



POMC AMPK α 1 deletion drives male-specific BAT thermogenesis and obesity resistance

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Abstract

Proopiomelanocortin (POMC) neurons in the arcuate nucleus of the hypothalamus (ARC) play a critical role in energy homeostasis. 5'-adenosine monophosphate-activated protein kinase (AMPK) is a serine/threonine kinase, which acts as the main energy sensor in the cell. The heterotrimeric AMPK results from the combination of a catalytic α subunit (α 1, α 2) with two regulatory subunits, β (β 1, β 2) and γ (γ 1, γ 2 and γ 3). Hypothalamic AMPK plays a key role in the control of energy balance, but current evidence shows that the precise combination of its heterotrimeric components determines its physiological action. Our findings show that AMPK α 1 ablation in POMC neurons has a sex-dependent protective effect against diet-induced obesity because of increased thermogenesis in brown adipose tissue (BAT) in males, but not in females. At the molecular level, deletion of AMPK α 1 in POMC neurons disrupts the ARC phosphoproteome and is associated with ameliorated endoplasmic reticulum (ER) stress and reduced ceramide content. These findings highlight the complex function of the different AMPK subunits in the hypothalamic regulation of energy balance.

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Introduction

The arcuate nucleus of the hypothalamus (ARC), anatomically located adjacent to the semipermeable blood–brain barrier (BBB) of the median eminence (ME) and surrounding the third ventricle (3 V), is a fundamental area to sense, integrate and respond to circulating hormonal and metabolic signals (Quarta et al, 2021; Quarta et al, 2020; Schneeberger et al, 2014). Within the ARC, two neuronal populations play critical but opposing roles in energy homeostasis. Agouti-related peptide (AgRP) neurons are activated by a negative energy balance and stimulate food intake and reduce energy expenditure by antagonizing melanocortin 4 receptor (MC4R) signaling in second-order neurons (Quarta et al, 2021; Quarta et al, 2020; Schneeberger et al, 2014). In contrast, proopiomelanocortin (POMC) neurons are activated by conditions of high energy availability and release the MC4R agonist α -melanocyte-stimulating hormone (α -MSH) onto second-order neurons to which they send projections (Leon et al, 2024; Quarta et al, 2021; Quarta et al, 2020; Schneeberger et al, 2014). Consequently, POMC neurons induce an anorectic effect and increase energy expenditure to maintain energy homeostasis. Accordingly, both genetic defects and metabolic challenges to the melanocortin system cause impairments, which can lead to the development of obesity (Ramadori et al, 2010; Sweeney et al, 2023; Yeo et al, 2021). Leptin resistance and impaired serotonin responses in POMC and/or AgRP neurons are considered a paradigmatic event in diet-induced obesity (Guo et al, 2019; Ramadori et al, 2010; Sweeney et al, 2023; Yeo et al, 2021). However, the molecular underpinnings causing the malfunction of this neuronal system in obesity are still under investigation.

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5' adenosine monophosphate-activated protein kinase (AMPK) is a serine/threonine kinase that acts as a major energy sensor in the hypothalamus (Lopez, 2022; López et al, 2016; Schneeberger and Claret, 2012), acting as a molecular mediator of several hormones (e.g., leptin, ghrelin, thyroid hormones, estradiol, and BMP8b), and drugs (e.g., nicotine, liraglutide, olanzapine) (Lopez, 2022; López et al, 2016; Schneeberger and Claret, 2012). Composed of a heterotrimeric complex, AMPK results from the combination of a catalytic α ($\alpha 1$ and $\alpha 2$) subunit, with a serine/threonine kinase domain, and two regulatory subunits β ($\beta 1$ and $\beta 2$) and γ ($\gamma 1$, $\gamma 2$, and $\gamma 3$), with each subunit encoded by different genes (Lopez, 2022; López et al, 2016; Schneeberger and Claret, 2012). The possible combinations of the heterotrimeric $\alpha\beta\gamma$ AMPK complex display a particular pattern of expression depending on the tissue and cellular population considered (Lopez, 2022; López et al, 2016; Schneeberger and Claret, 2012).

The role of AMPK subunits in different neuronal populations has revealed specific regulatory activities and functions (Lopez, 2022; López et al, 2016; Schneeberger and Claret, 2012). Genetically modified mouse lines have helped to precisely dissect those roles. Deletion of AMPK $\alpha 1$ in steroidogenic factor-1 (SF1) neurons of the ventromedial hypothalamic nucleus (VMH) increases brown adipose tissue (BAT) thermogenesis and energy expenditure, resulting in a lean phenotype resistant to diet-induced obesity (Martínez-Sánchez et al, 2017b; Seoane-Collazo et al, 2018). Consistent with this rationale, exosome-mediated targeting of AMPK $\alpha 1$ in SF1 neurons also represents an effective anti-obesity approach (Milbank et al, 2023; Milbank et al, 2021; Mukherjee et al, 2023). Conversely, loss of AMPK $\gamma 2$ in SF1 neurons causes obesity associated with reduced BAT thermogenesis (Freire-Agulleiro et al, 2025). Similarly, deletion of AMPK $\alpha 2$ in POMC neurons induces obesity through reduced energy expenditure and elevated food intake, whereas its loss in AgRP neurons leads to an age-dependent lean phenotype (Claret et al, 2007).

Overall, the current knowledge indicates that a precise and cell-specific study of the different hypothalamic AMPK subunits in the melanocortin system is necessary to understand their function. However, the specific role of AMPK α subunits in mediating neuronal adaptations to changes in energy homeostasis and body weight regulation remains unexplored. This study aimed to elucidate the role of AMPK $\alpha 1$ in hypothalamic POMC neurons in whole-body energy balance regulation. Our results unveil that genetic ablation of AMPK $\alpha 1$ in POMC neurons of the ARC induced a feeding-independent protection against diet-induced obesity coupled to an increased BAT thermogenesis in male, but not in female mice. This phenotype was linked to phosphoproteome alterations and reduced hypothalamic ER stress and ceramide content, highlighting the cell-specific roles of AMPK subunits in the metabolic adaptation to obesogenic diets and specifically implicating the $\alpha 1$ subunit in this process.

Results

Generation and validation of specific AMPK $\alpha 1$ null mice in POMC neurons

To investigate the functional relevance of the AMPK $\alpha 1$ subunit in POMC neurons, we generated a constitutive *Prkaa1* knockout (KO) mouse model

in those neurons (referred to as POMC-Cre AMPK $\alpha 1^{fl/fl}$). By crossing the POMC-Cre AMPK $\alpha 1^{fl/fl}$ mice with the Ai14-tdTomato reporter mouse line (Fig. 1A), we fluorescently labeled the Cre-recombined cells (Fig. 1B), allowing us to confirm the genetic targeting of POMC-derived cells in the ARC. Using the POMC-Cre AMPK $\alpha 1^{fl/fl}$ mouse, we detected by double in situ hybridization a clear decrease of *Prkaa1* transcripts in the POMC-derived cells (Fig. 1C,D), validating the knockout of *Prkaa1* in our mouse model. Together with this, we explored the presence of DNA recombination as a surrogate marker of the *Prkaa1* deletion in different tissues of the central nervous system and periphery. We detected amplicons compatible with effective recombination in the ARC, and to a lesser extent, in the nucleus tractus solitarius (NTS) (Fig. 1E). In contrast, non-expressing POMC tissues/areas, such as the cerebellum, cortex, liver, and white adipose tissue (WAT), showed a complete absence of the excised DNA material (Fig. 1E). These results confirm the AMPK $\alpha 1$ subunit deletion in POMC-derived neurons.

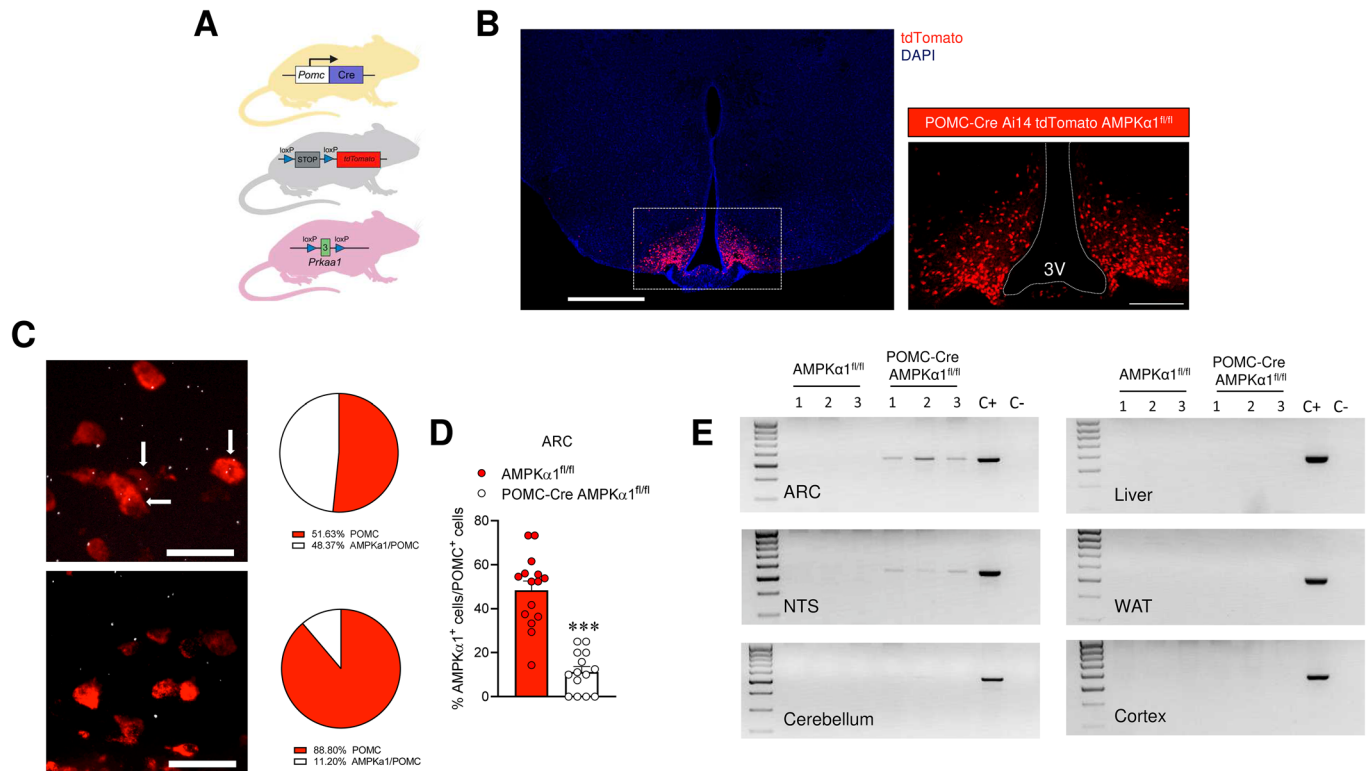
Loss of AMPK $\alpha 1$ in POMC neurons did not impact energy balance under a chow diet

To elucidate the metabolic implications of the AMPK $\alpha 1$ subunit ablation in POMC neurons, we subjected male and female POMC-Cre AMPK $\alpha 1^{fl/fl}$ mice, including cohorts phenotyped in parallel under standardized conditions, and their littermate controls to a complete metabolic phenotyping under a standard diet. Our data revealed that male and female POMC-Cre AMPK $\alpha 1^{fl/fl}$ mice attained the same body weight as the controls when fed chow (Figs. 2A and EV1A). The weight of fat pads (Figs. 2B and EV1B) and average daily food intake (Figs. 2C and EV1C) were similar between POMC-Cre AMPK $\alpha 1^{fl/fl}$ mice and controls. We also observed comparable body (Figs. 2D and EV1D) and BAT temperature (Figs. 2E,F and EV1E), as along with similar UCP1 levels in the BAT between genotypes (Figs. 2G,H and EV1F) and norepinephrine (NE, indicative of similar levels of sympathetic BAT activation), dopamine (DA) and serotonin (5-HT) levels (Fig. 2I). Both male and female controls and POMC-Cre AMPK $\alpha 1^{fl/fl}$ mice also exhibited similar levels of subcutaneous WAT (sWAT) browning, assessed by adipocyte area (Figs. EV1G and EV2A,B) and UCP1 staining (Figs. EV1H and EV2C,D).

Given the relevance of POMC neurons in the regulation of glucose homeostasis (Quarta et al, 2021; Quarta et al, 2020; Schneeberger et al, 2014), we characterized blood glucose levels in fed and fasted states (Figs. 2J and EV1I), glucose tolerance (Figs. 2K,L and EV1J,K) and insulin sensitivity (Figs. 2M,N and EV1L,M) of POMC-Cre AMPK $\alpha 1^{fl/fl}$ mice and controls without observing differences between genotypes either in males or females. To complete the metabolic phenotyping of these animals, we subjected them to indirect calorimetry. POMC-Cre AMPK $\alpha 1^{fl/fl}$ mice and controls displayed similar energy expenditure (EE; Figs. 2O,P and EV1N,O), respiratory exchange ratio (RER; Figs. 2Q and EV1P) and locomotor activity (LA; Figs. 2R and EV1Q). Overall, this evidence suggested that the lack of AMPK $\alpha 1$ in POMC neurons did not impact energy metabolism in conditions of a standard diet in either male or female mice.

Loss of AMPK $\alpha 1$ in POMC neurons conferred protection against HFD in male but not female mice

To gain further insights into the role of the AMPK $\alpha 1$ subunit in POMC neurons in the metabolic context of obesity, we characterized



male and female POMC-Cre *AMPK α 1^{fl/fl}* mice and their controls under a high-fat diet (HFD). Several reports have shown how POMC neurons participate in the pathophysiological mechanisms of diet-induced obesity (Claret et al, 2007; Quarta et al, 2021; Quarta et al, 2020; Ramadori et al, 2010; Souza et al, 2016). We observed that male, but not female, POMC-Cre *AMPK α 1^{fl/fl}* mice were protected against the body weight gain induced by HFD (Figs. 3A and EV3A). However, fat pads weight did not show major differences in either male or female POMC-Cre *AMPK α 1^{fl/fl}* mice (Figs. 3B and EV3B). When the different components of the energy balance were dissected, we observed no differences in either food intake (Figs. 3C and EV3C), or body temperature (Figs. 3D and EV3D). On the other hand, in males, but not females, BAT displayed an increased temperature (Figs. 3E,F and EV3E) and UCP1 expression (Figs. 3G,H and EV3F). In line with these data, NE levels were increased in the BAT of POMC-Cre *AMPK α 1^{fl/fl}* male mice (Fig. 3I), indicating enhanced sympathetic activation of the tissue. Of note, the thermogenic effect seemed restricted to the BAT since null mice and their control littermates displayed similar levels of sWAT browning, with no differences in adipocyte area (Figs. EV3G and EV4A,B) or UCP1 staining (Figs. EV3H and EV4C,D).

In keeping with the similar food intake observed between POMC-Cre *AMPK α 1^{fl/fl}* mice and controls fed a HFD, we detected no changes in glycemia in both fed and fasted conditions (Figs. 3J and EV3I). Glucose tolerance (Figs. 3K,L and EV3J,K), and insulin sensitivity (Figs. 3M,N and EV3L,M) were similar in POMC-Cre *AMPK α 1^{fl/fl}* male and female mice fed a HFD, when compared to control littermates. The observed changes in thermogenesis, including higher BAT temperature and UCP1 expression in POMC-Cre *AMPK α 1^{fl/fl}* male mice, led us to hypothesize that components of overall energy homeostasis would be affected. Indirect calorimetry analyses demonstrated that POMC-Cre *AMPK α 1^{fl/fl}* male, but not female, mice fed a HFD displayed a tendency toward higher EE when compared to controls (Figs. 3O,P and EV3N,O). However, both male and female mice presented similar RER (Figs. 3Q and EV3P) and LA (Figs. 3R and EV3Q) between genotypes. Overall, these results point that absence of *AMPK α 1* in POMC neurons has a protective effect under obesogenic conditions in male, but not female mice, and the reduction in body weight is driven by an increased BAT thermogenesis and EE. This sex-specific HFD response indicates that *AMPK α 1* in POMC neurons exerts diet-dependent, sexually

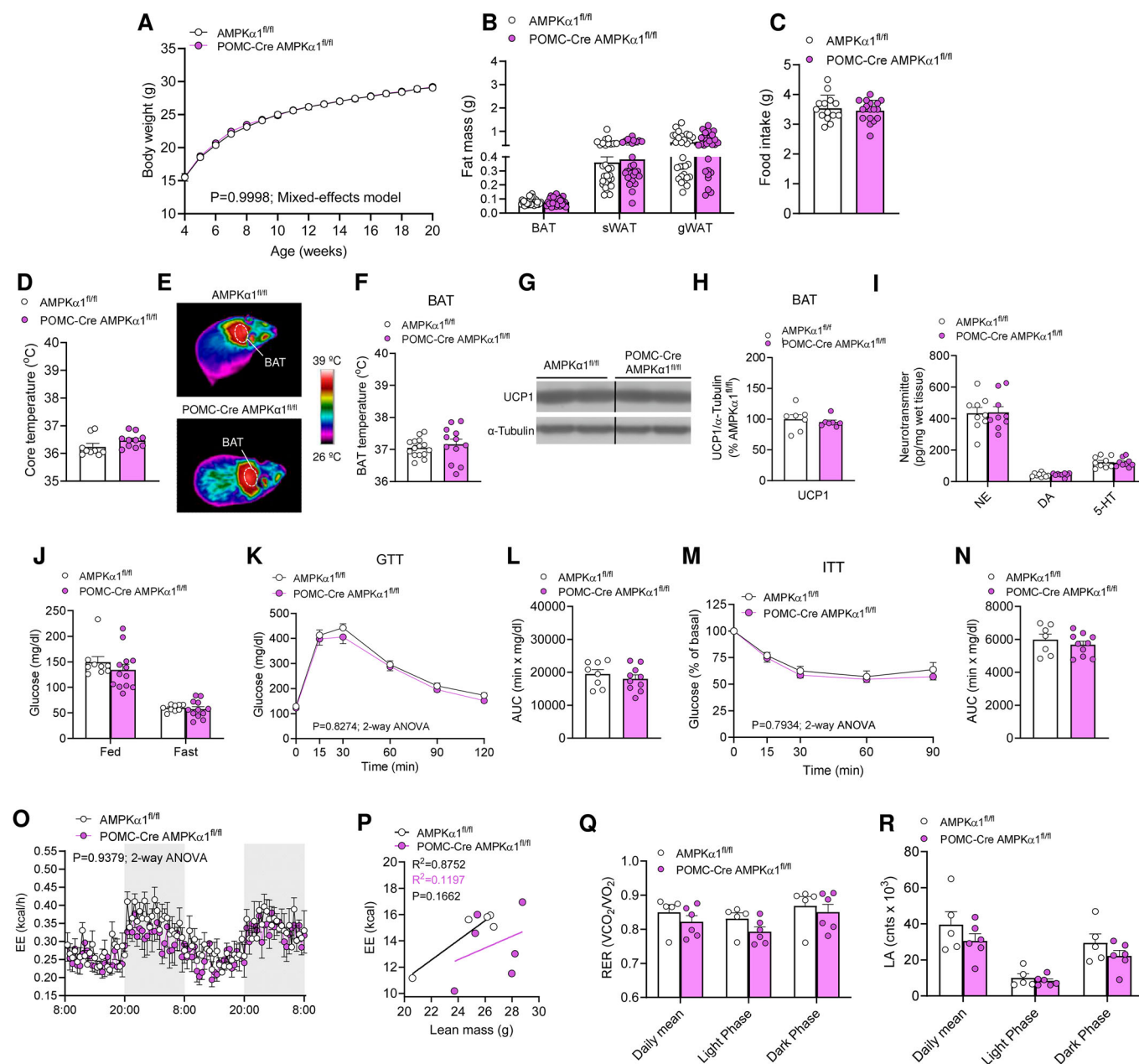


Figure 2. Effects of loss of AMPK α 1 in POMC neurons on energy balance, energy expenditure and glucose homeostasis in male mice fed a chow diet.

(A–D) Body weight (A; $n=33$ control and 37 KO), fat depot mass (B; $n=30$ control and 30 KO), daily food intake (C; $n=14$ control and 17 KO), and core body temperature (D; $n=9$ control and 10 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} male mice. (E–H) Representative BAT infrared thermographic images (E), BAT temperature (F; $n=15$ control and 12 KO), representative BAT UCP1 western blot images (G) and BAT UCP1 protein levels (H; $n=7$ control and 7 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} male mice. In the western blot analyses, α -tubulin was used as a control. A black line between the immunoblots depicts samples loaded in the same gel, but not next to each other. (I) BAT neurotransmitter levels ($n=9$ control and 9 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} male mice. (J–N) Fed and fasted blood glucose levels (J; $n=9$ control and 12–13 KO), glucose tolerance test and area under the curve (AUC) (K, L; $n=8$ control and 10 KO), insulin tolerance test, and AUC (M, N; $n=7$ control and 10 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} male mice. (O–R) Energy expenditure (EE; O; $n=5$ control and 6 KO), ANCOVA analysis of 48 h total energy expenditure using lean mass as a covariate (P; $n=5$ control and 6 KO), respiratory exchange ratio (RER; Q; $n=5$ control and 6 KO), locomotor activity (LA; R; $n=5$ control and 6 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} male mice. Data were expressed as mean $\pm$ SEM. Statistical significance was assessed by mixed-effect model (A), two-way ANOVA (K, M, O) and Student's t -test (B–D, F, H–J, L, N, Q, R) and ANCOVA (P). Source data are available online for this figure.

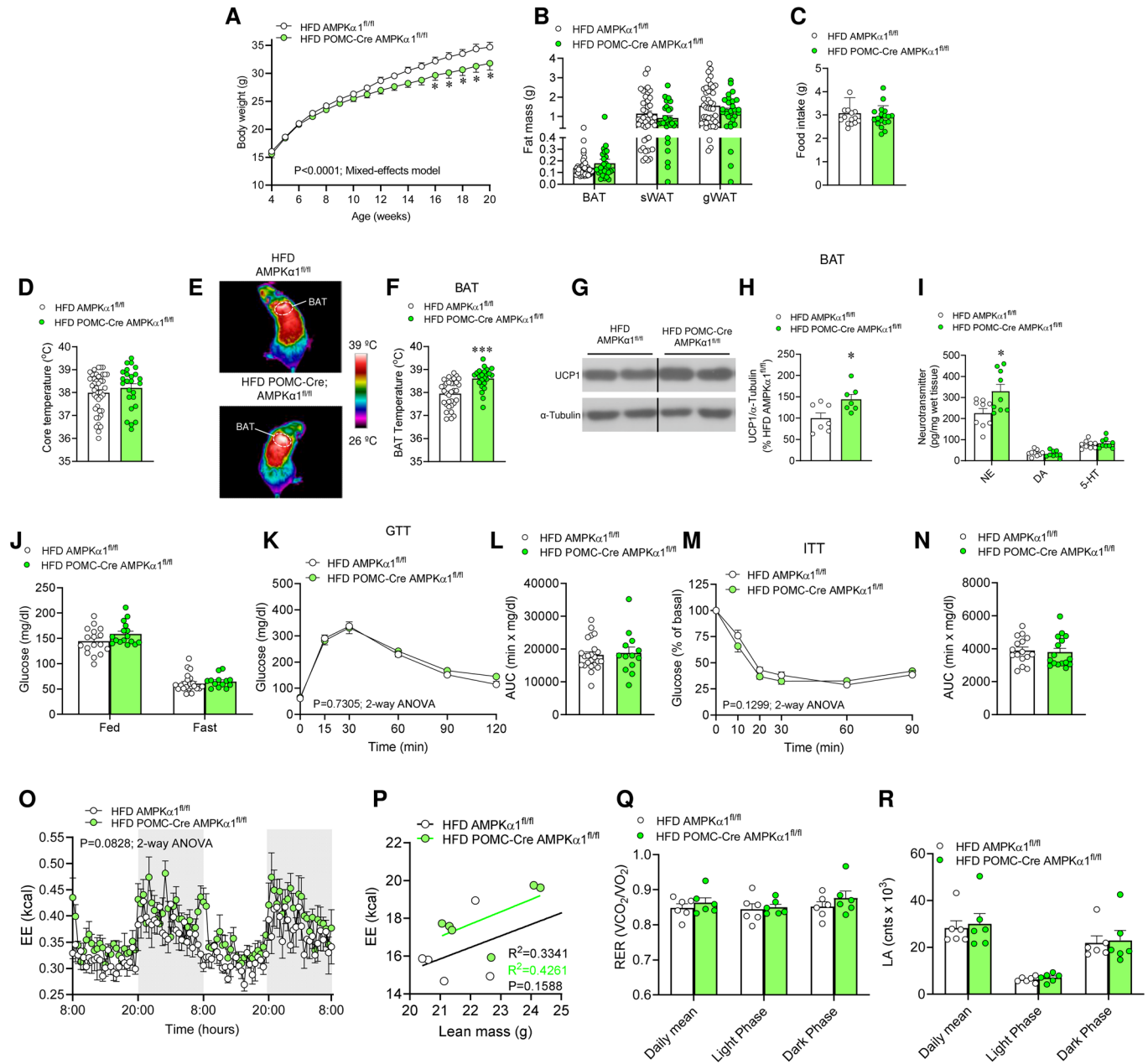


Figure 3. Effects of loss of AMPK α 1 in POMC neurons on energy balance, energy expenditure and glucose homeostasis in male mice fed a HFD.

(A–D) Body weight (A; $n = 44$ control and 31 KO), fat depot mass (B; $n = 43$ control and 29 KO), daily food intake (C; $n = 15$ control and 18 KO), core body temperature (D; $n = 43$ control and 25 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} male mice fed a HFD. (E–H) Representative BAT infrared thermographic images (E), BAT temperature (F; $n = 33$ control and 23 KO; $P < 0.0001$), representative BAT UCP1 western blot images (G) and BAT UCP1 protein levels (H; $n = 7$ control and 6 KO; $P = 0.0212$) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} male mice fed a HFD. In the western blot analyses, α -tubulin was used as a control. A black line between the immunoblots depicts samples loaded in the same gel, but not next to each other. (I) BAT neurotransmitter levels ($n = 9$ control and 9 KO; $P = 0.017622$ for NE) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} male mice fed a HFD. (J–N) Fed and fasted blood glucose levels (J; $n = 17$ –23 control and 13–16 KO), glucose tolerance test and area under the curve (AUC) (K, L; $n = 23$ control and 13 KO), insulin tolerance test, and AUC (M, N; $n = 16$ control and 16 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} male mice fed a HFD. (O–R) Energy expenditure (EE; O; $n = 6$ control and 6 KO), ANCOVA analysis of 48 h total energy expenditure using lean mass as a covariate (P; $n = 6$ control and 6 KO), respiratory exchange ratio (RER; Q; $n = 6$ control and 6 KO), locomotor activity (LA; R; $n = 6$ control and 6 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} male mice fed a HFD. Data were expressed as mean $\pm$ SEM. Statistical significance was assessed by mixed-effect model (A), two-way ANOVA (K, M, O) and Student's t -test (B–D, F, H–J, L, N, Q, R) and ANCOVA (P). Source data are available online for this figure.

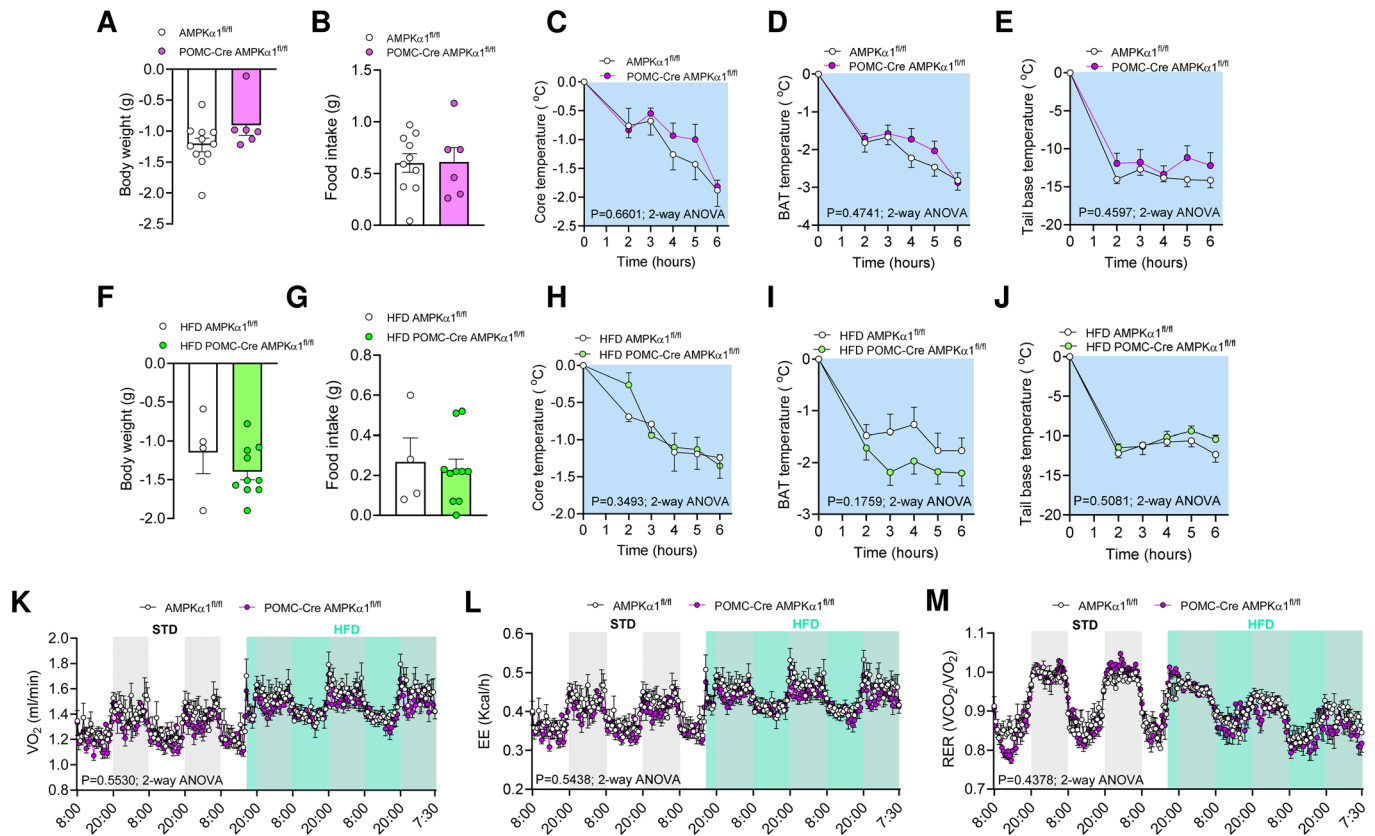


Figure 4. Effects of loss of AMPKα1 in POMC neurons in cold and short-term dietary responses in male mice.

(A–E) Body weight (A; $n = 11$ control and 6 KO), food intake (B; $n = 11$ control and 6 KO), change in: core body temperature (C; $n = 10$ control and 6 KO), BAT temperature (D; $n = 10$ control and 6 KO) and tail base temperature (E; $n = 10$ control and 6 KO) of cold exposed control (littermates AMPKα1^{fl/fl}) and POMC-Cre AMPKα1^{fl/fl} male mice. (F–J) Body weight (F; $n = 4$ control and 10 KO), food intake (G; $n = 4$ control and 9 KO), change in: core body temperature (H; $n = 4$ control and 10 KO), BAT temperature (I; $n = 4$ control and 10 KO) and tail base temperature (J; $n = 4$ control and 10 KO) of cold exposed control (littermates AMPKα1^{fl/fl}) and POMC-Cre AMPKα1^{fl/fl} male mice fed a HFD. (K–M) Oxygen consumption rate (VO₂; K; $n = 6$ control and 6 KO), energy expenditure (EE; L; $n = 6$ control and 6 KO), and respiratory exchange ratio (RER; M; $n = 6$ control and 6 KO) of control (littermates AMPKα1^{fl/fl}) and POMC-Cre AMPKα1^{fl/fl} male mice, during a chow-HFD switch. Cold exposure and diet switch experiments were performed once. Data were expressed as mean ± SEM. Statistical significance was assessed by two-way ANOVA (C–E, H–M) and Student's *t*-test (A, B, F, G). Source data are available online for this figure.

dimorphic control over energy balance, likely reflecting gonadal hormone modulation of hypothalamic energy sensing.

Loss of AMPKα1 in POMC neurons did not impact either cold- or diet-induced thermogenesis

To determine whether the elevated basal BAT activity in POMC AMPKα1 KO mice reflects enhanced adaptive thermogenic capacity, we challenged male POMC-Cre AMPKα1^{fl/fl} mice and their littermate controls with acute cold exposure (4 °C for 6 h) under both chow and HFD conditions. Neither body weight (Fig. 4A) nor food intake (Fig. 4B) was affected by cold challenge in chow mice. Core body temperature maintenance was identical between genotypes during cold challenge in chow-fed mice (Fig. 4C), accompanied by comparable intrascapular BAT temperature dynamics (Fig. 4D) and tail temperature as a vasoconstriction/heat dissipation proxy (Fig. 4E). These findings were replicated in HFD-fed mice, with no differences in body weight (Fig. 4F), food intake (Fig. 4G), core body temperature (Fig. 4H), BAT temperature kinetics (Fig. 4I), and tail temperature (Fig. 4J). Overall, cold tolerance outcomes confirmed preserved adaptive responses across

diets in null mice. We further assessed metabolic adaptation to dietary challenge through indirect calorimetry during the chow-to-HFD transition. Oxygen consumption rates (VO₂; Fig. 4K) and EE dynamics (Fig. 4L) were similar between genotypes across light/dark cycles. RER declined equivalently in both groups, reflecting a similar shift toward fat oxidation upon HFD introduction (Fig. 4M). Collectively, these data demonstrate that POMC AMPKα1 deletion elevates basal sympathetic nervous system (SNS)-driven BAT tone without altering cold- or diet-induced thermogenic adaptations. Notably, this uncouples POMC AMPKα1 from the dual basal/cold-adaptive role well-established for SF1 neuronal AMPKα1 (Martinez-Sanchez et al, 2017b; Milbank et al, 2021; Seoane-Collazo et al, 2018), highlighting hypothalamic neuronal subunit specificity.

Loss of AMPKα1 in POMC neurons impacted the ARC phosphoproteome

To investigate the molecular mechanisms potentially underlying our observed thermogenic phenotype, hypothalami were harvested from POMC-Cre AMPKα1^{fl/fl} male mice and littermate controls

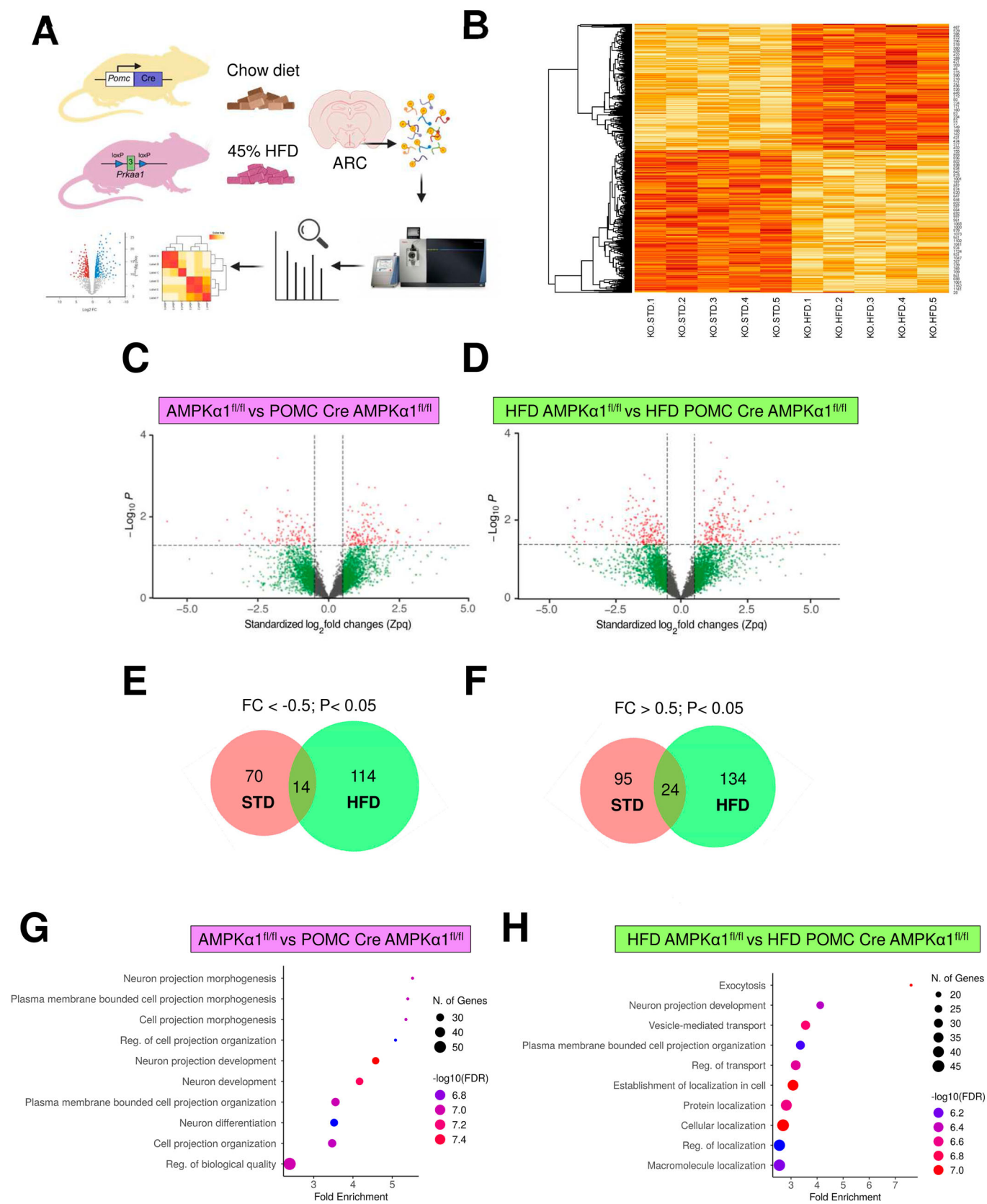


Figure 5. Impact of AMPK α 1 deletion in POMC neurons on the phosphoproteome of the ARC.

(A) Schematic representation of the phosphoproteomics workflow. (B) Hierarchical clustering of differentially phosphorylated peptides in the ARC of POMC-Cre AMPK α 1^{fl/fl} male mice under standard (STD) or high-fat diet (HFD). Relative abundances of phosphopeptides were expressed in relation to their corresponding protein using standardized log₂-ratios (Zpq values). (C) Volcano plot illustrating differential phosphorylation in the ARC [$n = 5$ control (ten total mice pulled by pairs) and 5 KO (ten total mice pulled by pairs)] between AMPK α 1^{fl/fl} and POMC-Cre AMPK α 1^{fl/fl} male mice. (D) Volcano plot depicting differential phosphorylation in the ARC [$n = 5$ control (ten total mice pulled by pairs) and five KO (ten total mice pulled by pairs)] between HFD AMPK α 1^{fl/fl} and HFD POMC-Cre AMPK α 1^{fl/fl} male mice. The phosphopeptides with the highest abundance changes ($P < 0.05$ and $Zpq > 0.5$ or < -0.5) in (C) and in (D) are highlighted in red. (E, F) Venn diagram showing the overlap between downregulated (E) and upregulated (F) phosphopeptides with the highest abundance change in STD POMC-Cre AMPK α 1^{fl/fl} and HFD POMC-Cre AMPK α 1^{fl/fl} male mice. (G) Bubble plot of the top ten enriched GO Biological Process terms associated with the proteins harboring the phosphopeptides selected in the comparison between AMPK α 1^{fl/fl} and POMC-Cre AMPK α 1^{fl/fl} male mice. (H) Bubble plot of the top ten enriched GO Biological Process terms for phosphopeptide-harboring proteins in HFD-fed AMPK α 1^{fl/fl} and POMC-Cre AMPK α 1^{fl/fl} male mice. In (F and G), circle size reflects the number of proteins, and color intensity indicates enrichment significance ($-\log_{10}(\text{FDR})$). Statistical significance was assessed by Student's *t*-test (C, D). Source data are available online for this figure.

that were fed either chow or HFD and subjected to unbiased phosphoproteomics (Fig. 5A). To assess the impact of AMPK α 1 deletion in POMC neurons, we performed unsupervised clustering of the differentially enriched phosphoproteins. This analysis clearly distinguished POMC-Cre AMPK α 1^{fl/fl} mice based on dietary conditions, demonstrating the robustness of our approach to capture metabolic effects on the phosphoproteomic profile (Fig. 5B). To further characterize the molecular signatures associated with the genetic loss of AMPK α 1 in POMC neurons, we performed differential expression analysis by comparing POMC-Cre AMPK α 1^{fl/fl} mice and littermate controls. Under chow diet, AMPK α 1 deletion in POMC neurons led to a profound alteration in the phosphorylation profile in the ARC, (Fig. 5C). The HFD also produced a clear modification of the phosphorylation pattern when comparing the null mice with the controls (Fig. 5D). However, when comparing between the diets the proteins harboring the affected phosphopeptides (acknowledging that a single protein may harbor multiple phosphorylation at different residues) most of them were of different nature, with few proteins being shared in both diets, suggesting that the phosphoproteome profile is highly dependent on the metabolic context (Fig. 5E,F). The fact that diet alters the pattern of phosphoproteome changes may explain why the phenotype we observed became apparent only under diet-induced obesity. To gain further insight into the downstream molecular effects of AMPK α 1 deletion in POMC neurons, particularly under HFD, we performed a post hoc Gene Ontology (GO) enrichment analysis on the non-overlapping phosphopeptide-containing protein sets. Under the chow diet, AMPK α 1 loss in POMC neurons enriched biological processes related to neuronal projection, morphogenesis and cell projection organization (Fig. 5G). Interestingly, categories related to neuron development and differentiation were also enriched, raising the possibility that AMPK α 1 deletion alters the neurotransmitter identity of POMC neurons. However, the percentage of glutamatergic and GABAergic POMC neurons was similar between genotypes (Fig. 6A,B), as well as the total number of POMC neurons, which was comparable (Fig. 6A,C), thus discarding any differentiation issue of our model. Applying a similar analysis on HFD in the POMC-Cre AMPK α 1^{fl/fl} mice, we found that affected phosphoproteins belonged to several biological processes associated with exocytosis and vesicle transport, as well as cell localization. We also found enriched projection-related processes (Fig. 5H). Taken together, these findings showed that AMPK α 1 deletion in POMC neurons drives specifically differential phosphorylation landscapes under chow and HFD conditions. Moreover, the enrichment of exocytosis-associated pathways in diet-induced obesity is particularly remarkable, as it

may carry significant functional implications given the peptidergic nature of POMC neurons.

Loss of AMPK α 1 in POMC neurons was associated with an amelioration of ER stress and ceramide levels in the ARC in HFD

Our GO enrichment analysis highlighted several projection developmental processes. Given that our mouse line undergoes AMPK α 1 deletion during embryogenesis, we decided to study POMC neuronal projections to the paraventricular nucleus of the hypothalamus (PVH) in male mice fed an HFD. We did not find differences in the PVH- α MSH fibers between the POMC-Cre AMPK α 1^{fl/fl} mice fed a HFD and the littermate controls, likely excluding the possibility that our phenotype is driven by a developmental alteration of the ARC-PVH melanocortin system (Fig. 7A,B).

Considering our previous findings, we decided to explore two possible AMPK-linked functions, namely ER stress and autophagy, that may contribute to the predicted alterations in exocytosis and vesicle transport. AMPK promotes autophagy (Li and Chen, 2019), a cell recycling process that has been described in ARC melanocortin neurons as a molecular mechanism controlling energy homeostasis (Coupe et al, 2012; Kaushik et al, 2012; Schneeberger et al, 2012). ATG7 (a key protein in the autophagy pathway) KO mice in AgRP neurons develop a decreased body weight and adipose mass (Kaushik et al, 2011). When autophagy related 7 (ATG7) was selectively ablated in POMC neurons, it leads to increased postweaning body weight, and higher adiposity (Coupe et al, 2012). We therefore hypothesized that autophagy may act as a downstream signal mediating the AMPK α 1 role in obesity in POMC-Cre AMPK α 1^{fl/fl} mice fed a HFD. To test this, we first confirmed that our model had a specific downregulation of the AMPK pathway (measured by a decreased pAMPK in the KO mice) in the ARC, but not in the VMH (Fig. 7C,D). No upregulation of AMPK α 2 subunit was found in the ARC of POMC-Cre AMPK α 1^{fl/fl} mice, either in chow diet (Fig. EV5A,B) or in HFD (Fig. EV5C,D) excluding the existence of any potential compensatory action between AMPK α subunits. Next, we investigated the hypothalamic mRNA expression of the main autophagy markers, namely *Atg7*, autophagy related 5 (*Atg5*), autophagy related 3 (*Atg3*), but no changes were detected (Fig. EV5E). Similarly, the protein levels of other autophagy markers, such as protein 62 (p62), and microtubule-associated protein light chain 3 (LC3-II/LC3-I) (Fig. EV5F,G) were also unchanged between null mice and the littermate controls fed a HFD. To achieve more cell-specificity in our analyses,

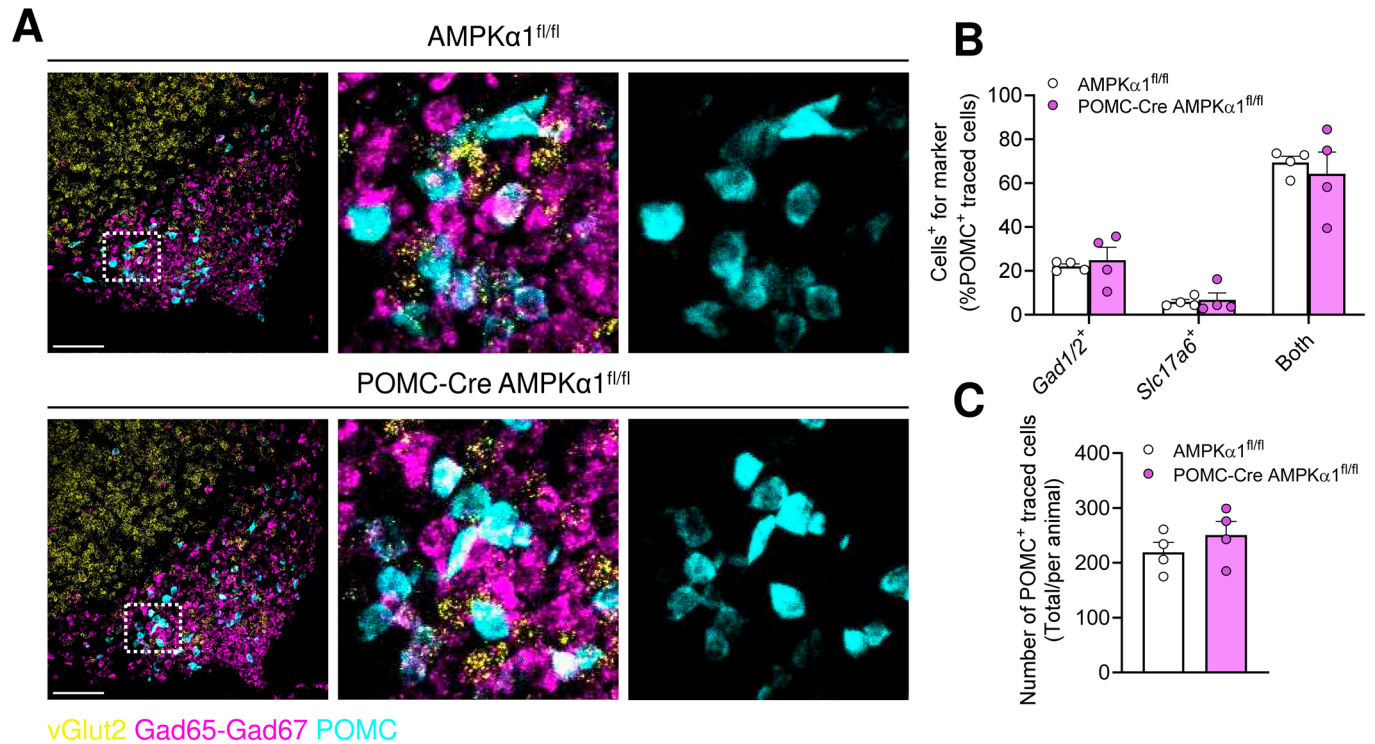


Figure 6. Effect of loss of AMPKα1 in POMC neurons on their neurotransmitter profile in male mice fed a HFD.

(A) Top: representative confocal images from the ARC of AMPKα1^{fl/fl} mice; bottom: representative confocal images from the ARC of POMC-Cre AMPKα1^{fl/fl} mice. Both, showing GABAergic (Gad65 (*Gad2*) and Gad67 (*Gad1*) in pink), and glutamatergic (vGlut2 (*Slc17a6*) in yellow) profile of POMC neurons (in blue). Scale bar 100 μm. Zoomed images come from the squared region in the first image, depicting details of the POMC neurons neurotransmitter profile. (B) Quantification of the number of POMC⁺ neurons that co-express either *Gad1/2* (GABAergic), *Slc17a6* (glutamatergic) or both markers (*n* = 4 control and 4 KO animals). (C) Total number of POMC neurons traced from AMPKα1^{fl/fl} mice, and POMC-Cre AMPKα1^{fl/fl} mice (*n* = 4 control and 4 KO animals). Data were expressed as mean ± SEM. Statistical significance was assessed by Student's *t*-test (B, C). Source data are available online for this figure.

we quantified p62 levels in POMC neurons from WT and POMC-Cre AMPKα1^{fl/fl} fed a HFD. Our data revealed no significant changes in p62 levels in POMC neurons of null mice (Fig. EV5H,I). Overall, this evidence did not support a primary role for autophagy in mediating the metabolic effects of POMC AMPKα1 deletion.

Hypothalamic ER stress also plays a prominent role in the regulation of energy balance (Contreras et al, 2014; Contreras et al, 2017; Dragano et al, 2024; Martinez-Sanchez et al, 2017b), being relevant in POMC neurons where it is associated with obese phenotypes (Dragano et al, 2024; He et al, 2021; Schneeberger et al, 2013; Williams et al, 2014; Xiao et al, 2017). To assess this potential mechanism in our model, we measured the protein levels of five key markers of the unfolded protein response (UPR): activating transcription factor 4 (ATF4), CHOP (C/EBP homologous protein), GRP78 (78 kDa glucose-regulated protein; a chaperone also known as BiP: binding immunoglobulin protein), and inositol-requiring enzyme 1α (IRE1α; an ER stress sensor), along with pEIF2α (phosphorylated eukaryotic initiation factor 2α). We observed a trend toward increased GRP78 expression, indicating enhanced capacity to handle unfolded proteins, accompanied by decreased IRE1α and a strong reduction in ATF4 in the ARC of POMC-Cre AMPKα1^{fl/fl} mice (Fig. 7E,F). These changes collectively reflect reduced ER stress. (Contreras et al, 2014; Contreras et al, 2017; Dragano et al, 2024; Martinez-Sanchez et al, 2017b). Of note, differences in ER stress were nucleus specific since the VMH had

comparable levels of the main UPR proteins, including phosphorylated protein kinase-like endoplasmic reticulum kinase (pPERK), between floxed and of POMC-Cre AMPKα1^{fl/fl} mice (Fig. EV5J,K). Consequently, our results suggest that the blunted weight gain associated with AMPKα1 ablation in POMC neurons is in part mediated by the ameliorated ER stress, specifically within the ARC.

Ceramide-induced lipotoxicity is a canonical mechanism inducing hypothalamic ER stress (Contreras et al, 2014; Gonzalez-Garcia et al, 2018; Heras et al, 2020; Lopez et al, 2025; Martinez-Sanchez et al, 2017b; Meneyrol et al, 2021). Therefore, we explored the mRNA expression of the ceramide synthesis pathway by measuring the expression of ceramide synthase 1 (*Cers1*), serine palmitoyltransferase long chain base subunit 1 (*Sptlc1*), sphingomyelin phosphodiesterase 2 (*Smpd2*) and sphingomyelin synthase 1 (*Sgms1*) in the ARC of POMC-Cre AMPKα1^{fl/fl} mice. We found a decrease in *Cers1* mRNA expression, which could account for a decreased ceramide synthesis (Hammerschmidt et al, 2023), in the ARC of POMC-Cre AMPKα1^{fl/fl} mice fed a HFD (Fig. 7G). To address this, we performed targeted lipidomics on mediobasal hypothalamus (MBH, containing the ARC) tissue from POMC-Cre AMPKα1^{fl/fl} mice and control male mice fed a HFD, focusing on ceramide species. The analysis identified widespread alterations in lipid classes including sphingomyelins and glycerophospholipids (Fig. 8A), with prominent reductions in ceramides (Fig. 8A,B; Dataset EV1). Short-chain (C16) ceramides were decreased,

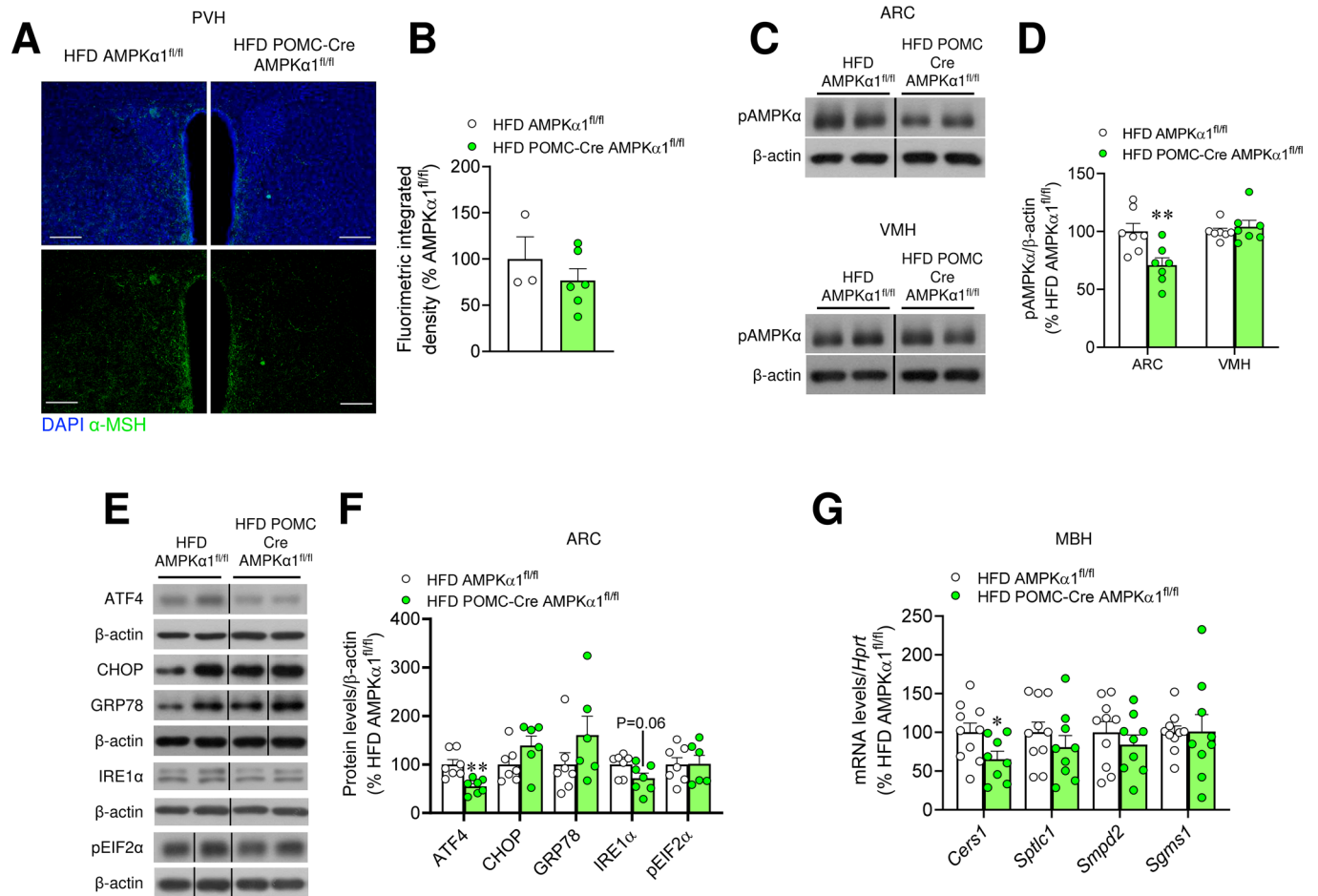


Figure 7. Effect of loss of AMPK $\alpha 1$ deletion in POMC neurons on the hypothalamus in HFD in male mice fed a HFD.

(A) Representative confocal images of the paraventricular nucleus of the hypothalamus (PVH), depicting α -MSH immunoreactivity (green), (B) Quantification of the mean intensity of α -MSH ($n = 3$ control and 6 KO) of control (littermates AMPK $\alpha 1^{fl/fl}$) and POMC-Cre AMPK $\alpha 1^{fl/fl}$ male mice fed a HFD. Scale bar 150 μ m. (C, D) Representative ARC and VMH pAMPK α western blot images (C) and pAMPK α protein levels in the ARC and VMH (D; $n = 7$ control and 7 KO; $P = 0.009217$) of HFD (littermates AMPK $\alpha 1^{fl/fl}$) and POMC-Cre AMPK $\alpha 1^{fl/fl}$ male mice fed a HFD. (E, F) Representative ARC ER stress pathway western blot images (E) and ER stress pathway protein levels in the ARC (F; $n = 7$ control and 6-7 KO; $P = 0.002429$ for ATF4; $P = 0.061881$ for IRE1 α) of control (littermates AMPK $\alpha 1^{fl/fl}$) and POMC-Cre AMPK $\alpha 1^{fl/fl}$ male mice fed a HFD. (G) mRNA expression of ceramide pathway enzymes in the MBH ($n = 10$ control and 9-10 KO; $P = 0.045272$ for *Cers1*) of control (littermates AMPK $\alpha 1^{fl/fl}$) and POMC-Cre AMPK $\alpha 1^{fl/fl}$ male mice fed a HFD. In the Western blot analyses, β -actin was used as control. A black line between the immunoblots depicts samples loaded in the same gel, but not next to each other. Data were expressed as mean $\pm$ SEM. Statistical significance was assessed by Student's *t*-test (B, D, F, G). * $P < 0.05$, ** $P < 0.01$ vs. AMPK $\alpha 1^{fl/fl}$. Source data are available online for this figure.

including Cer 18:2;O2/16:0 (Fig. 8C), Cer 18:1;O2/16:0 (Fig. 8D), and Cer 18:0;O2/16:0 (Fig. 8E). Medium-chain (C18) species such as Cer 18:0;O2/18:0 (Fig. 8F) and Cer 18:1;O2/18:1/Cer 18:2;O2/18:0 isomers (Fig. 8G,H) were also reduced; a biochemical change that perfectly mirrored the downregulation in *Cers1* (Fig. 7G), the enzyme responsible for C18 ceramide synthesis. Very long-chain ceramides showed marked declines, notably Cer 18:2;O2/22:0 (Fig. 8I), Cer 18:1;O2/22:0 (Fig. 8J), and Cer 18:1;O2/25:1 (Fig. 8K). These data indicate a global suppression of ceramide levels in the MBH upon AMPK $\alpha 1$ deletion in POMC neurons. These detailed lipidomics data unequivocally validate the functional consequences of the phosphoproteomic changes, demonstrating that POMC AMPK $\alpha 1$ deletion leads to reduced hypothalamic ceramide burden under obesogenic conditions. This would be consistent with the increased BAT thermogenesis in these animals (Contreras et al, 2014; Gonzalez-Garcia et al, 2018; Martinez-Sanchez et al, 2017b).

Overall, these results suggested that selective ablation of AMPK $\alpha 1$ in POMC neurons would lead to reduced ceramide-induced lipotoxicity in the ARC and, subsequently, to decreased hypothalamic ER stress, promoting increased BAT thermogenesis and weight loss under HFD.

Discussion

In recent years it has become clear that the different AMPK subunits display a complex functional and nuclei-dependent role controlling energy balance within the hypothalamus (Freire-Agulleiro et al, 2025; Martinez-Sanchez et al, 2017b; Milbank et al, 2023; Milbank et al, 2021; Okamoto et al, 2018; Seoane-Collazo et al, 2018). In the ARC, AMPK $\alpha 2$ deletion in AgRP neurons induced an age-dependent lean phenotype, while its

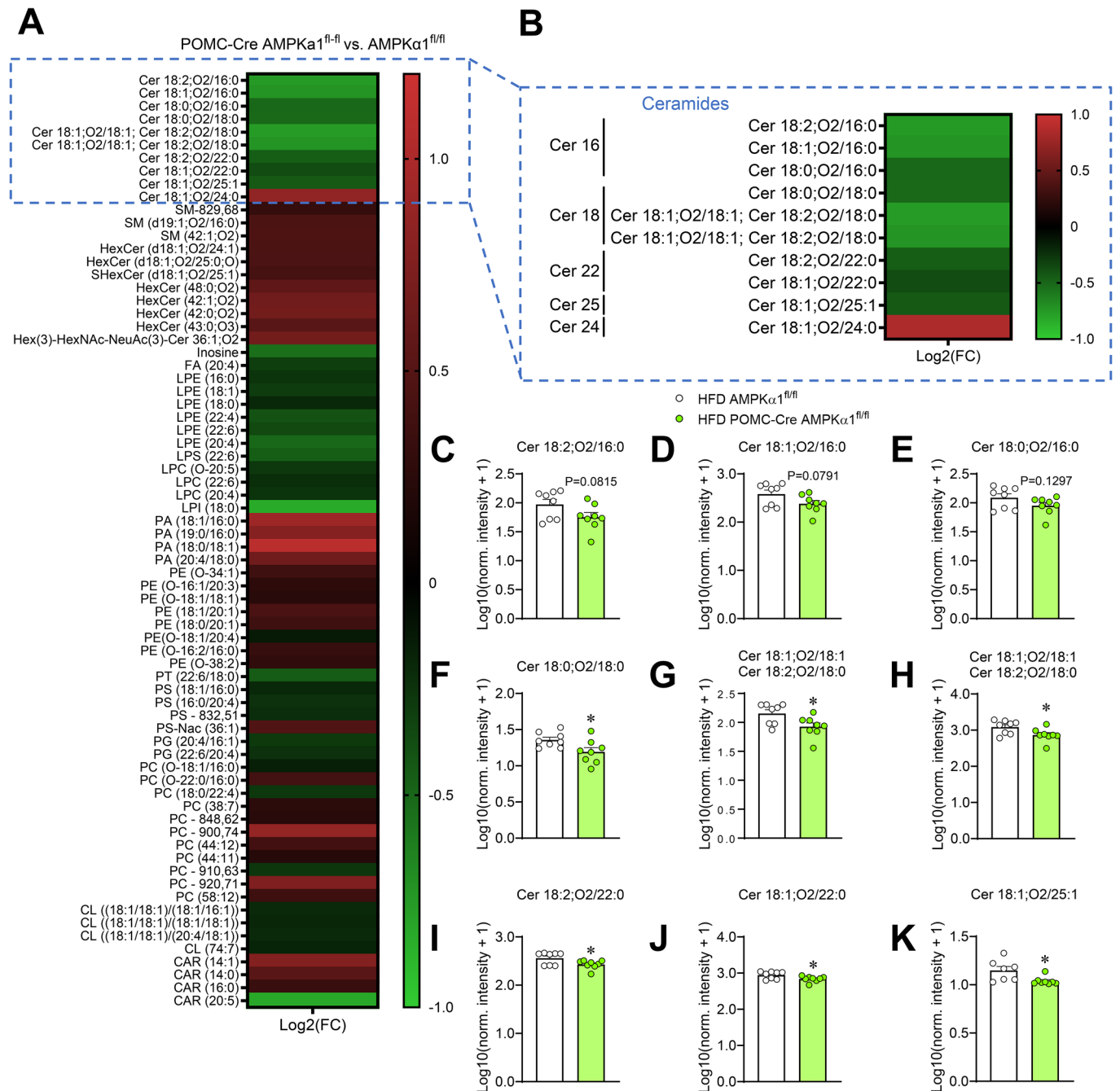


Figure 8. Effect of loss of AMPK $\alpha 1$ deletion in POMC neurons on the hypothalamic lipidomic profile in male mice fed a HFD.

(A) Heat map depicting lipid species detected by liquid chromatography coupled to mass spectrometry (LC/MS) and differentially enriched (significantly) in the mediobasal hypothalamus (MBH; containing the ARC) of control (littermates AMPK $\alpha 1^{fl/fl}$) and POMC-Cre AMPK $\alpha 1^{fl/fl}$ male mice fed a HFD. (B) Detailed zoom in showing species of ceramides with the highest Log2 (FC) values. (C–K) Individual ceramide species: (C) Cer 18:2;O2/16:0 ($P = 0.0815$), (D) Cer 18:1;O2/16:0 ($P = 0.0791$), (E) Cer 18:0;O2/16:0 ($P = 0.1289$), (F) Cer 18:0;O2/18:0 ($P = 0.0320$), (G, H) Cer 18:1;O2/18:1 and Cer 18:2;O2/18:0 isomers ($P = 0.0253$ and $P = 0.0349$), (I) Cer 18:2;O2/22:0 ($P = 0.0356$), (J) Cer 18:1;O2/22:0 ($P = 0.0308$) and (K) Cer 18:1;O2/25:1 ($P = 0.0195$). Data were expressed as mean $\pm$ SEM. Statistical significance was assessed by Student's t -test (C–K). * $P < 0.05$ vs. AMPK $\alpha 1^{fl/fl}$. Source data are available online for this figure.

deletion in POMC neurons promotes an increase in body weight both in chow and HFD due to reduced energy expenditure and exacerbated refeeding response after fasting (Claret et al, 2007). Interestingly, these AMPK α loss-of-function models previously described contrast with the one reported herein, where AMPK $\alpha 1$ ablation in the same POMC neuronal population, specifically in

males, drives an increased thermogenesis, coupled to a protection against diet-induced obesity. Several aspects might explain the differences found. (i) Firstly, the subcellular location of AMPK $\alpha 1$ and AMPK $\alpha 2$. AMPK $\alpha 2$, but not AMPK $\alpha 1$, has a nuclear localization signal (NLS) which is key for its nuclear translocation (Afinanisa et al, 2021; Suzuki et al, 2007). This suggests that

AMPK α 1-containing heterotrimers mainly regulate cytosolic substrates, while those AMPK α 2-based would control transcriptional machinery that regulates gene expression (Afinanisa et al, 2021; Suzuki et al, 2007). This might also explain why in our model we observe changes in ER stress, while the AMPK α 2 POMC KO mice reported by Claret and colleagues exhibited altered neuropeptide expression (Claret et al, 2007). (ii) Secondly, POMC neuronal heterogeneity should also be considered. This neuronal population is composed of different subclusters, which respond to diverse combinations of endocrine and metabolic signals (Biglari et al, 2021; Campbell et al, 2017; Chen et al, 2017; Lam et al, 2017; Leon et al, 2024; Quarta et al, 2021; Quarta et al, 2020; Saucisse et al, 2021). Given that AMPK α 1 and AMPK α 2 display a heterogeneous expression in POMC neurons (Steuernagel et al, 2022), they might integrate different cues and exert different mechanisms through specific neuronal projections. (iii) Thirdly, the *Pomc*-Cre lines used to generate the AMPK α 2 and AMPK α 1 knockout mice were different (Claret et al, 2007). This might have resulted in the genetic target of different POMC subpopulations (Leon et al, 2024; Quarta et al, 2021; Quarta et al, 2020; Saucisse et al, 2021), triggering the mentioned differences in signal integration and downstream projections, not only during postnatal life but also during embryogenesis. Given its role as gene expression regulator, AMPK α 2 deletion might induce differences in POMC neurons development, something that seems unlikely in our model, since no differences were detected in GABAergic and glutamatergic POMC neurons subpopulations in our analyses.

The constitutive POMC-Cre driver may introduce developmental compensation or circuit alterations, a known limitation of non-inducible systems. Inducible POMC-Cre models will enable adult-onset deletion, enabling clearer attribution to mature AMPK α 1 and AMPK α 2 signaling. Regardless of these considerations, the overall conclusion from the comparison between our data with the former literature indicates that the role of hypothalamic AMPK is not only nucleus-dependent but rather subunit-dependent (Seoane-Collazo et al, 2018). This finding reinforces the idea that within the hypothalamus AMPK α 1 and AMPK α 2 are indeed different protein kinases (Seoane-Collazo et al, 2018), are regulated in different ways, and engage divergent downstream signaling pathways. This is further supported by the fact that none of those changes seem to be mediated by a compensatory action of the non-ablated subunit (Claret et al, 2007; Seoane-Collazo et al, 2018).

Notably, our comprehensive phenotyping revealed a striking sexual dimorphism in POMC neuronal AMPK α 1 function: whereas males exhibit robust HFD resistance through elevated basal BAT thermogenesis and SNS outflow, females display no significant metabolic alterations even under obesogenic challenge. This sex-dependent phenotype likely reflects gonadal hormone modulation of hypothalamic nutrient sensing, with estrogen potentially affecting AMPK α 1-dependent signaling or SNS output in POMC circuits, which is consistent with the known sexual dimorphisms in ARC NPY/AgRP-POMC interactions (Gonzalez-Garcia et al, 2023; López and Tena-Sempere, 2017; Martínez de Morentin et al, 2014; Martínez de Morentin et al, 2015).

Our phosphoproteomics identified enrichment in exocytosis and vesicle transport in the POMC-Cre AMPK α 1^{flox/flox} mice when compared to the littermate controls in HFD. Together with this, the same animal model and experimental paradigm [HFD is a classical

inductor of ER stress in the hypothalamus (Contreras et al, 2014; Contreras et al, 2017; Dragano et al, 2024; Schneeberger et al, 2013)] displayed a downregulated ER stress in the ARC. In line with this, we have previously demonstrated that AMPK inhibition decreases ER stress by reducing ceramide-induced lipotoxicity in the VMH (Contreras et al, 2014; Gonzalez-Garcia et al, 2018; Lopez et al, 2025; Martinez-Sanchez et al, 2017b). Therefore, it was plausible to conclude that the same molecular mechanism might operate in neighboring POMC neurons within the ARC. Our expression and lipidomic data confirmed decreased ceramide synthesis in the ARC of POMC-Cre AMPK α 1^{flox/flox} mice, demonstrating that the regulation of complex lipid metabolism by AMPK α 1 represents a canonical mechanism shared by both SF1 (Martinez-Sanchez et al, 2017b) and POMC neurons. Interestingly, high ER stress levels in the hypothalamus and more specifically in POMC neurons have been associated with obese phenotypes (Dragano et al, 2024; He et al, 2021; Schneeberger et al, 2013; Williams et al, 2014; Xiao et al, 2017) and their amelioration drives an increase in BAT thermogenesis, which improves the energy imbalance. Notably, one of the downregulated ER stress markers we found, ATF4, was previously studied in hypothalamic POMC neurons and its specific ablation in this population reproduces a similar phenotype to our mice, where body weight is reduced because of increased energy expenditure (Xiao et al, 2017).

All this evidence might suggest that changes in ER stress, exocytosis and vesicle-mediated transport could induce an enhancement in the peptidergic output of POMC neurons facilitating the release of α -MSH in pre-autonomic sympathetic areas, other than the PVH, projecting to BAT. In this sense, it is known that POMC neurons modulate BAT thermogenesis through their control of the SNS (Bell et al, 2018; Jiang et al, 2020; Quarta et al, 2021). Several studies have demonstrated how these neurons project to pre-autonomic regions in the brainstem, where they modulate SNS tone to BAT (Bell et al, 2018; Joly-Amado et al, 2014; Quarta et al, 2021). The POMC-BAT regulation seems particularly relevant in the obesity context, as several groups have found that under diet-induced obesity paradigms, genetic loss-of-function models trigger an alteration in BAT thermogenesis, which leads to weight gain (Dodd et al, 2015; Qiu et al, 2024; Schneeberger et al, 2013; Tang et al, 2024). It is interesting to highlight how ER stress induced by HFD acts as a causal event leading to a downregulation in BAT thermogenesis and energy expenditure, which contributes to the increased body weight (Contreras et al, 2017; Schneeberger et al, 2013). Our POMC-Cre AMPK α 1^{flox/flox} mice fit in this pathophysiological mechanism, since we found a decreased ATF4 and IRE1 α expression in the ARC together with an increase in GRP78, all proteins components of the UPR, that indicate a decrease in two of the molecular branches of ER stress signaling and a higher capability to buffer the unfolded proteins in the ER, respectively (Martinez de Morentin and Lopez, 2010). Accordingly, the POMC-Cre AMPK α 1^{flox/flox} mice exhibit increased BAT thermogenesis compared to their littermate controls, but only in the HFD context. This confirms that POMC neurons are key elements in the energy imbalance and hypothalamic ER stress induction that characterizes obesity.

In summary, our study reveals that AMPK α 1 ablation in POMC neurons has a protective effect over diet-induced obesity, specifically in males. The reduced body weight observed in null male mice is associated with increased BAT thermogenesis and

decreased ceramide-induced hypothalamic ER stress. These findings highlight **i**) the complex nature of the different AMPK subunits in the hypothalamic regulation of energy balance and **ii**) emphasize the existence of sex-dependent differences in hypothalamic energy sensing. Overall, these findings reinforce the notion that AMPK α 1 in POMC neurons represents both a key component of the pathophysiological mechanism driving to obesity and a

promising target for therapeutic interventions, and suggests that AMPK α 1-targeted therapies for obesity (Milbank et al, 2023; Milbank et al, 2021; Mukherjee et al, 2023) may require sex-specific optimization to achieve efficacy across diverse patient populations.

Methods

Reagents and tools table

Reagent/resource	Reference or source	Identifier or catalog number
Experimental models		
POMC-Cre mice	The Jackson Laboratory	B6.FVB-Tg(Pomc-cre)1Lowl/J - Cat. 010714
AMPKα1 floxed mice	The Jackson Laboratory	Prkaa1 ^{tm1.1Sjnv/J} - Cat. 014141
Ai14-tdTomato mice	The Jackson Laboratory	B6.Cg-Gt(ROSA)26Sortm ^{14(CAG-tdTomato)Hze/J} - Cat. 007914
Antibodies		
α-tubulin	Sigma-Aldrich	T5168
β-actin	Sigma-Aldrich	A5316
ATF4	Sigma-Aldrich	WH0000468M1
CHOP	Santa Cruz	sc-793
GRP78	Cell Signaling	3183S
IRE1α	Cell Signaling	3294S
LC3-I/II	Cell Signaling	4180S
p62	Abcam	ab56416
pACCa (Ser79)	Cell Signalling	3661S
pAMPKα (Trh172)	Cell Signalling	2535S
pEIF2α (Ser52)	Santa Cruz	sc-101670
pPERK (Thr980)	Cell Signalling	3179S
UCP1	Abcam	ab10983
Polyclonal Rabbit anti-mouse immunoglobulinHPR	Dako	P0260
Polyclonal Rabbit anti-rabbit immunoglobulinHPR	Dako	P0448
PCR genotyping primers		
AMPKα1 floxed alleles	Forward 5'- CCC ACC ATC ACT CCA TCT CT - 3' Reverse 5' - AGC CTG CTT GGC ACA CTT AT - 3'	
AMPKα1 erased allele	Forward 5'- CCC ACC ATC ACT CCA TCT CT - 3' Reverse 5'- CCCACATAGGAAAGCGTGTT -3'	
qPCR primers and probes		
Cers1	Applied Biosystems TaqMan	Mm03024093_mH
Sptlc1	Applied Biosystems TaqMan	Mm00447343_m1
Smpd2	Applied Biosystems TaqMan	Mm01188195_g1
Smgs1	Applied Biosystems TaqMan	Mm00522643_m1
Atg7	Forward primer 5'-AGCGGCGGCTGGTAAGAA-3'; Reverse primer 5'-TCTTCTGGGTCAGTTCGTGC-3'	
Atg5	Forward primer 5'-GCTTTTGCCAAGAGTCAGCTA-3'; Reverse primer 5'-TCTGGAAAACTCATTTCAAGGGT-3'	
Atg3	Forward primer 5'-GCAGAGTCAGGGTGCTATTGA-3'; Reverse primer 5'-GCACAGAGCAGATGTAGGGA -3'	
Hprt	Forward Primer 5'-AGCCGACCGGTTCTGTCAT-3'; Reverse Primer 5'-GGTCATAACCTGGTTCATCATCAC-3'; Probe FAM-5'-CGACCCTCAGTCCCAGCGTCGTGAT-3'-TAMRA	

Reagent/resource	Reference or source	Identifier or catalog number
Probes for in situ hybridization and RNAScope		
<i>PrKaa1</i> -ex3 riboprobe	Forward T3-AMPKex3-sense (5'-CAGAGATGCAATTAACCCCTCACTAAAGGGAGAGTACCAGGTCATCAGTACACCATCT-3' Reverse T7-AMPKex3-as (5'-CCAAGCCTTCTAATACGACTCACTATAGGGAGACCTTCCATTTTACAGATATAATCA -3')	
<i>Pomc</i> riboprobe	Forward - 3-POMC-sense (5'-CAGAGATGCAATTAACCCCTCACTAAAGGGAGACCTCCTGCTTCAGACCTCCAT-3' Reverse T7-POMC-as (5'-CCAAGCCTTCTAATACGACTCACTATAGGGAGATGCTCCATGGAGTAGGAGCGC-3')	
<i>Pomc</i> probe (RNA Scope)	Advanced Cell Diagnostics	314081-C3
<i>Gad1</i> probe (RNA Scope)	Advanced Cell Diagnostics	400951-C1
<i>Gad2</i> probe (RNA Scope)	Advanced Cell Diagnostics	439371-C1
<i>Slc17a6</i> probe (RNA Scope)	Advanced Cell Diagnostics	456751-C2
Chemicals, diets and other reagents		
High-fat diet	Research Diets	D12451
Chow diet	Global Diet	2918
D-Glucose	Sigma-Aldrich	G8270
Insulin	Novo Nordisk	Actrapid 100 UI/ml,
10% Neutral Buffered Formalin	Sigma-Aldrich	HT501128
RNAscope Fluorescent Multiplex Detection Reagent kit	Advanced Cell Diagnostics	323110
Opal650	Akoya Biosciences	FP1496001KT
Opal520	Akoya Biosciences	FP1487001KT
Opal570	Akoya Biosciences	FP1488001KT
ProLong Gold Antifade Mountant	Thermo Fisher Scientific	P36930
TRIzol	Invitrogen	15596026
SuperScript First-Strand Synthesis System	Invitrogen	18091050
Protease inhibitor	Roche	Complete Mini EDTA-free and phosSTOP
Protein detection kit	Bio-Rad Laboratories	RC DC Protein Assay Kit
Software		
FLIR Tools	FLIR	FLIR Tools - Version 5.13.18031.2002
Leica Application Suite	Leica	v3.5.7
FIJI	NIH	
Shimadzu LC solution	Shimadzu	
Proteome Discoverer 2.5	Thermo Scientific	
RStudio		v1.2.1335
MassFrontiers	Thermo Scientific	version 8.0 SR1
Graphpad Prism	Graphpad	8.0.2
Other		
Infrared thermal camera	FLIR	B335
Glucometer	Roche	Accu-Check Guide
Calorimetric system	TSE Systems	LabMaster
Nuclear magnetic resonance	EchoMRI	EchoMRI-100H
Cryostat	Leica	NX50
Superfrost Plus Gold slides	Thermo Fisher Scientific	J1800AMNZ
HybEZ oven	Advanced Cell Diagnostics	HybEZ™ II Hybridization System
HybEZ Humidity Control Tray	Advanced Cell Diagnostics	HybEZ Humidity Control Tray
Confocal microscope	Leica	Leica DM6 CFS TCS SP8

Reagent/resource	Reference or source	Identifier or catalog number
Thermocycler	Applied Biosystems	QuantStudio 5 Real-Time PCR
High-performance liquid chromatography (HPLC)	Shimadzu Corporation	Shimadzu LC Prominence
Analytical columns	Waters Symmetry	300C18
Coulometric electrochemical detector	Thermo Scientific	ESA Coulochem III
Liquid chromatograph	Thermo Scientific	Thermo Scientific EASY-nLC 1000
Mass spectrometer	Thermo Scientific	Tribrid Orbitrap
Column	Thermo Scientific	PepMap100 C18 LC 75 µm ID, 2 cm
Liquid chromatography coupled to mass spectrometry	Thermo Scientific Waltham	Vanquish Horizon UHPLC Orbitrap ID-X Tribrid Mass Spectrometer

Animals

To generate ARC-specific POMC neuron AMPKα1 knock-out mice, POMC-Cre mice (B6.FVB-Tg(Pomc-cre)1Lowl/J, catalog number 010714; The Jackson Laboratory) (Dragano et al, 2024; Quinones et al, 2018) were crossed with AMPKα1 floxed mice (Prkaa1tm1.1Sjm/J, catalog number 014141; The Jackson Laboratory) (Martinez-Sanchez et al, 2017b; Seoane-Collazo et al, 2018). Mice were bred to generate both POMC-Cre AMPKα1^{fl/fl} (KO mice) and AMPKα1^{fl/fl} (control littermates). POMC-Cre AMPKα1^{fl/fl} were also bred with Ai14-tdTomato animals (B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J, catalog number 007914; The Jackson Laboratory) to obtain POMC-Cre Ai14-tdTomato AMPKα1^{fl/fl}, for histological characterization and validation of Cre-mediated recombination. Animal genotyping was performed according to the primers and PCR protocols provided by The Jackson Laboratory.

Male and female mice were housed (Centro de Biomedicina Experimental, CEBEGA University of Santiago de Compostela, USC) under a 12-h artificial light cycle (08:00 to 20:00), maintaining a controlled temperature (22–24 °C; with the exception of the cold exposure, in which animals were exposed to 4 °C for 6 h) and humidity (55%), and provided with free access (ad libitum) to either standard laboratory chow (2918 Global Diet) or high-fat diet (45 kcal% from fat; D12451, Research Diets) and tap water. All mice were group-housed, except for periods designated for food intake analyses, temperature measurements, infrared thermography measurements for BAT temperature, and indirect calorimetry. Food intake was measured for 7 consecutive days, and the average daily food intake for the whole period was plotted. The experiments were conducted in accordance with the International Law on Animal Experimentation and were approved by USC Ethical Committee (Project ID 15012/2020/010). No blinding was performed for the conduct of experiments. The study was designed, conducted, and reported in accordance with the ARRIVE guidelines to ensure transparent and reproducible reporting of animal research.

Temperature measurements

Core body temperature was measured daily for 7 days with a rectal probe connected to a digital thermometer (BAT-12; Microprobe-Thermometer) (Freire-Agulleiro et al, 2025; Lopez-Rey et al, 2026; Seoane-Collazo et al, 2018). Skin temperature surrounding the interscapular BAT was recorded with a B335 compact infrared

thermal imaging camera (FLIR) and analyzed with FLIR Tools software (FLIR Systems), as previously described (Dragano et al, 2024; Freire-Agulleiro et al, 2025; Gonzalez-Garcia et al, 2022; Milbank et al, 2023; Milbank et al, 2021).

Glucose and insulin tolerance test

Basal blood glucose levels were measured after an overnight fast (12 h during the dark phase) for the glucose tolerance test (GTT), and after 6 h (during the light phase) for the insulin tolerance test (ITT), with a glucometer (Accu-Chek, Roche). The GTT and ITT were performed after an intraperitoneal injection of 2 g/kg D-glucose (Sigma-Aldrich) or 0.7 (STD mice) and 1 (HFD mice) U/kg insulin (Novo Nordisk), respectively. Blood glucose measured at baseline (0 min) and post-injection time points 15, 30, 60, 90, and 120 min for GTT and 10/15, 20, 30, 60, and 90 min for ITT. Glucose levels and area under the curve (AUC) values were determined as previously described (Freire-Agulleiro et al, 2025; Gonzalez-Garcia et al, 2022; Seoane-Collazo et al, 2018). Fed glucose levels were assessed during the light phase and at the end of food intake measurements over 7 days.

Indirect calorimetry and nuclear magnetic resonance

Animals were analyzed for VO₂ EE, RER, and LA using calorimetric systems (LabMaster; TSE Systems) as previously described (Dragano et al, 2024; Freire-Agulleiro et al, 2025; Gonzalez-Garcia et al, 2022; Milbank et al, 2021). Continuous 48-h or 120-h monitoring of VO₂, EE, RER, and LA were recorded every 30 min across light/dark cycles. For body composition analysis, nuclear magnetic resonance (NMR; EchoMRI) was employed (Dragano et al, 2024; Gonzalez-Garcia et al, 2018; Milbank et al, 2021; Seoane-Collazo et al, 2019).

Sample processing

Mice were killed by cervical dislocation and decapitation. From each mouse, the mediobasal hypothalamus (MBH), the VMH and ARC [dissected from the hypothalamus, as shown (Gonzalez-Garcia et al, 2018; Milbank et al, 2021; Seoane-Collazo et al, 2018)], as well as the cerebellum, nucleus tractus solitarius (NTS), cortex, liver, BAT, sWAT and gonadal WAT (gWAT) were immediately homogenized on ice. Liver, BAT, sWAT, and gWAT were weighed. Samples and serum were stored at –80 °C until further processing.

PCR for recombined allele detection

Cre-mediated recombination was assessed by PCR in both *Pomc* expressing (ARC and NTS) and non-expressing tissues (cerebellum, cortex, WAT and liver), for both control littermates and POMC-Cre AMPK $\alpha 1^{fl/fl}$. A specific antisense primer 5'-CCCACATAG-GAAAGCGTGTT -3' was employed in combination with the forward primer used for genotyping to detect the recombined *Prkaa1* allele (531 bp) over the floxed one, as shown (Martinez-Sanchez et al, 2017b; Seoane-Collazo et al, 2018).

Double in situ hybridization

In situ hybridization (ISH) analyses were performed as previously shown (Manfredi-Lozano et al, 2016) using specific antisense riboprobes complementary to the coding sequence of the whole exon 3 of mice *Prkaa1* mRNA (*PrKaa1*-ex3 riboprobe; from 278- to 371-nt; NM_001013367.3) and *Pomc* mRNA (*Pomc* riboprobe; from 376- to 717-nt; NM_001278581). For the generation of the template, primer sequences were as follows: *PrKaa1*-ex3 riboprobe [forward T3-AMPKex3-sense (5'-CAGAGATGCAATTAACCCCTC ACTAAAGGGAGAGTACCAGGTCATCAGTACACCATCT-3'); reverse T7-AMPKex3-as (5'-CCAAGCCTTCTAATACGACTCAC-TATAGGGAGACCTTCCATTTTACAGATATAATCA -3')]; *Pomc* riboprobe [forward T3-POMC-sense (5'-CAGAGATGC AATTAACCCCTCACTAAAGGGAGACCTCCTGCTTCAGACCT CCAT-3'); reverse T7-POMC-as (5'-CCAAGCCTTCTAATAC GACTCACTATAGGGAGATGCTCCATGGAGTAGGAGCGC-3')]. For double ISH *PrKaa1*-ex3 riboprobe was radiolabelled with [^{33} P]-UTP (Perkin Elmer), while *Pomc* riboprobe was labeled with UTP-digoxigenin using DIG labeling mix (Roche). Following hybridization, sections were processed for the detection of *Pomc* DIG-labeled riboprobe using an anti-DIG antibody conjugated to alkaline phosphatase (Roche; dilution 1:200; incubation overnight at room temperature). Next, staining detection was developed by incubating with ImmPACT Vector Red alkaline phosphatase substrate following the manufacturer's instructions (Vector Laboratories). After, the slices were dried, dipped in Kodak Autoradiography Emulsion type NTB (Kodak) and exposed for 3 weeks at 4°C in dark for *PrKaa1*-ex3-radiolabelled riboprobe detection. After this period, the sections were developed and fixed. For evaluation of colocalization, DIG-labeled cells (POMC neurons) were located under the microscope and considered positive for *PrKaa1*-ex3 expression when the number of silver grains deposited on POMC neurons exceeded that of the background by threefold (Kauffman et al, 2007). Data were expressed as the percentage of POMC neurons expressing *PrKaa1*-ex3 in each brain slice.

Immunohistochemistry

Adult male POMC-Cre Ai14-tdTomato AMPK $\alpha 1^{fl/fl}$, POMC-Cre AMPK $\alpha 1^{fl/fl}$, and AMPK $\alpha 1^{fl/fl}$ mice were transcardially perfused with a saline solution followed by 4% neutral buffered paraformaldehyde (PFA). Brains were dissected and post-fixed for 24 h with 4% PFA, followed by immersion in a 30% sucrose solution for dehydration. When brains sank, they were sectioned at 30 μ m on a cryostat (Leica Biosystems). Sections were washed in 0.1 M TBS and blocked with 0.1 M TBS containing gelatin (0.25%) and Triton X-100 (0.5%) for 2 h at room temperature followed by incubation

with primary antibody (Sheep anti- α -MSH; 1:10000; #AB5087 RRID: AB_91683; Merck) overnight at 4°C. Sections were washed with 0.1 M PBS and incubated with secondary antibody (Alexa Fluor 555 donkey anti-sheep 1:1000; #A-21436; RRID: AB_2535857; Invitrogen) for 2 h at room temperature with 0.1 M TBS containing gelatin (0.25%) and Triton X-100 (0.5%) and further incubated with DAPI for nuclear staining. Images were obtained using a Leica AOBSP5X Confocal Microscope (Leica Microsystems), and analysis was performed using Fiji 2.0.0 (ImageJ; NIH). For α -MSH stainings maximum projections were further skeletonized and integrated density was calculated. For the p62/POMC staining, brain sections were washed in 0.1 M TBS and blocked with 0.1 M TBS containing donkey serum (5%) and Triton X-100 (0.25%) for 2 h at room temperature followed by incubation with primary antibody (Rabbit Anti-p62 antibody; 1:250; RRID: AB_2810880, #AB109012; Abcam) overnight at 4°C. Sections were washed with 0.1 M TBS and incubated with secondary antibody (Donkey anti-Rabbit Alexa Fluor 647; RRID: AB_2536183; #A31573; Invitrogen) for 2 h at room temperature with 0.1 M TBS containing donkey serum (5%) and Triton X-100 (0.25%). Next, sections were washed with 0.1 M TBS and further incubated with Rabbit Anti-POMC antibody (1:1000; RRID: AB_2753188; #H-029-030; Phoenix Pharmaceutical) overnight at 4°C with 0.1 M TBS containing donkey serum (5%) and Triton X-100 (0.25%). Finally, sections were washed with 0.1 M TBS and incubated with secondary antibody (Donkey anti-Rabbit Alexa Fluor 488; RRID: AB_2535792; #A21206; Invitrogen) for 2 h at room temperature with 0.1 M TBS containing donkey serum (5%) and Triton X-100 (0.25%) and further incubated with DAPI for nuclear staining. Images were obtained using a Leica AOBSP5X Confocal Microscope (Leica Microsystems), and analysis was performed using Fiji 2.0.0 (ImageJ; NIH). For p62/POMC staining, max projections were used to semi-automatically define POMC neurons and p62 intensity was calculated within the area of the selected POMC neurons.

sWAT samples were processed as shown (Contreras et al, 2017; Gonzalez-Garcia et al, 2022; Martinez-Sanchez et al, 2017a; Seoane-Collazo et al, 2019). For the adipocyte area, images were analyzed with the Adiposoft plugin in Fiji Software (NIH). sWAT sections were used for immunohistochemistry detection of uncoupling protein 1 (UCP1). UCP1-stained area quantified with Fiji image analysis software, as described elsewhere (Contreras et al, 2017; Gonzalez-Garcia et al, 2022; Martinez-Sanchez et al, 2017a; Seoane-Collazo et al, 2019). Three pictures per image section were analyzed for histological analysis.

RNAscope

Adult male POMC-Cre AMPK $\alpha 1^{fl/fl}$ and AMPK $\alpha 1^{fl/fl}$ mice were transcardially perfused with ice-cold PBS, pH 7.4, followed by 4% paraformaldehyde (Sigma-Aldrich). Brains were extracted and post-fixed in 4% PFA overnight at 4°C, then cryoprotected in 30% sucrose in PBS at 4°C. Coronal sections (30 μ m) were cut using a cryostat (CM1950; Leica). All reagents were purchased from Advanced Cell Diagnostics (ACD), except for the Opal fluorophores, which were obtained from Akoya Biosciences. The *Pomc* probe (314081-C3) contained ten oligonucleotide pairs targeting region 19-995 (NM_008895.3) of the *Pomc* transcript; the *Gad1* probe (400951-C1) contained 15 oligonucleotide pairs targeting

region 62-3113 (NM_008077.4) of the *Gad1* transcript; the *Gad2* probe (439371-C1) contained 20 oligonucleotide pairs targeting region 522-1506 (NM_008078.2) of the *Gad2* transcript; the *Slc17a6* probe (456751-C2) contained 19 oligonucleotide pairs targeting region 3-1240 (NM_080853.3) of the *Slc17a6* transcript. Two brain sections per mouse were selected, based on the Allen Brain Atlas. Sections were mounted on SuperFrost Plus Gold slides (J1800AMNZ; Thermo Fisher Scientific), dried at room temperature for 1 h, baked at 60 °C for 1 h, briefly rehydrated in ultrapure (Milli-Q® water), air-dried overnight, and baked again at 60 °C for 1 h the following day. For all baking steps, the slides were placed in a dry HybEZ Humidity Control Tray (without lid or humidifying paper), and incubated in the HybEZ oven at 60 °C. Subsequent steps—including tissue pretreatment, probe hybridization, and signal detection and amplification were carried out according to the manufacturer's protocol for fixed-frozen samples (RNAscope Fluorescent Multiplex Detection Reagent kit; 323110). During pretreatment, slides were incubated in target retrieval solution for 5 min and in protease III for 30 min. Probes against *Pomc*(-C3) and *Slc17a6*(-C2) were each diluted 1:50 into a 1:1 mix of *Gad1*(-C1) and *Gad2*(-C1) probes, which were combined to label GABAergic neurons. Probes were then detected using tyramide signal amplification with the following fluorophores, each diluted 1:750: Opal650 (FP1496001KT; for *Gad1/Gad2*), Opal570 (FP1488001KT; for *Slc17a6*), and Opal520 (FP1487001KT; for *Pomc*). Sections were counterstained with DAPI and mounted with ProLong Gold Antifade Mountant (P36930; Thermo Fisher Scientific), with glass coverslips (thickness 0.13–0.17 mm). Slides were stored in the dark until imaging. Images were acquired using Leica Application Suite (v3.5.7) and a Leica DM6 CFS TCS SP8 confocal microscope equipped with an X20/1 dry objective available at the Bordeaux Imaging Centre (BIC). Optical sectioning was set at 0.9 µm to perform Z-stacks. A total of 4 hemi-ARC were imaged per mouse (two sections). Images were imported into FIJI (NIH) for analysis. For each image maximum intensities were projected and POMC positive cells were segregated using visual threshold. Then the plugin “Analyze Particle” was used to define single-cell ROIs. Mean fluorescence intensities for every channel were measured in each cell and positivity for each marker was assessed by individual thresholds.

RT-qPCR

RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. About 500 ng of total RNA were used for each RT reaction, and cDNA synthesis was performed using the SuperScript First-Strand Synthesis System (Invitrogen) and random primers. Negative control reactions, containing all reagents except the sample were used to ensure specificity of the PCR amplification. For Real-time PCR, we used a fluorescent temperature cycler (Applied Biosystems) following the manufacturer's instructions and TaqMan (Applied Biosystems) commercially available validated probes for ceramide synthase 1 (*Cers1*; assay ID: Mm03024093_mH), serine palmitoyltransferase long chain base subunit1 (*Splcl1*; assay ID: Mm00447343_m1), sphingomyelin phosphodiesterase 2 (*Smpd2*; assay ID: Mm01188195_g1), sphingomyelin synthase 1 (*Sngs1*; assay ID: Mm00522643_m1). For autophagy related 7 (*Atg7*; Forward primer 5'-AGCGGCGGCTGGTAA-GAA-3'; Reverse primer 5'-TCTTCTGGGTCAGTTCGTGC-3'), autophagy related 5 (*Atg5*, forward primer 5'-GCTTTTGCCAA

GAGTCAGCTA-3'; reverse primer 5'- TCTGGAAAACATCATTT-CAGGGGT-3'), and autophagy related 3 (*Atg3*, forward primer 5'-GCAGAGTCAGGGTGCTATTGA-3'; reverse primer 5'- GCACAGAG-CAGATGTAGGGA -3'), we employed SYBR green. Cycling conditions included an initial denaturation at 95 °C for 3 min followed by 40 cycles at 95 °C for 5 s and 60 °C for 32 s, with a holding stage of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Primers values were expressed relative to hypoxanthine-guanine phosphoribosyl-transferase levels (*Hprt*; Forward Primer 5'-AGCCGACCGGTTCTGTCAT-3'; Reverse Primer 5'-GGTCATAACCTGGTTCATCATCAC-3'; Probe FAM-5'-CGACCCT-CAGTCCCAGCGTCGTGAT-3'-TAMRA). All reactions were performed in duplicate using the QuantStudio 5 Real-Time PCR (qPCR) as shown (Freire-Agulleiro et al, 2025; Gonzalez-Garcia et al, 2022; Milbank et al, 2021).

Western blot

Protein lysates from VMH, ARC and BAT were separated by SDS-PAGE, electrotransferred to polyvinylidene difluoride membranes (PVDF; Merck Millipore) with a semidry blotter and probed with the following antibodies against: α-tubulin (1:5000; T5168; RRID: AB_330337; Sigma), β-actin (1:5000; A5316; RRID: AB_476743; Sigma), ATF4 (1:1000; WH0000468M1; RRID: AB_1839944; Sigma), CHOP (1:1000; sc-793; RRID:AB_631364; Santa Cruz), GRP78 (1:1000; 3183S; RRID:AB_10695864; Cell Signaling), IRE1α (14C10; 1:1000; 3294S; RRID:AB_823545; Cell Signaling), LC3-I/II (1:1000; 4180S; RRID: AB_2137703; Cell Signaling), p62 (1:1000; ab56416; RRID: AB_945626; Abcam), pACCα (Ser79; 1:1000; 3661S; RRID: AB_330337; Cell Signalling), pAMPKα (Thr172; 1:1000; 2535S; RRID: AB_331250; Cell Signalling), pEIF2α (Ser52; 1:1000; sc-101670; AB_2096507; Santa Cruz), pPERK (Thr980; 1:500; 3179S; RRID:AB_2095853; Cell Signalling) and, UCP1 (1:10,000; ab10983; RRID: AB_2241462; Abcam) as previously described (Dragano et al, 2024; Freire-Agulleiro et al, 2025; Gonzalez-Garcia et al, 2022; Seoane-Collazo et al, 2018). Primary antibodies were incubated with the following secondary antibodies: polyclonal goat anti-rabbit immunoglobulin/HRP (1:5000; Dako; P0448; RRID: AB_2617138) and polyclonal rabbit anti-mouse immunoglobulin/HRP (1:5000; Dako; P0260; RRID: AB_2636929). Autoradiographic films (Fujifilm) were scanned, and band signals were quantified by densitometry using ImageJ-1.44 software (NIH) as reported (Dragano et al, 2024; Freire-Agulleiro et al, 2025; Gonzalez-Garcia et al, 2022; Seoane-Collazo et al, 2018). Values were expressed relative to α-tubulin, or β-actin. Representative images for all proteins are displayed, with all bands in each picture derived from the same gel, although spliced for clarity. A black line was inserted on the immunoblots when samples were loaded on the same gel, but not side by side.

HPLC-based neurotransmitter quantification

BAT, sWAT, and gWAT samples were dissected on a chilled plate, and immediately flash-frozen in dry ice before being stored at −80 °C until further analysis. The frozen tissue was subsequently homogenized, sonicated, and centrifuged at 14,000×g for 20 min at 4 °C. The resulting supernatant fraction was filtered and then injected into a high-performance liquid chromatography (HPLC) system (Shimadzu LC Prominence; Shimadzu Corporation) as shown (Dragano et al, 2024; Fernandez-Gonzalez et al, 2025; Gonzalez-Garcia et al, 2022; Seoane-Collazo et al, 2019).

Mass-spectrometry-based quantitative phosphoproteomics analysis

Protein extracts were prepared using a lysis buffer composed of 50 mM Tris-HCl (pH 7.6), 2% SDS, and 10 mM Tris-(2-Carboxyethyl) phosphine (TCEP), supplemented with protease and phosphatase inhibitor cocktails (Complete Mini EDTA-free and phosSTOP, Roche). The samples were heated to boiling for 5 min and then left at room temperature for 20 min to allow for protein solubilization and reduction. Following centrifugation at 15,000×g for 15 min, the supernatants were collected. Protein concentrations were measured using the RC DC Protein Assay Kit (Bio-Rad Laboratories). Proteins (120 µg) were digested using the FASP method. Protein extracts were diluted in UA Buffer (7 M urea in 0.1 M Tris-HCl, pH 8.5) and loaded onto 30 kDa centrifugal filter devices (NanoSep 10k Omega, Pall Life Sciences). Proteins were alkylated with 20 mM iodoacetamide in UA for 30 min in the dark, followed by three washes with UA and three washes with 50 mM ammonium bicarbonate. Digestion was carried out overnight at 37 °C using modified trypsin (Promega) in 50 mM ammonium bicarbonate at a protein-to-trypsin ratio of 40:1 (w/w). Trifluoroacetic acid (TFA) was added to a final concentration of 1%, and the peptides were desalted using Oasis-HLB columns (Waters) and dried using a Speed Vac system for further analysis. The tryptic peptides were dissolved in 150 mM Triethylammonium bicarbonate (TEAB) buffer. Peptide concentration was determined by measuring amide bonds with the Direct Detect system (Millipore). Equal amounts of each peptide sample were labeled using TMT 11plex reagents (Thermo Fisher) according to the manufacturer's protocol with minor modifications. Peptides were labeled with 0.8 mg of TMT reagents reconstituted in 70 µL of acetonitrile. After a 1-h incubation at room temperature, the reaction was stopped with 0.5% TFA and incubated for 10 min. Peptides were combined, concentrated using a Speed Vac, and excess labeling reagents were removed and desalted using Oasis-HLB columns. The samples were dried for further analysis. A fraction of the TMT-labeled sample underwent phosphopeptide enrichment using Ti4+IMAC HP (Immobilized Metal Affinity Chromatography) microparticles (Resyn Biosciences) following the manufacturer's protocol with slight modifications. Magnetic particles were equilibrated with loading/binding solvent (0.1 M glycolic acid in 80% acetonitrile, 5% TFA) for 5 min and incubated with the reconstituted TMT-labeled sample for 20 min. Bead-bound peptides were washed sequentially with 80% ACN-1% TFA and 10% ACN-0.2% TFA. Phosphopeptides were eluted using 1% ammonium hydroxide, acidified with TFA, desalted on C18 spin microcolumns (Nest Group), and dried in a vacuum centrifuge. A portion of the enriched phosphopeptides was fractionated by using the High pH Reversed-Phase Peptide Fractionation Kit (Pierce). LC-MS analysis of TMT-labeled peptides was performed using a Thermo Scientific EASY-nLC 1000 liquid chromatograph (Thermo Scientific) coupled to a Tribrid Orbitrap mass spectrometer (Orbitrap Fusion, Thermo Scientific). Peptides were suspended in Buffer (0.2% formic acid - 2% acetonitrile), loaded onto a pre-column (PepMap100 C18 LC 75 µm ID, 2 cm, Thermo Scientific), and separated online on a NanoViper PepMap100 C18 LC analytical column (75 µm ID, 50 cm, Thermo Scientific) using a continuous gradient of 5–32% B for 240 min and 32–90% B for 5 min (solvents A and B were 0.1% FA in water and 0.1% FA in acetonitrile, respectively) at a flow rate

of 200 nL/min. Each MS run included enhanced FT-resolution spectra (60,000 resolution) in the 400–1500 m/z range, followed by data-dependent MS/MS spectra of the most intense parent ions with charge states 2–4. The AGC target value in the Orbitrap was set to 200,000, with a maximum injection time of 50 ms. Fragmentation was performed at 34% normalized collision energy, targeting 50,000 ions, with 30,000 resolution, 120 ms injection time, and dynamic exclusion set to 40 s. For peptide identification, MS/MS spectra were searched using Proteome Discoverer 2.1 (Thermo Scientific; with the Sequest HT algorithm against the Uniprot database containing all mouse sequences (Uniprot SwissProt and TrEMBL; September 2018; 53,780 sequences), with decoy sequences generated using DecoyPyrat (Wright and Choudhary, 2016). Parameters included trypsin digestion with up to two missed cleavage sites, precursor and fragment mass tolerances of 2 Da and 0.03 Da, respectively, carbamidomethyl (+57.021) cysteine, and TMT modifications (+229.1629) at peptide N-terminal and Lys residues as fixed modifications. Methionine oxidation (+15.995) and serine/threonine/tyrosine phosphorylation (+79.096) were selected as dynamic modifications. The precursor charge range was set to 2–4. The false discovery rate (FDR) was calculated applying an additional filter for precursor mass tolerance of 12 ppm as described (Bonzon-Kulichenko et al, 2015). Identified peptides had an FDR of 1% or lower. Quantitative analysis of phosphopeptides was performed using the SanXoT program (Perez-Riverol et al, 2025; Rodriguez et al, 2024; Trevisan-Herraz et al, 2019). Quantitative information from TMT reporter intensities was integrated from the spectrum level to the peptide level, and then to the protein level, to quantify the relative abundance changes of each peptide within the corresponding protein. This was done using the WSPP (weighted spectrum, peptide, and protein) statistical model (Navarro et al, 2014) and the Generic Integration Algorithm (GIA) (Garcia-Marques et al, 2016). This model standardizes relative peptide abundances (log2-ratios) expressing the quantitative values in units of standard deviation (Z_{pq}) according to their estimated variances. Changes in mean Z_{pq} values between groups were analyzed using a two-sided t-test using the Limma approach (Ritchie et al, 2015). RStudio v1.2.1335 was used for the determination of differentially regulated phosphopeptides and generation of volcano plots using the R package EnhancedVolcano, (<https://github.com/kevinblighe/EnhancedVolcano>), with cutoff values for the adjusted *P* value <0.05 and fold change <−0.5, >0.5.

Lipidomics

Lipids were extracted from MBH by adding 100 µL of cold isopropanol as extraction solvent. Samples were vortexed previously to five cycles of incubation in liquid nitrogen for 30 s, sonicated for 30 s in a water bath and vortexed for 30 s. Samples were kept for 1 h to enable protein precipitation in the freezer. Subsequently, samples were centrifuged for 10 min at 4 °C and 12,000 rpm. The supernatants were transferred to LC vials. Quality control (QC) samples consisting of pooled samples from each condition were injected at the beginning and periodically through the workflow. Liquid chromatography coupled to mass spectrometry (LC/MS) was performed with a Thermo Scientific Vanquish Horizon UHPLC system interfaced with a Thermo Scientific Orbitrap ID-X Tribrid Mass Spectrometer (Waltham). Lipids were

separated by reverse-phase chromatography with an Acquity UPLC C18-RP (ACQUITY UPLC BEH C18 1.7 μ M; Waters). Mobile phase A was acetonitrile/water (60:40/v:v) (10 mM ammonium formate), and mobile phase B was isopropanol/acetonitrile (90:10/v:v) (10 mM ammonium formate). Solvent modifiers were used to enhance ionization and to improve the LC resolution in positive ionization mode. Separation was conducted under the following gradient: 0–2 min, 15–30% B; 2–2.5 min, 48% B; 2.5–11 min, 82% B; 11–11.5 min, 99% B; 11.5–12 min, 99% B; 12–12.1 min, 15% B; 12.1–15 min, 15% B. The injection volumes were 2 and 3 μ L in positive and negative mode, respectively. Data were collected with the following settings: spray voltage, 3.5 kV and –2.8 kV in positive and negative mode, respectively; sheath gas, 50; auxiliary gas, 10; sweep gas, 1; ion transfer tube temperature, 300 °C; vaporizer temperature, 300 °C; mass range, 150–1800 Da; RF lens, 60%; resolution, 120,000 (MS1); AGC target, 2e5; maximum injection time, 200 ms (MS1), isolation window, 1 Da. An LC/MSMS method using a DDA dynamic Inclusion List (IL) method was used for metabolite identification. The instrument settings were identical to those from the untargeted experiments except for resolution, 15,000; AGC target, 5e4; HCD fragmentation energy in Stepped mode combining 10–20–30–40%; and the injection time that was defined by HERMES (Gine et al, 2021) exported IL in csv format. Metabolite identification was performed by HERMES (Gine et al, 2021) using two strategies: cosine spectral matching using an in-house DB containing MS/MS spectra from MassBankEU, MoNA, HMDB, Riken and NIST23 databases; and using MassFrontiers version 8.0 SR1 (Thermo Scientific) matching against the mzCloud database. Spectra hits with high similarity scores (>0.8) were manually revised to assess correct metabolite identifications, hits were restricted to compounds with formulas present in the HERMES formula database. Lipidomics data processing and variable quantification were performed using Rhermes and XCMS R packages. Data quality were evaluated using PCA to check for outliers and run stability was measured through the coefficient of variation of QC samples' total intensity. Lipidomic variables were normalized using the total sum of quantified intensity per sample and scaled by $\log_{10}(x + 1)$.

Statistical analysis

Data were expressed as mean $\pm$ SEM. Normalized data were presented as percentages of the corresponding control group. Data points outside ± 2 standard deviations from the group mean were excluded as outliers from the primary analysis; this criterion was pre-specified before data collection. Statistical significance was determined using two-sided tests, including Student's *t*-test (for two-group comparisons), two-way ANOVA or mixed-effects analysis (when data were missing), followed by Sidak's or Bonferroni's post hoc tests (for >2 groups), or ANCOVA (for calorimetric analyses vs. body weight). Effect sizes were quantified using Cohen's *d*. *P* < 0.05 was considered statistically significant. Analyses were performed using Prism 8.0.2 (GraphPad Software).

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<https://www.proteomexchange.org/>) via the PRIDE partner repository (<https://www.ebi.ac.uk/pride/>) with the dataset identifier PXD068034 (<https://www.ebi.ac.uk/pride/archive/projects/PXD068034>). The lipidomic data from this publication have been deposited to the Zenodo database (<https://zenodo.org/>) and assigned the identifier 21025207 (<https://zenodo.org/records/21025207>).

The source data of this paper are collected in the following database record: [biostudies:S-SCDT-10_1038-S44319-026-00915-0](https://doi.org/10.1038/s44319-026-00915-0).

Expanded view data, supplementary information, appendices are available for this paper at <https://doi.org/10.1038/s44319-026-00915-0>.

Peer review information

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References

- Afinanisa Q, Cho MK, Seong HA (2021) AMPK localization: a key to differential energy regulation. *Int J Mol Sci* 22:10921
- Bell BB, Harlan SM, Morgan DA, Guo DF, Cui H, Rahmouni K (2018) Differential contribution of POMC and AgRP neurons to the regulation of regional autonomic nerve activity by leptin. *Mol Metab* 8:1–12
- Biglari N, Gaziano I, Schumacher J, Radermacher J, Paeger L, Klemm P, Chen W, Corneliusen S, Wunderlich CM, Sue M et al (2021) Functionally distinct POMC-expressing neuron subpopulations in hypothalamus revealed by intersectional targeting. *Nat Neurosci* 24:913–929
- Bonzon-Kulichenko E, Garcia-Marques F, Trevisan-Herraz M, Vazquez J (2015) Revisiting peptide identification by high-accuracy mass spectrometry: problems associated with the use of narrow mass precursor windows. *J Proteome Res* 14:700–710
- Campbell JN, Macosko EZ, Fenselau H, Pers TH, Lyubetskaya A, Tenen D, Goldman M, Verstegen AM, Resch JM, McCarroll SA et al (2017) A molecular census of arcuate hypothalamus and median eminence cell types. *Nat Neurosci* 20:484–496
- Chen R, Wu X, Jiang L, Zhang Y (2017) Single-cell RNA-seq reveals hypothalamic cell diversity. *Cell Rep* 18:3227–3241
- Claret M, Smith MA, Batterham RL, Selman C, Choudhury AI, Fryer LG, Clements M, Al Qassab H, Heffron H, Xu AW et al (2007) AMPK is essential for energy homeostasis regulation and glucose sensing by POMC and AgRP neurons. *J Clin Invest* 117:2325–2336
- Contreras C, González-García I, Martínez-Sánchez N, Seoane-Collazo P, Jacas J, Morgan DA, Serra D, Gallego R, González F, Casals N et al (2014) Central ceramide-induced hypothalamic lipotoxicity and ER stress regulate energy balance. *Cell Rep* 9:366–377
- Contreras C, Gonzalez-Garcia I, Seoane-Collazo P, Martinez-Sanchez N, Linares-Pose L, Rial-Pensado E, Ferno J, Tena-Sempere M, Casals N, Dieguez C et al (2017) Reduction of hypothalamic endoplasmic reticulum stress activates browning of white fat and ameliorates obesity. *Diabetes* 66:87–99
- Coupe B, Ishii Y, Dietrich MO, Komatsu M, Horvath TL, Bouret SG (2012) Loss of autophagy in pro-opiomelanocortin neurons perturbs axon growth and causes metabolic dysregulation. *Cell Metab* 15:247–255
- Dodd GT, Decherf S, Loh K, Simonds SE, Wiede F, Bolland E, Merry TL, Munzberg H, Zhang ZY, Kahn BB et al (2015) Leptin and insulin act on POMC neurons to promote the browning of white fat. *Cell* 160:88–104
- Dragano NRV, Milbank E, Haddad-Tovoli R, Garrido-Gil P, Novoa E, Fondevilla MF, Capelli V, Zanesco AM, Solon C, Morari J et al (2024) Hypothalamic free

- fatty acid receptor-1 regulates whole-body energy balance. *Mol Metab* 79:101840
- Fernandez-Gonzalez I, Freire-Agulleiro O, Ferreira V, Silveira-Loureiro M, Rial-Pensado E, Garrido-Gil P, Martinez G, Rada P, Labandeira-Garcia JL, Mittag J et al (2025) JNK1 in SF1 neurons regulates the central action of thyroid hormones on hepatic lipid metabolism. *Mol Metab* 98:102170
- Freire-Agulleiro O, Estevez-Salguero A, Ferreira V, Holleman CL, Garcia-Curra J, Gonzalez-Garcia I, Nogueiras R, Tena-Sempere M, Garcia-Caceres C, Dieguez C et al (2025) SF1-specific deletion of the energy sensor AMPK γ 2 induces obesity. *Mol Metab* 92:102091
- Garcia-Marques F, Trevisan-Herraz M, Martinez-Martinez S, Camafeita E, Jorge I, Lopez JA, Mendez-Barbero N, Mendez-Ferrer S, Del Pozo MA, Ibanez B et al (2016) A novel systems-biology algorithm for the analysis of coordinated protein responses using quantitative proteomics. *Mol Cell Proteomics* 15:1740–1760
- Gine R, Capellades J, Badia JM, Vughs D, Schwaiger-Haber M, Alexandrov T, Vinaixa M, Brunner AM, Patti GJ, Yanes O (2021) HERMES: a molecular-formula-oriented method to target the metabolome. *Nat Methods* 18:1370–1376
- Gonzalez-Garcia I, Contreras C, Estevez-Salguero A, Ruiz-Pino F, Colsh B, Pensado I, Linares-Pose L, Rial-Pensado E, Martinez de Morentin PB, Ferno J et al (2018) Estradiol regulates energy balance by ameliorating hypothalamic ceramide-induced ER stress. *Cell Rep* 25:413–423
- Gonzalez-Garcia I, Freire-Agulleiro O, Nakaya N, Ortega FJ, Garrido-Gil P, Linares-Pose L, Ferno J, Labandeira-Garcia JL, Dieguez C, Sultana A et al (2022) Olfactomedin 2 deficiency protects against diet-induced obesity. *Metabolism* 129:155122
- Gonzalez-Garcia I, Garcia-Clave E, Cebrian-Serrano A, Le Thuc O, Contreras RE, Xu Y, Gruber T, Schriever SC, Legutko B, Lintelmann J et al (2023) Estradiol regulates leptin sensitivity to control feeding via hypothalamic Cited1. *Cell Metab* 35:438–455.e437
- Guo DF, Lin Z, Wu Y, Searby C, Thedens DR, Richerson GB, Usachev YM, Grobe JL, Sheffield VC, Rahmouni K (2019) The BBSome in POMC and AgRP neurons is necessary for body weight regulation and sorting of metabolic receptors. *Diabetes* 68:1591–1603
- Hammerschmidt P, Steculorum SM, Bandet CL, Del Rio-Martin A, Steuernagel L, Kohlhaas V, Feldmann M, Varela L, Majcher A, Quatorze Correia M et al (2023) CerS6-dependent ceramide synthesis in hypothalamic neurons promotes ER/mitochondrial stress and impairs glucose homeostasis in obese mice. *Nat Commun* 14:7824
- He Z, Lieu L, Dong Y, Afrin S, Chau D, Kabahizi A, Wallace B, Cao J, Hwang ES, Yao T et al (2021) PERK in POMC neurons connects celastrol with metabolism. *JCI Insight* 6:e145306
- Heras V, Castellano JM, Fernandois D, Velasco I, Rodriguez-Vazquez E, Roa J, Vazquez MJ, Ruiz-Pino F, Rubio M, Pineda R et al (2020) Central ceramide signaling mediates obesity-induced precocious puberty. *Cell Metab* 32:951–966.e958
- Jiang J, Morgan DA, Cui H, Rahmouni K (2020) Activation of hypothalamic AgRP and POMC neurons evokes disparate sympathetic and cardiovascular responses. *Am J Physiol Heart Circ Physiol* 319:H1069–H1077
- Joly-Amado A, Cansell C, Denis RG, Delbes AS, Castel J, Martinez S, Luquet S (2014) The hypothalamic arcuate nucleus and the control of peripheral substrates. *Best Pract Res Clin Endocrinol Metab* 28:725–737
- Kauffman AS, Gottsch ML, Roa J, Byquist AC, Crown A, Clifton DK, Hoffman GE, Steiner RA, Tena-Sempere M (2007) Sexual differentiation of Kiss1 gene expression in the brain of the rat. *Endocrinology* 148:1774–1783
- Kaushik S, Arias E, Kwon H, Lopez NM, Athonvarangkul D, Sahu S, Schwartz GJ, Pessin JE, Singh R (2012) Loss of autophagy in hypothalamic POMC neurons impairs lipolysis. *EMBO Rep* 13:258–265
- Kaushik S, Rodriguez-Navarro JA, Arias E, Kiffin R, Sahu S, Schwartz GJ, Cuervo AM, Singh R (2011) Autophagy in hypothalamic AgRP neurons regulates food intake and energy balance. *Cell Metab* 14:173–183
- Lam BYH, Cimino I, Poley-Wolf J, Nicole Kohnke S, Rimmington D, Iyemere V, Heeley N, Cossetti C, Schulte R, Saraiva LR et al (2017) Heterogeneity of hypothalamic pro-opiomelanocortin-expressing neurons revealed by single-cell RNA sequencing. *Mol Metab* 6:383–392
- Leon S, Simon V, Lee TH, Steuernagel L, Clark S, Biglari N, Leste-Lasserre T, Dupuy N, Cannich A, Bellocchio L et al (2024) Single cell tracing of Pomc neurons reveals recruitment of ‘Ghost’ subtypes with atypical identity in a mouse model of obesity. *Nat Commun* 15:3443
- Li Y, Chen Y (2019) AMPK and autophagy. *Adv Exp Med Biol* 1206:85–108
- Lopez M (2022) Hypothalamic AMPK as a possible target for energy balance-related diseases. *Trends Pharmacol Sci* 43:546–556
- Lopez M, Dieguez C, Tena-Sempere M, Gonzalez-Garcia I (2025) Ceramides in the central control of metabolism. *Trends Endocrinol Metab* 36:11–14
- López M, Nogueiras R, Tena-Sempere M, Dieguez C (2016) Hypothalamic AMPK: a canonical regulator of whole-body energy balance. *Nat Rev Endocrinol* 12:421–432
- López M, Tena-Sempere M (2017) Estradiol effects on hypothalamic AMPK and BAT thermogenesis: a gateway for obesity treatment? *Pharmacol Ther* 178:109–122
- Lopez-Rey N, Cabaleiro A, Pata MP, Peyrou M, Estevez-Salguero A, Fernandez-Sanmartin P, Morgan DA, Ferreira V, Sanchez-Pintos P, Folgueira C et al (2026) Increased brown adipose tissue thermogenesis in phenylketonuria. *MedComm* 7:e70820
- Manfredi-Lozano M, Roa J, Ruiz-Pino F, Piet R, Garcia-Galiano D, Pineda R, Zamora A, Leon S, Sanchez-Garrido MA, Romero-Ruiz A et al (2016) Defining a novel leptin-melanocortin-kisspeptin pathway involved in the metabolic control of puberty. *Mol Metab* 5:844–857
- Martínez de Morentin PB, González-García I, Martins L, Lage R, Fernández-Mallo D, Martínez-Sánchez N, Ruiz-Pino F, Liu J, Morgan DA, Pinilla L et al (2014) Estradiol regulates brown adipose tissue thermogenesis via hypothalamic AMPK. *Cell Metab* 20:41–53
- Martinez de Morentin PB, Lage R, Gonzalez-Garcia I, Ruiz-Pino F, Martins L, Fernandez-Mallo D, Gallego R, Ferno J, Senaris R, Saha AK et al (2015) Pregnancy induces resistance to the anorectic effect of hypothalamic malonyl-CoA and the thermogenic effect of hypothalamic AMPK inhibition in female rats. *Endocrinology* 156:947–960
- Martinez de Morentin PB, Lopez M (2010) “Mens sana in corpore sano”: exercise and hypothalamic ER stress. *PLoS Biol* 8:e1000464
- Martinez-Sanchez N, Moreno-Navarrete JM, Contreras C, Rial-Pensado E, Ferno J, Nogueiras R, Dieguez C, Fernandez-Real JM, Lopez M (2017a) Thyroid hormones induce browning of white fat. *J Endocrinol* 232:351–362
- Martinez-Sanchez N, Seoane-Collazo P, Contreras C, Varela L, Villarroya J, Rial-Pensado E, Buque X, Aurrekoetxea I, Delgado TC, Vazquez-Martinez R et al (2017b) Hypothalamic AMPK-ER stress-JNK1 axis mediates the central actions of thyroid hormones on energy balance. *Cell Metab* 26:212–229.e212
- Meneyrol K, Estevez-Salguero A, Gonzalez-Garcia I, Guitton J, Taouis M, Benomar Y, Magnan C, Lopez M, Le Stunff H (2021) Ovarian insufficiency impairs glucose-stimulated insulin secretion through activation of hypothalamic de novo ceramide synthesis. *Metabolism* 123:154846
- Milbank E, Dragano N, Vidal-Gomez X, Rivas-Limeres V, Garrido-Gil P, Wertheimer M, Recoquillon S, Pata MP, Labandeira-Garcia JL, Dieguez C et al (2023) Small extracellular vesicle targeting of hypothalamic AMPK α 1 promotes weight loss in leptin receptor deficient mice. *Metabolism* 139:155350
- Milbank E, Dragano NRV, Gonzalez-Garcia I, Garcia MR, Rivas-Limeres V, Perdomo L, Hilaiet G, Ruiz-Pino F, Mallegol P, Morgan DA et al (2021) Small

- extracellular vesicle-mediated targeting of hypothalamic AMPK α 1 corrects obesity through BAT activation. *Nat Metab* 3:1415-1431
- Mukherjee S, Dieguez C, Ferno J, Lopez M (2023) Obesity wars: hypothalamic sEVs a new hope. *Trends Mol Med* 29:622-634
- Navarro P, Trevisan-Herraz M, Bonzon-Kulichenko E, Nunez E, Martinez-Acedo P, Perez-Hernandez D, Jorge I, Mesa R, Calvo E, Carrascal M et al (2014) General statistical framework for quantitative proteomics by stable isotope labeling. *J Proteome Res* 13:1234-1247
- Okamoto S, Sato T, Tateyama M, Kageyama H, Maejima Y, Nakata M, Hirako S, Matsuo T, Kyaw S, Shiuchi T et al (2018) Activation of AMPK-regulated CRH neurons in the PVH is sufficient and necessary to induce dietary preference for carbohydrate over fat. *Cell Rep* 22:706-721
- Perez-Riverol Y, Bandla C, Kundu DJ, Kamatchinathan S, Bai J, Hewapathirana S, John NS, Prakash A, Walzer M, Wang S et al (2025) The PRIDE database at 20 years: 2025 update. *Nucleic Acids Res* 53:D543-D553
- Qiu S, Wu Q, Wang H, Liu D, Chen C, Zhu Z, Zheng H, Yang G, Li L, Yang M (2024) AZGP1 in POMC neurons modulates energy homeostasis and metabolism through leptin-mediated STAT3 phosphorylation. *Nat Commun* 15:3377
- Quarta C, Claret M, Zeltser LM, Williams KW, Yeo GSH, Tschop MH, Diano S, Bruning JC, Cota D (2021) POMC neuronal heterogeneity in energy balance and beyond: an integrated view. *Nat Metab* 3:299-308
- Quarta C, Fioramonti X, Cota D (2020) POMC neurons dysfunction in diet-induced metabolic disease: hallmark or mechanism of disease? *Neuroscience* 447:3-14
- Quinones M, Al-Massadi O, Folgueira C, Bremser S, Gallego R, Torres-Leal L, Haddad-Tovoli R, Garcia-Caceres C, Hernandez-Bautista R, Lam BYH et al (2018) p53 in AgRP neurons is required for protection against diet-induced obesity via JNK1. *Nat Commun* 9:3432
- Ramadori G, Fujikawa T, Fukuda M, Anderson J, Morgan DA, Mostoslavsky R, Stuart RC, Perello M, Vianna CR, Nilni EA et al (2010) SIRT1 deacetylase in POMC neurons is required for homeostatic defenses against diet-induced obesity. *Cell Metab* 12:78-87
- Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK (2015) limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 43:e47
- Rodriguez JM, Jorge I, Martinez-Val A, Barrero-Rodriguez R, Magni R, Nunez E, Laguillo A, Devesa CA, Lopez JA, Camafeita E et al (2024) iSanXoT: a standalone application for the integrative analysis of mass spectrometry-based quantitative proteomics data. *Comput Struct Biotechnol J* 23:452-459
- Saucisse N, Mazier W, Simon V, Binder E, Catania C, Bellocchio L, Romanov RA, Leon S, Matias I, Zizzari P et al (2021) Functional heterogeneity of POMC neurons relies on mTORC1 signaling. *Cell Rep* 37:109800
- Schneeberger M, Altirriba J, Garcia A, Esteban Y, Castano C, Garcia-Lavandeira M, Alvarez CV, Gomis R, Claret M (2012) Deletion of miRNA processing enzyme Dicer in POMC-expressing cells leads to pituitary dysfunction, neurodegeneration and development of obesity. *Mol Metab* 2:74-85
- Schneeberger M, Claret M (2012) Recent insights into the role of hypothalamic AMPK signaling cascade upon metabolic control. *Front Neurosci* 6:185
- Schneeberger M, Dietrich MO, Sebastian D, Imbernon M, Castano C, Garcia A, Esteban Y, Gonzalez-Franquesa A, Rodriguez IC, Bortolozzi A et al (2013) Mitofusin 2 in POMC neurons connects ER stress with leptin resistance and energy imbalance. *Cell* 155:172-187
- Schneeberger M, Gomis R, Claret M (2014) Hypothalamic and brainstem neuronal circuits controlling homeostatic energy balance. *J Endocrinol* 220:T25-T46
- Seoane-Collazo P, Linares-Pose L, Rial-Pensado E, Romero-Pico A, Moreno-Navarrete JM, Martinez-Sanchez N, Garrido-Gil P, Iglesias-Rey R, Morgan DA, Tomasini N et al (2019) Central nicotine induces browning through hypothalamic kappa opioid receptor. *Nat Commun* 10:4037
- Seoane-Collazo P, Roa J, Rial-Pensado E, Linares-Pose L, Beiroa D, Ruiz-Pino F, Lopez-Gonzalez T, Morgan DA, Pardavila JA, Sanchez-Tapia MJ et al (2018) SF1-specific AMPK α 1 deletion protects against diet-induced obesity. *Diabetes* 67:2213-2226
- Souza GF, Solon C, Nascimento LF, De-Lima-Junior JC, Nogueira G, Moura R, Rocha GZ, Fioravante M, Bobbo V, Morari J et al (2016) Defective regulation of POMC precedes hypothalamic inflammation in diet-induced obesity. *Sci Rep* 6:29290
- Steuernagel L, Lam BYH, Klemm P, Dowsett GKC, Bauder CA, Tadross JA, Hitschfeld TS, Del Rio Martin A, Chen W, de Solis AJ et al (2022) HypoMap-a unified single-cell gene expression atlas of the murine hypothalamus. *Nat Metab* 4:1402-1419
- Suzuki A, Okamoto S, Lee S, Saito K, Shiuchi T, Minokoshi Y (2007) Leptin stimulates fatty acid oxidation and peroxisome proliferator-activated receptor alpha gene expression in mouse C2C12 myoblasts by changing the subcellular localization of the α 2 form of AMP-activated protein kinase. *Mol Cell Biol* 27:4317-4327
- Sweeney P, Gimenez LE, Hernandez CC, Cone RD (2023) Targeting the central melanocortin system for the treatment of metabolic disorders. *Nat Rev Endocrinol* 19:507-519
- Tang M, Zhang Y, Zhang R, Zhang Y, Zheng J, Wang D, Wang X, Yan J, Hu C (2024) GPSM1 in POMC neurons impairs brown adipose tissue thermogenesis and provokes diet-induced obesity. *Mol Metab* 79:101839
- Trevisan-Herraz M, Bagwan N, Garcia-Marques F, Rodriguez JM, Jorge I, Ezkurdia I, Bonzon-Kulichenko E, Vazquez J (2019) SanXoT: a modular and versatile package for the quantitative analysis of high-throughput proteomics experiments. *Bioinformatics* 35:1594-1596
- Williams KW, Liu T, Kong X, Fukuda M, Deng Y, Berglund ED, Deng Z, Gao Y, Liu T, Sohn JW et al (2014) Xbp1s in Pomc neurons connects ER stress with energy balance and glucose homeostasis. *Cell Metab* 20:471-482
- Wright JC, Choudhary JS (2016) DecoyPy: fast non-redundant hybrid decoy sequence generation for large scale proteomics. *J Proteomics Bioinform* 9:176-180
- Xiao Y, Deng Y, Yuan F, Xia T, Liu H, Li Z, Liu Z, Ying H, Liu Y, Zhai Q et al (2017) ATF4/ATG5 signaling in hypothalamic proopiomelanocortin neurons regulates fat mass via affecting energy expenditure. *Diabetes* 66:1146-1158
- Yeo GSH, Chao DHM, Siebert AM, Koerperich ZM, Ericson MD, Simonds SE, Larson CM, Luquet S, Clarke I, Sharma S et al (2021) The melanocortin pathway and energy homeostasis: from discovery to obesity therapy. *Mol Metab* 48:101206

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Disclosure and competing interests statement

ML serves as Scientific Director of Gazella Biotech (<https://gazellabiotech.com/>) and Lyrea Biotech. RN serves in the Advisory Board of Albor Biotech (<https://alborbiotech.com/>). The rest of the authors have no other conflicts of interest.

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Expanded View Figures

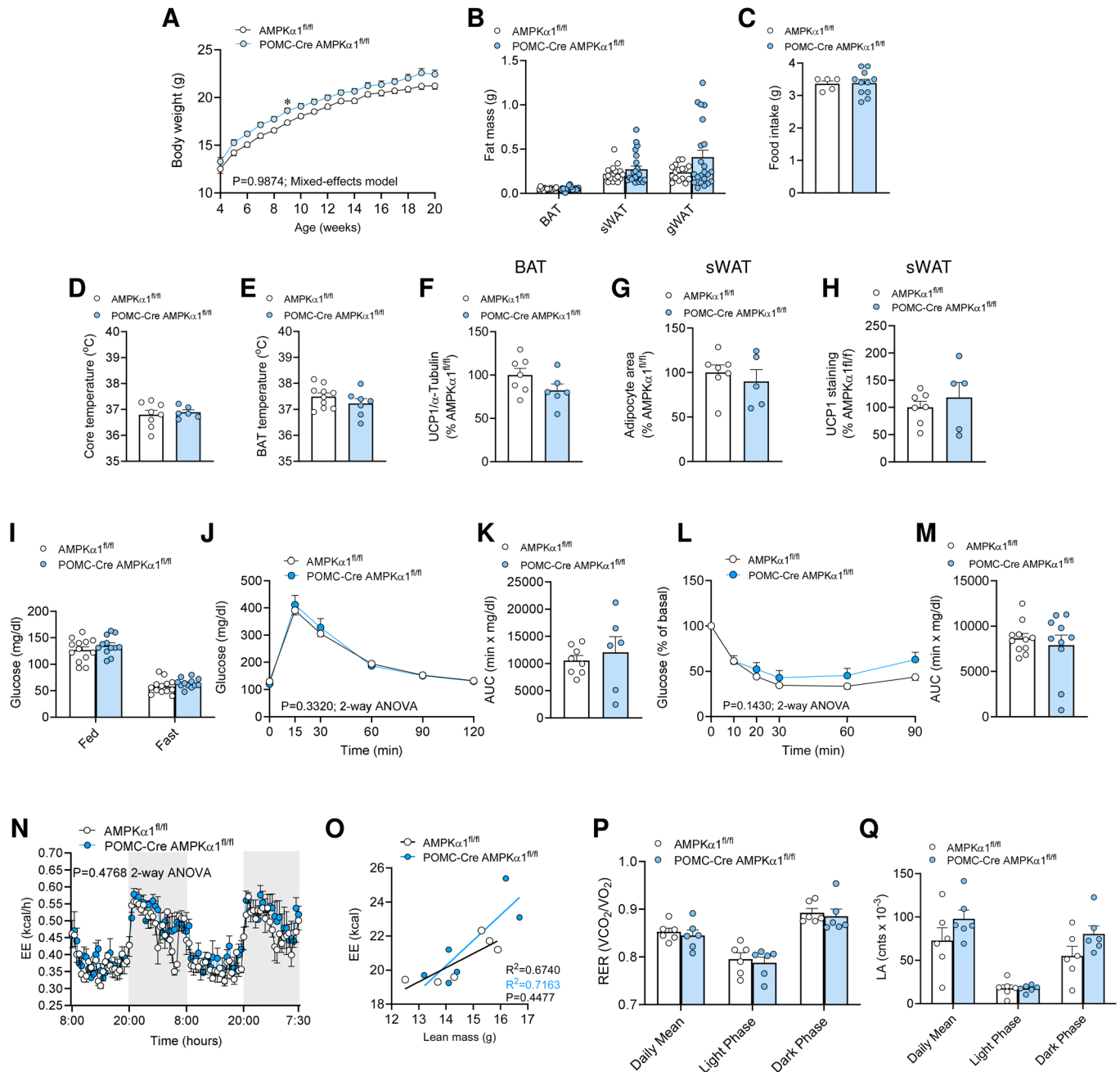


Figure EV1. Effects of loss of AMPK α 1 in POMC neurons on energy balance, energy expenditure and glucose homeostasis in female mice fed a chow diet.

(A–E) Body weight (A; $n=20$ control and 31 KO), fat depots mass (B; $n=17$ control and 22 KO), and daily food intake (C; $n=5$ control and 11 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} female mice. (D–H) Core body temperature (D; $n=8$ control and 6 KO) BAT temperature (E; $n=10$ control and 7 KO), BAT UCP1 protein levels (F; $n=7$ control and 6 KO), quantification of adipocyte area (G; $n=7$ control and 5 KO) and quantification of UCP1-stained area (H; $n=7$ control and 5 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} female mice. (I–M) Fed and fasted blood glucose levels (I; $n=13$ control and 12 KO), glucose tolerance test and area under the curve (AUC) (J, K; $n=8$ control and 6 KO), insulin tolerance test, and AUC (L, M; $n=11$ control and 9 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} female mice. (N–Q) Energy expenditure (EE; N; $n=6$ control and 6 KO), ANCOVA analysis of 48 h total energy expenditure using body weight as a covariate (O; $n=6$ control and 6 KO), respiratory exchange ratio (RER; P; $n=6$ control and 6 KO), locomotor activity (LA; Q; $n=6$ control and 6 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} female mice. Data were expressed as mean $\pm$ SEM. Statistical significance was assessed by mixed-effect model (A), two-way ANOVA (J, L, N) and Student's *t*-test (B–I, K, M, P, Q) and ANCOVA (O). * $P < 0.05$ vs. female AMPK α 1^{fl/fl}.

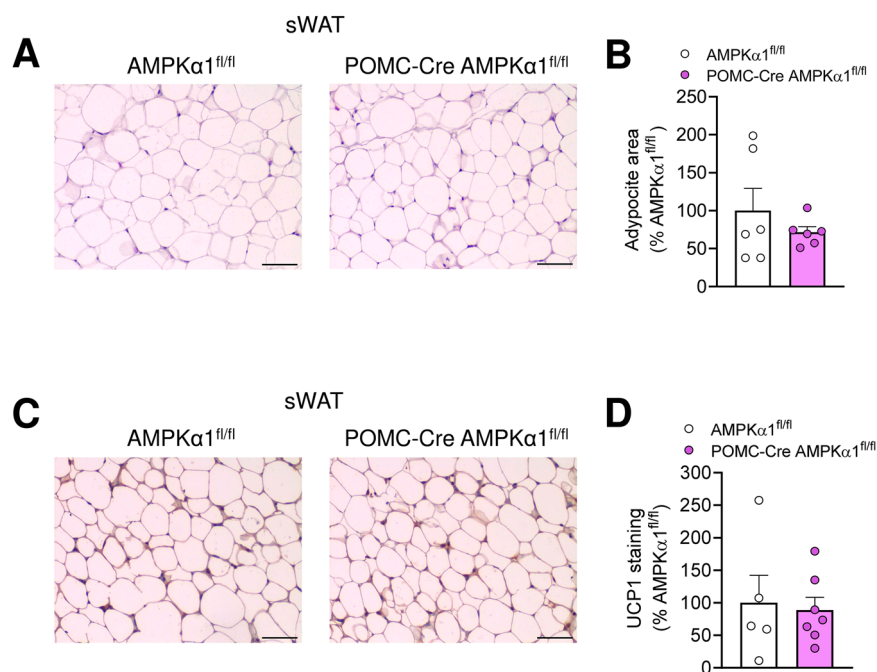


Figure EV2. Effects of loss of AMPK α 1 in POMC neurons on WAT in male mice fed a chow diet.

(A, B) Quantification of the adipocyte area (A), and representative subcutaneous white adipose tissue (sWAT) hematoxylin-eosin staining (B; $n = 6$ control and 6 KO) of AMPK α 1^{fl/fl} mice, and POMC-Cre AMPK α 1^{fl/fl} mice. Scale bar 50 μ m. The mean value of each animal was obtained from 3 photos of each tissue. (C, D) Quantification of UCP1-stained area (C) and representative sWAT immunohistochemistry with anti-UCP1 antibody showing UCP1 staining (D; $n = 5$ control and 7 KO) of AMPK α 1^{fl/fl} mice, and POMC-Cre AMPK α 1^{fl/fl} mice. Scale bar 50 μ m. Data were expressed as mean $\pm$ SEM. Statistical significance was assessed by Student's *t*-test (B, D).

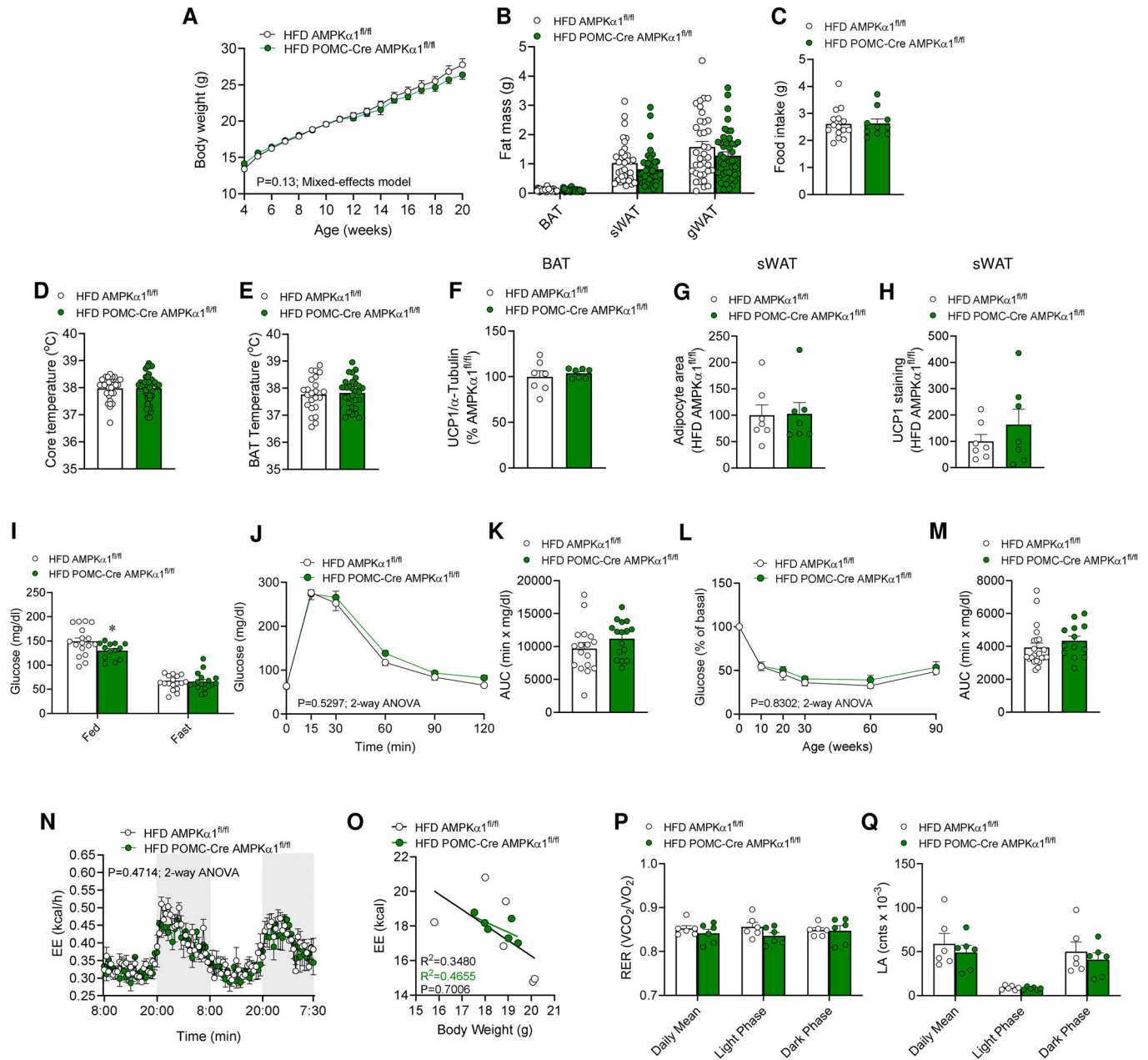


Figure EV3. Effects of loss of AMPK α 1 in POMC neurons on energy balance, energy expenditure and glucose homeostasis in female mice fed a HFD.

(A–E) Body weight (A; $n = 31$ control and 42 KO), fat depot mass (B; $n = 36$ control and 43 KO), and daily food intake (C; $n = 15$ control and 10 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} female mice in HFD. (D–H) Core body temperature (D; $n = 28$ control and 38 KO), BAT temperature (E; $n = 23$ control and 29 KO), BAT UCP1 protein levels (F; $n = 7$ control and 7 KO), quantification of adipocyte area (G; $n = 7$ control and 7 KO) and quantification of UCP1-stained area (H; $n = 7$ control and 7 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} female mice in HFD. (I–M) Fed and fasted blood glucose levels (I; $n = 17$ control and 13–17 KO; $P = 0.047664$ for fed), glucose tolerance test and area under the curve (AUC) (J, K; $n = 17$ control and 16 KO), insulin tolerance test, and area under the curve (L, M; $n = 22$ control and 13 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} female mice in HFD. (N–Q) Energy expenditure (EE; N; $n = 6$ control and 6 KO), ANCOVA analysis of 48 h total energy expenditure using body weight as a covariate (O; $n = 6$ control and 6 KO), respiratory exchange ratio (RER; P; $n = 6$ control and 6 KO), locomotor activity (LA; Q; $n = 6$ control and 6 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} female mice in HFD. Data are expressed as mean $\pm$ SEM. Statistical significance was assessed by Mixed-effect model (A), two-way ANOVA (J, L, N), Student's t -test (B–I, K, M, P, Q) and ANCOVA (O). * $P < 0.05$ vs. HFD female AMPK α 1^{fl/fl}.

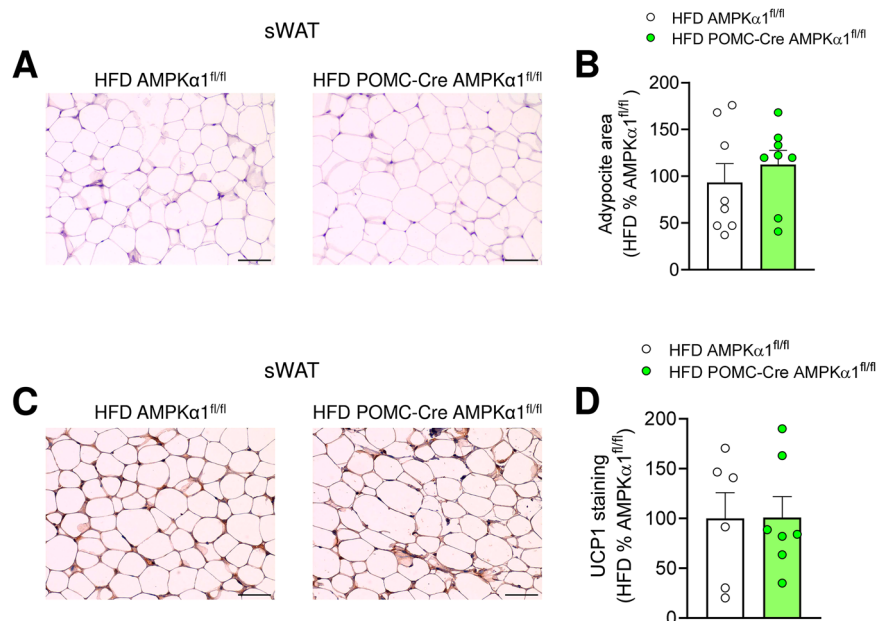


Figure EV4. Effects of loss of AMPK α 1 in POMC neurons on WAT in male mice fed a HFD.

(A, B) Quantification of the adipocyte area (A), and representative subcutaneous white adipose tissue (sWAT) hematoxylin-eosin staining (B; $n = 8$ control and 8 KO) of AMPK α 1^{fl/fl} mice, and POMC-Cre AMPK α 1^{fl/fl} mice fed a HFD. Scale bar 50 μ m. The mean value of each animal was obtained from three photos of each tissue. (C, D) Quantification of UCP1-stained area (C) and representative sWAT immunohistochemistry with anti-UCP1 antibody showing UCP1 staining (D; $n = 7$ control and 6 KO) of AMPK α 1^{fl/fl} mice, and POMC-Cre AMPK α 1^{fl/fl} mice fed a HFD. Scale bar 50 μ m. Data are expressed as mean $\pm$ SEM. Statistical significance was assessed by Student's t -test (B, D).

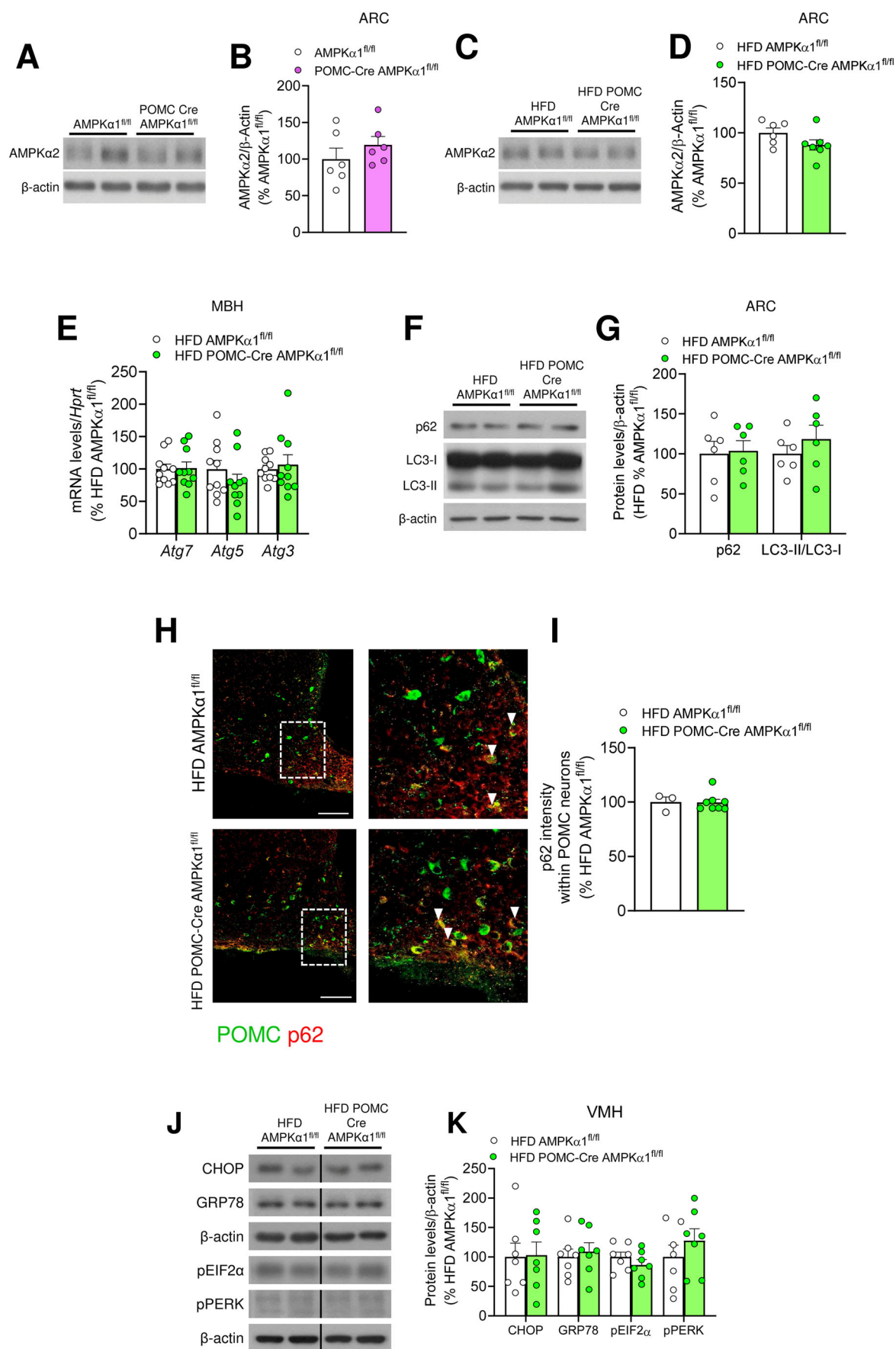


Figure EV5. Effects of loss of AMPK α 1 in POMC neurons on hypothalamic AMPK α 2, autophagy and ER stress.

(A, B) Representative ARC AMPK α 2 western blot images (A) and AMPK α 2 protein levels in the ARC (B; $n = 6$ control and 6 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} male mice. (C, D) Representative ARC AMPK α 2 western blot images (C) and AMPK α 2 protein levels in the ARC (D; $n = 6$ control and 7 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} male mice in HFD. (E) mRNA expression of autophagy-related proteins in the MBH ($n = 10$ control and 10 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} male mice fed a HFD. (F, G) Representative ARC autophagy markers western blot images (F) and autophagy markers protein levels in the ARC (G; $n = 6$ control and 6 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} male mice fed a HFD. (H, I) Representative confocal images from the ARC of HFD AMPK α 1^{fl/fl} mice and HFD POMC-Cre AMPK α 1^{fl/fl} mice showing p62 (red) and POMC (green) immunofluorescent staining (white arrowheads indicate cells coexpressing POMC and p62; scale bar 100 μ m; zoomed images come from the squared region in the first image, depicting details of POMC neurons colocalization with p62) and quantification of the p62 intensity within POMC neurons (I; $n = 3$ control and 7 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} male mice in HFD. (J, K) Representative VMH ER stress pathway western blot images (J) and ER stress pathway protein levels in the VMH (K; $n = 7$ control and 6-7 KO) of control (littermates AMPK α 1^{fl/fl}) and POMC-Cre AMPK α 1^{fl/fl} male mice fed a HFD. In the western blot analyses, β -actin was used as control. A black line between the immunoblots depicts samples loaded in the same gel, but not next to each other. Data were expressed as mean $\pm$ SEM. Statistical significance was assessed by Student's *t*-test (B, D, E, G, I, K).