity was low in the ARC of control mice after over night starvation. These finding suggest that the induction of *Agrp* and *NPY*

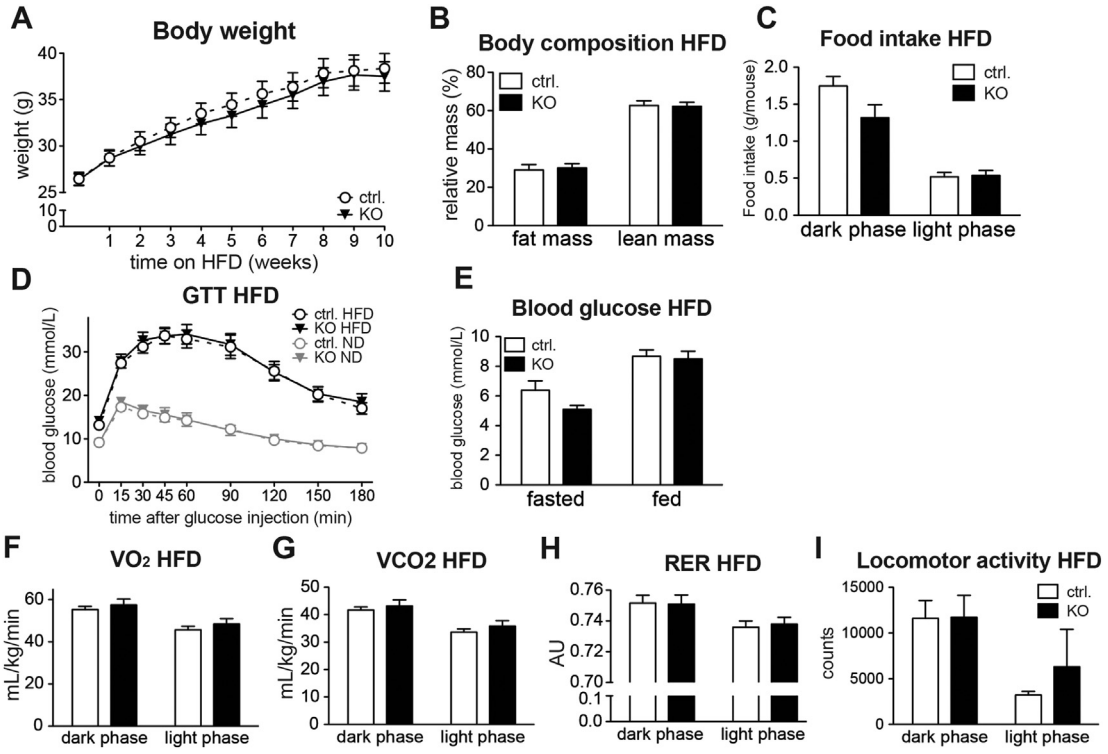

Fig. 4. Agrp-*raptor* KO mice respond to metabolic stress. (A) Body weight of HFD-fed Agrp-*raptor* KO and control mice. (B) Body composition of HFD-fed Agrp-*raptor* KO and control mice. (C) Food intake of HFD-fed Agrp-*raptor* KO and control mice. (D) Glucose tolerance test (GTT) of standard diet and HFD-fed Agrp-*raptor* KO and control mice. (E) Fasted and fed *ad libitum* blood glucose levels of HFD-fed Agrp-*raptor* KO and control mice. (F) Oxygen consumption (VO_2) of HFD-fed Agrp-*raptor* KO and control mice. (G) Carbon dioxide production (VCO_2) of HFD-fed Agrp-*raptor* KO and control mice. (H) Respiratory exchange ratio (RER) of HFD-fed Agrp-*raptor* KO and control mice. (I) Locomotor activity of HFD-fed Agrp-*raptor* KO and control mice. Data represent mean ± SEM.

mRNA expression after over night starvation is mTORC1-independent and thus mechanistically different compared to the circadian regulation of *Agrp* and *NPY* mRNA expression. Future studies should be aimed at identifying the molecular basis for this difference in the regulation of *NPY* and *Agrp* expression.

Acknowledgments

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