SF1-specific AMPKα1 deletion protects against diet-induced obesity

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Running title: Ablation of AMPK in SF1 neurons and obesity

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Word count: 4505
Figures: 7 (7 color)
Supplemental Figures: 3
Supplemental Tables: 1
ABSTRACT

AMP-activated protein kinase (AMPK) is a cellular gauge that is activated under conditions of low energy, increasing energy production and reducing energy waste. Current evidence links hypothalamic AMPK with the central regulation of energy balance. However, it is unclear whether targeting hypothalamic AMPK has beneficial effects in obesity. Here, we show that genetic inhibition of AMPK in the ventromedial nucleus of the hypothalamus (VMH) protects against high fat diet (HFD)-induced obesity by increasing brown adipose tissue (BAT) thermogenesis and subsequently energy expenditure. Notably, this effect depends upon the AMPKα1 isoform in steroidogenic factor 1 (SF1) neurons of the VMH, since mice bearing selective ablation of AMPKα1 in SF1 neurons display resistance to diet-induced obesity (DIO), increased BAT thermogenesis, browning of white adipose tissue (WAT) and improved glucose and lipid homeostasis. Overall, our findings point to hypothalamic AMPK in specific neuronal populations as a potential druggable target for the treatment of obesity and associated metabolic disorders.
AMP-activated protein kinase (AMPK) is a serine/threonine kinase which is highly conserved throughout evolution. AMPK is a heterotrimer complex comprising a catalytic $\alpha$ subunit ($\alpha_1$, $\alpha_2$), with a conventional serine/threonine protein kinase domain, and two regulatory subunits, $\beta$ ($\beta_1$, $\beta_2$) and $\gamma$ ($\gamma_1$, $\gamma_2$, $\gamma_3$) (1-3). AMPK is activated by phosphorylation on Thr172 of the $\alpha$ subunit which can be allosterically induced by AMP and catalyzed by several upstream kinases such as liver kinase B1 (LKB1) and calmodulin-dependent kinase kinases, especially CaMKK$\beta$ (1-3). Consequently, by detecting changes in the ratio of adenine nucleotides AMPK is activated by stresses that deplete cellular energy status. By turning off ATP-consuming processes whilst turning on catabolic processes, AMPK mediates a global counter-regulatory response (1-3) that maintain cellular energy homeostasis.

Hypothalamic AMPK plays a major role in the regulation of food intake and energy expenditure (EE), as well as glucose and lipid homeostasis at the whole-body level (4-10). These differential effects of AMPK seem to have an anatomical basis. While the regulatory effect of AMPK on food intake emanate from the arcuate nucleus of the hypothalamus (ARC) (4,6,11,12), its effect on EE stems from the ventromedial nucleus of the hypothalamus (VMH), where AMPK affects the sympathetic nervous system (SNS) to regulate brown adipose tissue (BAT) thermogenesis (7,10,11,13-16). The fact that hypothalamic AMPK controls both feeding and EE, as well as leptin resistance (17,18), points it as an interesting candidate for the treatment of obesity (9,19,20). The aim of this study is to investigate the VMH targeting of AMPK in diet-induced obese (DIO) models. Our data show that ablation of AMPK$\alpha_1$ in steroidogenic factor 1 (SF1) neurons of the VMH protects against high fat diet (HFD)-induced obesity and that this effect is mediated by increased SNS-driven BAT thermogenesis and browning of white adipose tissue (WAT).
RESEARCH DESIGN AND METHODS

Animals

Adult male Sprague-Dawley rats (8-10 weeks old, 200-250 g; Animalario General USC, Santiago de Compostela, Spain) and adult male null SF1-Cre AMPKα1^floxed/floxed^ mice (mixed background; 20 weeks old for the standard diet (SD) experiments and 34 weeks old for the HFD experiments) and their littermates were used. All experiments were performed in agreement with the International Law on Animal Experimentation and were approved by the USC Ethical Committee (Project ID 15010/14/006) and the University of Iowa Institutional Animal Care and Use Committee. To generate SF1 neuron-specific AMPKα1 knock-out mice (SF1-Cre AMPKα1^floxed/floxed^), SF1-Cre mice (Tg(Nr5a1-cre)Lowl/J, stock number 012462) were crossed with AMPKα1 floxed mice (AMPKα1^floxed/floxed^ mice, Prkaa1tm1.1Sjm/J; stock number 014141; both strains from The Jackson Laboratory; Bar Harbor, ME, USA) that possess loxP sites flanking exon 3 of Prkaa1 gene. Cre-negative, floxed (AMPKα1^floxed/floxed^) littermates were used as controls (10). The animals were housed with an artificial 12-hour light (8:00 to 20:00)/12-hour dark cycle, under controlled temperature and humidity conditions and allowed to free access to standard laboratory chow (STD, SAFE A04 Scientific Animal Food & Engineering; Nantes, France) or 45% high fat diet (HFD, D12451; Research Diets, Inc; New Brunswick, US) and tap water.

Generation of lentiviral particles

The protein-coding sequence of AMPKα1-DN was cloned from pVQAd Sf1-AMPKalpha1-DN (ViraQuest Inc.#MSRN=24603; Viraquest; North Liberty, IA, USA) into the pSIN-Flag vector. To generate lentiviral particles the pSIN-Flag vector containing AMPKα1-DN was co-transfected with packaging vectors (psPAX2 and pMD2G) into
HEK293T as previously described (21). pMD2G and psPAX2 were a gift from Didier Trono (Addgene Plasmids #12259 and #12260 respectively; Cambridge, MA, USA)

**Stereotaxic microinjection of viral vectors**

Adenoviral (GFP, AMPKα1-DN, AMPKα2-DN and AMPKα1-CA; Viraquest; North Liberty, IA, USA) and lentiviral (Null and AMPKα1-DN) vectors were delivered in the VMH of rats or mice as previously reported (7,10,11,13-16,22,23).

**Peripheral treatments**

The adrenergic receptor beta 3 (β3-AR) specific antagonist SR59230A (3 mg/Kg/day; Tocris Bioscience; Bristol, UK) was administrated subcutaneously (SC), as previously reported (7,10,11,24).

**Glucose and insulin tolerance tests**

Glycaemia was measured with a glucometer (Accucheck; Roche; Barcelona, Spain) after insulin or glucose administration, after an intraperitoneal injection of 0.75 U/kg insulin (Actrapid, Novonordisk; Bagsvaerd, Denmark) for insulin tolerance test (ITT) or 1 mg/g D-glucose (Sigma; St Louis, MO, USA) for glucose tolerance test (GTT), as previously shown (23,24). HOMA insulin resistance (IR) was calculated as reported (24).

**Calorimetric system and nuclear magnetic resonance**

Animals were analyzed for EE, oxygen consumption (VO₂), respiratory quotient (RQ) and locomotor activity (LA) using a calorimetric system (LabMaster; TSE Systems; Bad Homburg, Germany) as previously shown (10,11,13,16).
Positron emission tomography–computed tomography

Whole-body microPET/CT (positron emission tomography–computed tomography) images were acquired with the *Albira PET/CT Preclinical Imaging System* (Bruker Biospin; Woodbridge, CT, US). Mice received an injection of \((7.4\pm1.85)\) MBq of \(2-^{18}\text{F}-\text{Fluoro-2-Deoxy-2-Glucose}\) (\(^{18}\text{F}-\text{FDG}\)) on the tail vein. The acquisition was performed \(45\pm10\) minutes after the \(^{18}\text{F}-\text{FDG}\) injections. Images were generated by using the *Bruker Albira Suite Software Version 5.0*. The brown fat and liver areas were delineated by using image tools implemented the *AMIDE Software* ([http://amide.sourceforge.net/](http://amide.sourceforge.net/)) to generate a three-dimensional spherical volume of interest with radius of \(6\) mm. Thus, mean standardized uptake values (SUV) were calculated (10).

BAT Temperature measurements

Skin temperature surrounding BAT was recorded with an infrared camera (*B335: Compact-Infrared-Thermal-Imaging-Camera*; FLIR; West Malling, Kent, UK) as previously shown (10,11,13,14,16,23,24).

Sympathetic nerve activity recording

Multi-fiber recording of sympathetic nerve activity (SNA) was obtained from the nerve subserving BAT as previously described (7,10,11,14,23).

Sample processing

From each animal, the VMH, the BAT, the subcutaneous WAT and the liver were immediately homogenized on ice to preserve phosphorylated protein levels. Samples were stored at \(-80^\circ\text{C}\) until further processing. The specificity of the VMH dissections was
confirmed by analyzing the mRNA of SF1 and proopi melanocortin (POMC) (data not shown).

**Blood biochemistry**

Levels of insulin and leptin were measured using mice ELISA kits (*Merck Millipore; Billerica, MA, USA*), triglyceride and cholesterol were measured by using *Spinreact Kits* (*Spinreact S.A.; San Esteve de Bas, Spain*) and NEFA were measured by using *Wako Kit* (*Wako Chemicals GmbH; Neuss, Germany*) on fed animals. Plasma glucagon levels were measured using a mouse ELISA kit (*Mercodia* AB, Uppsala, Sweden), corticosterone (CORT) levels were analyzed using a competitive enzyme immunoassay kit (*Enzo Life Sciences, Farmingdale, NY, USA*) and epinephrine levels were measured using an ELISA kit (*CUSABIO, Houston, TX, USA*) in 24h fasted HFD mice.

**Real-time quantitative RT-PCR**

Real-time PCR (*TaqMan; Applied Biosystems; Foster City, CA, USA*) was performed using specific primers and probes (*Supplemental Table 1*) as previously described (7,10,11,16,23).

**Immunohistochemistry**

Detection of uncoupling protein 1 (UCP1) in WAT was performed using anti-UCP1 (1:500; ab10983; *Abcam, Cambridge, UK*) as previously reported (24-26). Digital images for WAT were quantified with *ImageJ Software (National Institutes of Health; USA)*, as previously shown (24-26). Direct detection of GFP fluorescence was performed as reported (10,24,26).
Double immunohistochemistry/in situ hybridization

*In situ* hybridization (ISH) analyses were performed as previously shown (27) using a specific antisense riboprobe complementary to the coding sequence of the whole exon 3 of mice *Prkaa1* mRNA (*PrKaal-ex3* riboprobe; 278- and 371-nt; NM_001013367.3). For the generation of the template, primer sequences were as follows: forward T3-AMPKex3-sense (5’-
CAGAGATGCAATTAACCCTCACTAAAGGGAGAGTACCAGGTCATCAGTACACCATCT-3’); reverse T7-AMPKex3-as (5’-CCAAGCCTTCTAATACGACTCACTATAGGGAGACCTTCCATTTTTACAGATATAATCA -3’). Following hybridization, sections were processed for immunohistochemistry (IHC) of SF1 with anti-steroidogenic factor 1 antibody (1:500; *Abcam*; Cambridge, UK). Brain sections were incubated against a biotinilated donkey anti-rabbit secondary antibody (1:500; *Jackson Immunoresearch Laboratories;* West Grove, PA, USA) and peroxidase reaction was carried out using *VECTASTAIN Elite ABC-HRP Kit* (*Vector Labs;* Burlingame, CA, USA) and 3,3’-diaminobenzidine-tetrahydrochloride (DAB). Slides were dipped in *Kodak Autoradiography Emulsion type NTB* (*Kodak;* Rochester, NY, USA) and exposed for 3 weeks at 4°C in dark. After this period, the sections were developed and fixed.

Western blotting

Protein lysates from the VMH, BAT and liver were subjected to SDS-PAGE, electrotransferred and probed with antibodies against acetyl-CoA carboxylase α (ACCα), AMPKα2 (*Merck Millipore;* Billerica, MA, USA), phosphorylated acetyl-CoA carboxylase α (pACCα; Ser79), phosphorylated hormone sensitive lipase (pHSL; Ser660), tumor necrosis factor α (TNFα), forkhead box protein O1 (FOXO1) and its phosphorylated form (pFOXO1) (*Cell Signaling;* Danvers; MA, USA); activating transcription factor 6 β (ATF6β), C/EBP
homologous protein (CHOP), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) p65, phosphorylated IkB kinase α/β (p-IKKα/β), phosphorylated protein kinase RNA-like endoplasmic reticulum kinase (pPERK; Thr981), glucokinase (GCK) (Santa Cruz Biotechnology, Dallas, TX, USA), hormone sensitive lipase (HSL), interleukin 1 beta (IL1β), interleukin 6 (IL6), phosphorylated inositol requiring enzyme 1 (pIREα; Ser724), UCP1, glucose-6-phosphatase (G6pase) and pyruvate carboxykinase (PCK1), (Abcam; Cambridge, UK); α-tubulin or β-actin (Sigma-Aldrich; St. Louis, MO, USA) as described (7,10,11,14,23,24). Autoradiographic films (Fujifilm, Tokyo, Japan) were scanned and the bands signal was quantified by densitometry using ImageJ-1.33 software (NIH; Bethesda, MD, USA), as previously shown (7,10,11,13-16,22,23). Values were expressed in relation to β-actin (VMH) or α-tubulin (BAT). Representative images for all proteins are shown; in the case of the loading controls a representative gel is displayed, although each protein was corrected by its own internal control (β-actin or α-tubulin). In all the Figures showing images of gels, all the bands for each picture come always from the same gel, although they may be spliced for clarity.

**Statistical analysis**

Data are expressed as mean±SEM; when data are relativized, they are given as percentage of the appropriate controls. Error bars represent SEM. Statistical significance was determined by t-Student (when two groups were compared) or ANOVA (when more than two groups were compared) followed of post-hoc Bonferroni test. P<0.05 was considered significant; the exact P values are specified in the Results Section.
RESULTS

Inhibition of AMPKα in the VMH reverses HFD-induced obesity

We stereotaxically injected into the VMH of HFD-induced obese rats a combination of adenoviruses harboring dominant negative isoforms of the catalytic AMPKα1 and α2 (AMPKα1α2-DN) subunits. The infection efficiency was demonstrated by assaying the hypothalamic expression of GFP into the VMH (Figure 1A), as previously reported (7,10,11,13,14,16,22). Treatment with an adenovirus expressing AMPKα1α2-DN induced a feeding-independent weight loss in rats fed HFD (day 1 P=0.004, day 2 P=0.0006, day 3 P=0.00007, day 4 P=0.00007, day 5 P=0.00001) (Figures 1B-C) and SD controls (day 1 P=0.08, day 2 P=0.014, day 3 P=0.001, day 4 P=0.0001, day 5 P=0.0002) (Supplemental Figures 1A-B). This effect was associated with VMH decreased levels of pACCα (HFD P=0.04, SD P=0.005) and/or endoplasmic reticulum (ER) stress markers (pIRE P=0.009, pPERK P=0.04, ATF6β P=0.0007) (Figures 1D-E and Supplemental Figure 1C). AMPKα1α2-DN elicited an increase in BAT temperature (P=0.04) (Figure 1F), as well as in the protein (P=0.001) and/or mRNA expression (P=0.01) of UCP1 and peroxisome-proliferator-activated-receptor-gamma co-activator 1 alpha (P=0.01) and beta (P=0.06) (PGC1α and PGC1β) in the BAT of HFD fed rats (Figures 1G-H). These data indicate that inhibition of AMPKα within the VMH decreases DIO.

Specific inhibition of AMPKα1 isoform in the VMH reverses HFD-induced obesity

Next, we treated rats stereotaxically within the VMH with adenoviruses expressing AMPKα1-DN or AMPKα2-DN. Although both isoforms induced a significant decrease in body weight (AMPKα1-DN: day 1 P=0.01, day 2=P=0.018, day 3 P=0.04, day 4 P=0.008, day 5 P=0.005; AMPKα2-DN: day 1 P=0.03, day 2 P=0.07, day 3 P=0.06, day 4 P=0.02, day 5 P=0.01), AMPKα1-DN, but not AMPKα2-DN, caused a feeding-independent weight loss.
(AMPKα2-DN: P=0.03) (Figures 2A-C). Since BAT thermogenesis is mainly controlled by the SNS via beta 3 adrenoceptors (β3-AR) (28), we investigated whether BAT activity following administration of AMPKα1-DN or AMPKα2-DN adenoviral particles in the VMH was mediated by the SNS. Pharmacological inactivation of β3-adrenergic receptor by SC administration of SR59230A (7,10,11,24) prevented the effect on BAT (ANOVA P=0.018, F=4.45; GFP vs. AMPKα1-DN P=0.007; AMPKα1-DN vs. AMPKα1-DN + SR59230A P=0.04) (Figure 2D) and body temperature (ANOVA P=0.0084, F=5.35; GFP vs. AMPKα1-DN P=0.006; AMPKα1-DN vs. AMPKα1-DN + SR59230A P=0.002) (Figure 2E) associated with AMPKα1-DN injection into the VMH. Consistent with the increased thermogenesis, the treatment with SR59230A blunted the weight-reducing effect of AMPKα1-DN (GFP: -9.76±2.62 g; AMPKα1-DN: -21.86±3.71 g, P<0.01 vs. GFP; AMPKα1-DN + SR59230A: -12.71±2.93 g, P<0.05 vs. AMPKα1-DN).

Next, we evaluated the impact of AMPKα1-DN in rats fed a HFD. Administration of AMPKα1-DN adenovirus into the VMH of HFD-induced obese rats induced feeding-independent weight loss (day 1 P=0.04; day 2 P=0.002; day 3 P=0.006; day 4 P=0.006; day 5 P=0.001) (Figures 2F-G), associated with increased BAT temperature (P=0.0004) (Figure 2H). Since our adenoviral treatments lasted 5 days, which correspond to the maximal effect of the viral infection (7,10,11,14-16,22-24,26,29), we investigated the effect of virogenic targeting of AMPKα1 in HFD obese rats during longer time. For this purpose, we used lentiviruses harboring the AMPKα1-DN cDNA. Lentiviruses allow the prolonged expression of the transgene due to the integration in the host genome (30). Our data show that a single VMH administration with AMPKα1-DN lentiviruses after 40 days induced a marked feeding-independent weight loss (Body weight change ANOVA P=0.018, F=4.99; SD Lv Null vs. HFD Lv Null P=0.005; HFD Lv Null vs. HFD Lv AMPKα1-DN P=0.04; Food intake ANOVA P=0.0043, F=7.24; SD Lv Null vs. HFD Lv Null P=0.006; SD Lv Null vs. HFD Lv
AMPKα1-DN P=0.002; HFD Lv Null vs. HFD Lv AMPKα1-DN P=0.2 non-significant) (Figure 2I-J). Importantly, this was associated with increased BAT thermogenesis in HFD animals which lasted during the 40-day time period (ANOVA P=0.00002, F=20.19; HFD Lv Null vs. HFD Lv AMPKα1-DN P=0.0007) (Figure 2K). Together, these data indicated that AMPKα1, but not AMPKα2, is the key isoform modulating BAT thermogenesis within the VMH and that its inhibition decreases HFD-induced obesity.

**Ablation of AMPKα1 in SF1 neurons of the VMH increases BAT thermogenesis and energy expenditure**

Next, we aimed to identify the specific VMH neuronal population of neurons mediating those effects. To this end, we generated a SF1 specific AMPKα1 null mouse line (SF1-Cre AMPKα1^{flox/flox}) (10) by crossing floxed AMPKα1 mice (31) with SF1-Cre mice, which express Cre recombinase under SF1 promoter (32). Allele-specific PCR demonstrated the successful ablation of AMPKα1 in the VMH, a hypothalamic area highly enriched for SF1 neurons, whereas no evidence for ablation of this allele was detected in the cerebral cortex or the cerebellum (Figure 3A). In addition, AMPKα1 allele ablation was detected in peripheral tissues where SF1 is also expressed, such as the adrenal gland, the pituitary and the testis, but not in SF1-negative tissues, as the liver (Figure 3A). To further characterize the SF1-specific deletion of AMPKα1 in the VMH, we performed a dual procedure for simultaneous detection of SF1 immunoreactivity and AMPKα1 mRNA, the later using a probe directed against the deleted sequence of the allele (flanked by the lox-P sites). Unambiguous co-localization of AMPKα1 mRNA was detected in a substantial proportion of SF1 neurons in the VMH. In contrast, this proportion significantly dropped to nearly negligible levels in SF1-Cre AMPKα1^{flox/flox} mice, therefore confirming the effective ablation of AMPKα1 in SF1 neurons of the VMH. This was further confirmed by quantification, showing the low degree of
colocalization of AMPKα1 mRNA and SF1 positive staining in the null mice (only 11.26 %) in relation to WT littermates (P=2.10^{-9}) (Figure 3B). Next, we investigated the effect of AMPKα1 deletion on hypothalamic AMPKα2; SF1-Cre AMPKα1^flox/flox mice exhibited a significant upregulation of the AMPKα2 isoform within the VMH, likely to compensate for the deficiency in AMPKα function (P=0.02) (Figure 3C).

Interestingly, SF1-Cre AMPKα1^flox/flox mice displayed reduced body weight (week 4 P=0.02; week 8 P=0.001; week 12 P=0.003; week 16 P=0.005; week 20 P=0.02) (Figure 3D) and adiposity (P=0.009) (Figure 3E). Body length was comparable between SF1-Cre AMPKα1^flox/flox mice and littermate controls (Figure 3F). Food intake was also not different in SF1-Cre AMPKα1^flox/flox mice (Figure 3G). SF1-Cre AMPKα1^flox/flox mice exhibited higher EE and VO_2 (P<0.001) (Figure 3H-I). LA was decreased (P=0.007-0.04) (Figure 3J), whereas RQ remained unchanged (Figure 3K). Analysis of plasma levels showed lower triglycerides (P=0.04), non-esterified fatty acids (NEFAs) (P=0.02) and leptin (P=0.005), but unchanged cholesterol (Figure 3L-O), in SF1-Cre AMPKα1^flox/flox mice.

In keeping with the increased EE, SF1-Cre AMPKα1^flox/flox showed BAT activation, as demonstrated by elevated sympathetic nerve activity subserving this tissue (P=0.002) (Figure 4A) and the increased BAT temperature (P=0.008) (Figure 4B) and UCP1 protein expression (P=0.008) (Figure 4C) and higher ^18F-FDG uptake in the BAT, when compared to liver (P=0.03) (Figure 4D), indicating higher BAT function. Of note, pharmacological inactivation of β3-AR by SC administration of SR59230A (7,10,11,24), reverted the weight loss (P=0.004) in a feeding-independent manner and decreased BAT temperature (P=0.0009) of SF1-Cre AMPKα1^flox/flox mice (Supplemental. Figures 2A-C). Finally, SF1-Cre AMPKα1^flox/flox mice displayed higher expression of thermogenic markers in subcutaneous WAT (Ucp1 P=0.007; Pparγ1a P=0.04; Pparγ1b P=0.01; Cidea P=0.005) (Supplemental Figure 3A), which was indicative of browning.
SF1 is also expressed in peripheral organs. Therefore, it is possible that the phenotype of SF1-Cre AMPKα1\textsuperscript{flox/flox} mice may be driven by the loss of AMPKα1 in those tissues. To investigate such possibility, SF1-Cre AMPKα1\textsuperscript{flox/flox} mice were treated with an adenovirus encoding a constitutive active AMPKα1 (AMPKα1-CA) into the VMH. This gain of function treatment promoted feeding-independent weight gain of SF1-Cre AMPKα1\textsuperscript{flox/flox} mice (day 1 \(P=0.09\); day 2 \(P=0.15\); day 3 \(P=0.05\); day 4 \(P=0.02\); day 5 \(P=0.002\)) (Figures 4E-F), associated with decreased BAT temperature (\(P=0.04\)) (Figure 4G) and UCP1 expression (\(P=0.01\)) (Figure 4H). Finally, SF1-Cre AMPKα1\textsuperscript{flox/flox} mice were exposed to 4ºC for 6 hours. This cold challenge demonstrated that null mice defended their body temperature (2 hours \(P=0.009\); 3 hours \(P=0.011\); 4 hours \(P=0.011\); 5 hours \(P=0.02\); 6 hours \(P=0.02\)) (Figure 4I), average body temperature (\(P=0.003\)) (Figure 4J) and BAT temperature (Figure 4K) (0 hours \(P=0.01\); 2 hours \(P=0.003\); 3 hours \(P=0.02\); 4 hours \(P=0.01\); 5 hours \(P=0.003\)) (Figure 4K) better than the littermate controls. Overall, this evidence demonstrated that specific ablation of AMPKα1 in SF1 neurons of the VMH promoted a negative energy balance through a process that involved activation of BAT thermogenesis and subsequent increase in EE.

**SF1 AMPKα1 null mice are resistant to HFD-induced obesity**

We evaluated whether SF1-Cre AMPKα1\textsuperscript{flox/flox} mice were protected against obesity. Conditional null mice fed a HFD showed a feeding-independent decrease in body weight (\(P=0.006\)) and adiposity (iWAT, \(P=0.02\)) (Figures 5A-D), associated with increased EE (\(P<0.05-0.001\)) and VO\(_2\) (\(P<0.05-0.01\)) (Figure 5E-F), a slight tendency of elevated LA (Figure 5G) and no change in RQ (Figure 5H). Plasmatic levels of NEFAs (\(P=0.005\)) and leptin (\(P=0.03\)), but neither triglycerides nor cholesterol, were decreased in SF1-Cre AMPKα1\textsuperscript{flox/flox} fed a HFD (Figures 5I-L).
Analysis of the hypothalami of SF1-Cre AMPKα1<sup>flox/flox</sup> mice fed a HFD demonstrated decreased pACCα levels in the VMH (P=0.04) (Figure 6A). Recent evidence demonstrated that hypothalamic inflammation and ER stress induce obesity and inhibit BAT thermogenesis and browning of WAT (23,24,26,33-35). Our result show reduced ER stress (pIRE P=0.02; pPERK P=1x10<sup>-5</sup>; ATF6β P=0.01; CHOP P=0.03) (Figure 6B) and inflammatory markers (IL1β P=0.02; pIKKαβ P=0.001; NF-κB P=0.04) (Figure 6C) in the VMH of HFD fed SF1-Cre AMPKα1<sup>flox/flox</sup> mice. Notably, this effect was associated with higher BAT temperature (P=1x10<sup>-6</sup>) (Figure 6D) and elevated mRNA and protein expression of thermogenic markers, such as UCP1, PGC1α, PGC1β (Ucp1 P=0.0015; Ucp3 P=0.06; Ppargc1 P=0.015; UCP1 protein P=0.005) and pHSL (P=0.0005; pHSL/HSL P=0.003) (Figure 6E-F) in BAT, as well as browning of subcutaneous WAT, as indicated by decreased adipocyte area (P=0.0007) (Figure 6G) and increased UCP1 staining (P=1x10<sup>-12</sup>) (Figure 6H).

**SF1 AMPKα1 null mice show improved glucose homeostasis**

Finally, we evaluated the impact of AMPKα1 ablation in SF1 neurons on peripheral glucose homeostasis in the context of obesity. HFD SF1-Cre AMPKα1<sup>flox/flox</sup> mice showed decreased glucose levels in the fast and fed states (fast P=0.001; fed P=0.004) (Figure 7A), improved glucose tolerance (0 min P=0.009; 15 min P=0.05; 30 min P=0.07; 60 min P=0.004; 90 min P=0.006; 120 min P=0.003; AUC P=0.004) (Figures 7B-C) and unchanged insulin sensitivity and insulin levels (Figures 7D-F). HOMA-IR confirmed that HFD-induced insulin resistance was ameliorated in SF1-Cre AMPKα1<sup>flox/flox</sup> (P=0.06) (Figure 7G).

On this context, the lack of effect in insulin sensitivity was intriguing. It has been reported that AMPK in the VMH plays a major role in counterregulatory responses to hypoglycemia by modulating the release of glucagon, CORT and epinephrine (5,36). Our results showed that when fasted, HFD AMPKα1 null animals displayed lower levels of
glucagon (non-significant trend, P=0.06), decreased CORT (P=0.008) and no changes in epinephrine (Figures 7H-J), suggesting an altered counterregulatory response and that hepatic release of glucose is likely also diminished. Finally, we examined the levels of key enzymes involved in hepatic glucose metabolism. Our data showed that the protein levels of pFOXO1 were increased, while the protein levels of PCK1 and G6Pase were decreased (Supplemental Figure 4A), which was consistent with decreased hepatic gluconeogenesis in SF1-Cre AMPKα1<sup>flox/flox</sup> mice. On the other hand, the liver protein expression of GCK was increased, consistent with increased glycolysis in the liver of SF1-Cre AMPKα1<sup>flox/flox</sup> (Supplemental Figure 4A). Overall, these findings indicate that targeting of AMPKα1 in SF1 neurons not only ameliorated obesity but also reversed associated impaired glucose metabolism.
DISCUSSION

Hypothalamic AMPK has been implicated in the regulation of feeding, BAT thermogenesis and browning of WAT, muscle metabolism, hepatic function and glucose homeostasis (8-10,19,25,26), as well as being involved in diet-induced leptin resistance (17,18). From a therapeutic perspective, this evidence is relevant since several agents with potential anti-obesity and/or antidiabetic effects, such as nicotine (13,37), metformin (38) and liraglutide (15), some of which are even in clinical use, act through AMPK, either peripherally or centrally. Furthermore, the orexigenic and weight-gaining effects of antipsychotic drugs, such as olanzapine, are also mediated by hypothalamic AMPK (39). Therefore, hypothalamic AMPK might theoretically be considered as an interesting target for drug development, due to its potential to modulate both sides of the energy balance equation, namely food intake and EE (19,20). However, it is unclear 1) whether specific inhibition of hypothalamic AMPK could ameliorate obesity, 2) which AMPK isoform would be the best to target, and 3) which neuronal AMPK-expressing population should be targeted.

Global inhibition of AMPKα1 and AMPKα2 in the VMH ameliorated ovariectomy-induced obesity in female rats (11). Moreover, it has been reported that specific ablation of AMPKα2 in POMC or agouti-related peptide (AgRP) neurons of the ARC produce opposite phenotypes. Indeed, while POMC AMPKα2 null mice display hyperphagia and obesity, AgRP AMPKα2 null mice were hypophagic and lean (6). Our data show that global inhibition of AMPKα1 and AMPKα2 within the VMH markedly decreased HFD-induced obesity in a feeding-independent manner involving increased BAT thermogenesis and EE. These latter effects occurred also when AMPKα1 was inhibited in the VMH of diet-induced obese rats but were not mimicked by targeting of AMPKα2. Notably, the effect of global inhibition of both isoforms is recapitulated by selective ablation of AMPKα1 in SF1 neurons of the VMH, that play a major role in the sympathetic traffic to the BAT (10,40,41). The relevance of this data
is reinforced by findings in our animal model, namely the SF1-Cre AMPKα1\textsuperscript{flox/flox} mouse, which support our evidence demonstrating that AMPK signaling in the VMH, and specifically in SF1 neurons, is a canonical mechanism modulating energy balance (7,9-11,15,16,26,42). Importantly, our results also point to AMPKα1, but not AMPKα2, as the main catalytic AMPKα subunit in the VMH that mediate thermogenic control. This is supported by several findings. Firstly, both thyroid hormones and estradiol decrease the expression and/or activity of AMPKα1 but not AMPKα2 (7,11,42). Secondly, the catabolic effects of those hormones, bone morphogenetic protein 8B (BMP8B), nicotine and liraglutide are reversed by specific activation of AMPKα1 in the VMH (7,9-11,15,16,26,42). Thirdly, the hypothalamic effects of the α2 subunits are frequently linked to regulation of food intake (4,6,22,43,44) and not thermogenesis, as confirmed in our current experiments involving VMH administration of AMPKα2-DN isoforms. Finally, SF1-Cre AMPKα1\textsuperscript{flox/flox} mice exhibited a significant compensatory upregulation of the AMPKα2 isoform within the VMH, however, null mice still displayed a markedly thermogenic phenotype, indicating that the increase in AMPKα2 was unable to counteract the effect of AMPKα1 deficiency on BAT and WAT function. Overall, this evidence reinforces the idea that both AMPKα isoforms are playing different roles in the hypothalamus and that while AMPKα1 modulates thermogenesis, AMPKα2 does not. Further work, involving the specific knockdown of α1 or α2 isoforms in other specific hypothalamic population is warranted to better understand this pathway.

Activation of BAT and browning of WAT may represent a therapeutic strategy to combat obesity (45-47); however, the specific control of this process by central mechanism is still unclear. Therefore, our findings showing that specific targeting of the discrete neuronal population of SF1 neurons in VMH impacts obesity by modulating thermogenesis in feeding-independent manner is of translational relevance. Notably, that action also occurs in the absence of appetite compensatory changes in the SF1-Cre AMPKα1\textsuperscript{flox/flox} mice, which
excludes undesired rebound effects. Regarding this, HDF SF1-Cre AMPKα1\textsuperscript{flox/flox} mice also exhibit a clear amelioration of the diet-induced metabolic disorders, as demonstrated by improved glucose homeostasis. Although this may be related to the weight-reducing effects of AMPKα1 deletion in SF1 neurons, direct effects cannot be ruled out. The fact that these mice showed higher \(^{18}\)F-FDG uptake analyzed by PET-CT was indicative of a primary mechanism for increased glucose clearance, likely independent of the weight-reducing factor. In keeping with this, our results showed that when fasted, SF1-Cre AMPKα1\textsuperscript{flox/flox} animals displayed lower levels of glucagon, CORT and epinephrine, indicating an abnormal counterregulatory response to hypoglycemia. Moreover, biochemical data were consistent with decreased gluconeogenesis and increased glycolysis, and therefore lower hepatic glucose production in the liver of null mice.

Current evidence points to use multifactorial strategies for the treatment of obesity. The unimolecular combination of different compounds, such as peptide conjugates (with other peptides or steroid/thyroid hormones), has yielded very successful and promising preclinical and clinical (phases 1-2) results (48-50). These strategies target different molecules/receptors to broadly and simultaneously affect several aspects of energy homeostasis, such as feeding, EE and glucose metabolism (48-50). The fact that hypothalamic AMPK controls all those aspects makes it an interesting and unique candidate for obesity treatment (19,20). In this sense, it has been demonstrated that AgRP AMPKα2 null tend to have lower body weight when exposed to a HFD (6). This suggests that the concomitant targeting of AMPKα in SF1 neurons of the VMH and AgRP neurons in the ARC may allow to control both feeding and EE by inhibiting a single molecule, namely AMPK. However, this is challenging for several reasons. Firstly, the strategy must ensure the specific inhibition of hypothalamic AMPK given the differential regulation of this enzyme between the hypothalamus and the peripheral tissues (4,8,9,51), where it would have deleterious consequences, for example worsening insulin
resistance and diabetes. Secondly, the possible choices to specifically target hypothalamic AMPK seem limited. Optogenetic modulation of central AMPK has been successfully achieved in rodents (52), however, at the current stage its implementation in clinics appears distant. The utilization of nanoparticles (53) might be an interesting option. Finally, chimeras combining glucagon-like peptide-1 (GLP-1) with estradiol (54) or glucagon with T3 (55), all of which inhibit hypothalamic AMPK (7,11,12,15), would allow targeting of hypothalamic AMPK. Further work is warranted to answer these questions.

In conclusion, our data identify the AMPKα1 isoform in SF1 neurons of the VMH as the energy sensor regulating energy balance, being sufficient its inhibition to ameliorate obesity in a feeding-independent, but thermogenic-dependent manner. This finding also opens the need of further functional assessment of the two AMPKα isoforms in the different homeostatic processed controlled by the hypothalamus. Finally, these results suggest that targeting this energy sensor in specific sets of neurons may be a suitable strategy to combat obesity and related metabolic complications.
AUTHOR’S CONTRIBUTIONS

PS-C, ER-P, LL-P, TL-G and NM-S performed the in vivo experiments, analytical methods (NMR, blood biochemistry, RT-PCR and Western blotting), collected and analyzed the data
PS-C generated and phenotyped the SF1-Cre AMPKα1^flox/flox mouse model; JR, FR-P and MJS-T conducted part of the genotyping and characterization of this mouse model
DAM and KR performed and analyzed the sympathetic nerve activity studies
JAP and MF developed the lentiviruses encoding AMPKα1-DN isoforms
The PET-CT analysis was performed in the Molecular Imaging Unit of the Department of Nuclear Medicine of University of Santiago de Compostela
PS-C, JR, CD, RC, KR, RN, MT-S and ML analyzed, interpreted and discussed the data
All authors reviewed and edited the manuscript
PS-C and ML made the figures
PS-C and ML conceived and designed the experiments
ML developed the hypothesis, secured funding, coordinated and led the project and wrote the manuscript

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement nº 281854 -the ObERStress project (ML); Xunta de Galicia (MF: ED431F 2016/016; RN: 2015-CP080 and 2016-PG057; ML: 2015-CP079 and 2016-PG068); Junta de Andalucía (MT-S: P12-FQM-01943); Ministerio de Economía y Competitividad (MINECO) co-funded by the FEDER
Program of EU (MF: BFU2016-80899-P; CD: BFU2014-55871-P; RN: BFU2015-70664R; MTS: BFU2014-57581-P; ML: SAF2015-71026-R and BFU2015-70454-REDT/Adipoplast); US National Institutes of Health (KR: HL084207); American Heart Association (KR: EIA#14EIA18860041) and The University of Iowa Fraternal Order of Eagles Diabetes Research Center (KR) and Atresmedia Corporación (ML). ER-P is recipient of a fellowship from MINECO (BES-2015-072743); LL-P is recipient of a fellowship from Xunta de Galicia (ED481A-2016/094); MF is recipient of a Ramón y Cajal contract from MINECO (RYC-2014-16779). The CiMUS is supported by the Xunta de Galicia (2016-2019, ED431G/05). CIBER de Fisiopatología de la Obesidad y Nutrición is an initiative of ISCIII. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ML serves as the guarantor.
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FIGURE LEGENDS

FIGURE 1. Effect of AMPKα1α2-DN microinjection into the VMH on energy balance in HFD-induced obese rats

(A) Representative image of direct GFP fluorescence in the VMH after injection of adenovirus

(B) Body weight change (n=9 rats/group)

(C) Daily food intake (n=9 rats/group)

(D) Protein levels of pACCα and ACCα in the VMH (n=7 rats/group)

(E) Protein levels of ER stress pathway in the VMH (n=7 rats/group)

(F) BAT temperature (n=7 rats/group)

(G) Protein levels of UCP1 in the BAT (n=7 rats/group)

(H) mRNA levels of thermogenic markers in the BAT (n=9 rats/group)

of rats fed a HFD stereotaxically treated within the VMH with GFP or AMPKα1-DN and AMPKα2-DN adenoviruses.

*P<0.05, **P<0.01, ***P<0.001 vs. HFD GFP VMH. Statistical significance was determined by t-Student. Data expressed as mean±SEM. The bands in gels from panels D, E and G have been spliced from the same original gels, as indicated by vertical black lines.

FIGURE 2. Effect of AMPKα1-DN or AMPKα2-DN microinjection into the VMH on energy balance in rats

(A-B) Body weight change (n=14-17 rats/group)

(C) Daily food intake (n=14-17 rats/group)

(D) BAT temperature (n=14-17 rats/group)

(E) Body temperature (n=14-17 rats/group)
of rats fed a SD stereotaxically treated within the VMH with GFP or AMPKα1-DN or AMPKα2-DN adenoviruses and SC with SR59230A

(F) Body weight change (n=16-19 rats/group)

(G) Daily food intake (n=16-19 rats/group)

(H) BAT temperature (n=8 rats/group)

of rats fed a HFD stereotaxically treated within the VMH with GFP or AMPKα1-DN adenoviruses.

(I) Body weight change (n=7-8 rats/group)

(J) Food intake (n=7-8 rats/group)

(K) BAT temperature (n=7-8 rats/group)

of rats fed a HFD stereotaxically treated within the VMH with Null or AMPKα1-DN lentiviruses.

*P<0.05, **P<0.01, ***P<0.001 vs. GFP VMH or Null VMH. #P<0.05, ## P<0.01, ###P<0.001 vs. AMPKα1-DN VMH. Statistical significance was determined by t-Student (when two groups were compared) or ANOVA (when more than two groups were compared followed of post-hoc Bonferroni test. Data expressed as mean±SEM.

**FIGURE 3. Effect of AMPKα1 deletion in SF1 neurons on energy balance in mice**

(A) PCR for detection of the recombined Prkaa1 allele in the following tissues from AMPKα1^{flox/flox} and SF1-Cre AMPKα1^{flox/flox} mice: VMH, cortex, cerebellum, adrenal gland, pituitary, testis and liver.

(B) Double immunohistochemistry/in situ hybridization against SF1 and AMPKα1, respectively in the VMH of AMPKα1^{flox/flox} and SF1-Cre AMPKα1^{flox/flox} mice. White and red arrows indicate the presence and absence of colocalization, respectively. Scale bar: 20 µm.

(C) Protein levels of AMPKα2 in the VMH (n=7 mice/group)
(D) Body weight (n=12-23 mice/group)

(E) Fat pads mass (n=12-23 mice/group)

(F) Body length (n=7-8 mice/group)

(G) Daily food intake (n=8 mice/group)

(H) Energy expenditure (n=5-6 mice/group)

(I) Oxygen consumption (n=5-6 mice/group)

(J) Locomotor activity (n=5-6 mice/group)

(K) Respiratory quotient (n=5-6 mice/group)

(L-O) Circulating levels of triglycerides, cholesterol, NEFA and leptin (n=8-9 mice/group) of AMPKα1^flox/flox and SF1-Cre AMPKα1^flox/flox mice.

*P<0.05, **P<0.01, ***P<0.001 vs. AMPKα1^flox/flox. Statistical significance was determined by t-Student. Data expressed as mean±SEM. The bands in gels from panels C come from the same original gels.

FIGURE 4. Effect of deleting AMPKα1 in SF1 neurons on BAT thermogenesis in mice

(A) Sympathetic nerve activity recorded from the nerves subserving BAT (n=8 mice/group)

(B) BAT temperature (n=12-17 mice/group)

(C) Protein levels of UCP1 in the BAT (n=7 mice/group)

(D) PET-CT scan (n=6 mice/group)

of AMPKα1^flox/flox and SF1-Cre AMPKα1^flox/flox mice.

(E) Body weight change (n=8 mice/group)

(F) Daily food intake (n=8 mice/group)

(G) BAT temperature (n=8 mice/group)

(H) Protein levels of UCP1 in the BAT (n=7 mice/group)
of SF1-Cre AMPKα1^{flox/flox} mice stereotaxically treated within the VMH with GFP or AMPKα1-CA adenoviruses.

(I) Body temperature (n=5 mice/group)

(J) Average body temperature (n=5 mice/group)

(K) BAT temperature (n=5 mice/group)

of AMPKα1^{flox/flox} and SF1-Cre AMPKα1^{flox/flox} mice cold-challenged at 4°C for 6 hours.

*P<0.05, **P<0.01, vs. AMPKα1^{flox/flox}, SF1-Cre AMPKα1^{flox/flox} GFP VMH or AMPKα1^{flox/flox} at 4°C. Statistical significance was determined by t-Student. Data expressed as mean±SEM. The bands in gels from panels C and H have been spliced from the same original gels, as indicated by vertical black lines.

FIGURE 5. Effect of AMPKα1 deletion in SF1 neurons on HFD-induced obesity in mice

(A) Representative pictures of mice

(B) Body weight (n=10-11 mice/group)

(C) Fat pad mass (n=10-11 mice/group)

(D) Daily food intake (n=10-11 mice/group)

(E) Energy expenditure (n=5-8 mice/group)

(F) Oxygen consumption (n=5-8 mice/group)

(G) Locomotor activity (n=5-8 mice/group)

(G) Respiratory quotient (n=5-8 mice/group)

(I-L) Circulating levels of triglycerides, cholesterol, NEFA and leptin (n=8-9 mice/group)

of AMPKα1^{flox/flox} and SF1-Cre AMPKα1^{flox/flox} mice fed a HFD.

*P<0.05, **P<0.01, ***P<0.001 vs. HFD AMPKα1^{flox/flox}. Statistical significance was determined by t-Student. Data expressed as mean±SEM.
FIGURE 6. Effect of deleting AMPKα1 in SF1 neurons on the hypothalamus and BAT of HFD-induced obese mice

(A) Protein levels of pACCα and ACCα in the VMH (n=7 mice/group)
(B) Protein levels of ER stress pathway in the VMH (n=7 mice/group)
(C) Protein levels of inflammatory markers in the VMH (n=7 mice/group)
(D) BAT temperature (n=10 mice/group)
(E) mRNA levels of thermogenic markers in the BAT (n=9 rats/group)
(F) Protein levels of thermogenic markers in the BAT (n=7 mice/group)
(G) H&E staining (left panels; 20X, scale bar: 100 µm) and adipocyte area (right panels) in subcutaneous WAT (n=11-15 mice/group)
(H) UCP1 staining (left panels; 20X, scale bar: 100 µm) and UCP1 stained area (right panels) in subcutaneous WAT (n=8-11 mice/group)

of AMPKα1^floxflox^ and SF1-Cre AMPKα1^floxflox^ mice fed a HFD.

*P<0.05, **P<0.01, ***P<0.001 vs. HFD AMPKα1^floxflox^. Statistical significance was determined by t-Student. Data expressed as mean±SEM. The bands in gels from panels A, B, C and F have been spliced from the same original gels, as indicated by vertical black lines.

FIGURE 7. Effect of deleting AMPKα1 in SF1 neurons on glucose homeostasis in HFD-induced obese mice

(A) Fast and fed plasma glucose levels (n=9-11 mice/group)
(B-C) Glucose tolerance test and area under the curve (n=10-11 mice/group)
(D-E) Insulin tolerance test and area under the curve (n=10-11 mice/group)
(F) Plasma insulin levels (n=9 mice/group)
(G) HOMA-IR index (n=5 mice/group)
(H) Glucagon fasting levels (n=5 mice/group).
(I) Corticosterone fasting levels (n=5 mice/group).

(J) Epinephrine fasting levels (n=5-6 mice/group).

**P<0.01 vs. HFD AMPKα1^{flox/flox}.** Statistical significance was determined by t-Student. Data expressed as mean±SEM.
Figure 1

104x87mm (300 x 300 DPI)
Figure 2

(Seoane-Collazo et al)

135x175mm (300 x 300 DPI)
Figure 3
(Seoane-Collazo et al.)
Figure 4

(Seoane-Collazo et al)
Figure 5

(Seoane-Collazo et al)
Figure 6

Seoane-Collazo et al.
Figure 7

86x71mm (300 x 300 DPI)
## Supplemental Table 1: Primers and probes for real-time PCR (TaqMan)

| mRNA  | GenBank accession number | Fw Primer | Rv Primer | Probe                  |
|-------|--------------------------|-----------|-----------|------------------------|
| f8S   | M11188.1                 | 5'-CGGCTACCACATCCAAGGAA-3' | 5'-GCTGGAAATTACCCGGGCT-3' | 5'-AGGCGCAATCTGCTGAGCAGCA-3' |
| Adb3  | NM_013108                | 5'-TGCTAGATCTCCATCGTCCCTCTTA-3' | 5'-AAATCACCACGGCTGACTAGGTATGC-3' | 5'-TCCCTGTACGGACTGCTCTTACCTTAT-3' |
| Cidea | Assay ID ThermoFisher TaqMan® Gene Expression Assays | 5'-TGCCTTACGGACAGCTGCTCTTACCTTCT-3' |
| Hprt  | NM_012583                | 5'-AGCCGACGGTTTCTGTCAT-3' | 5'-GGTCTACAACCTGGGCTCTCATCACC-3' | 5'-CGACCCCTCAGCTCCAGGCTGGTAGAT-3' |
| Ppargc1a | NM_031347           | 5'-CGATCGACATTTCCAGGTAAGCTTACCTT-3' | 5'-CGATGGTTCGCTGCTCTGTAGTG-3' | 5'-AGGTCCCAGGGAGTAGCTCCTCTTCTGAGA-3' |
| Ppargc1b | Assay ID Applied Biosystems TaqMan® Gene Expression Assays | 5'-AGGCCGCGGTTTCTGTCAT-3' |
| Ucp1  | NM_012682                | 5'-CAGATACCATGTACACCAAGGAA-3' | 5'-GATCCGACCGCAGAAGGAA-3' | 5'-CAATGACCATGTACACCAAGGAA-3' |
| Ucp1  | NM_009463                | 5'-CGATGGTTCGCTGCTCTGTAGTG-3' | 5'-GACCCGAGCGCAAGAAGAAGAA-3' | 5'-GACCCGAGCGCAAGAAGAAGAA-3' |
| Ucp3  | Assay ID Applied Biosystems TaqMan® Gene Expression Assays | 5'-CAATGACCATGTACACCAAGGAA-3' | 5'-GATCCGACCGCAGAAGGAA-3' | 5'-CAATGACCATGTACACCAAGGAA-3' |
SUPPLEMENTAL FIGURE 1. Effect of microinjection of AMPKα1α2-DN into the VMH on energy balance in rats
(A) Body weight change (n= 9 rats/group)
(B) Daily food intake (n= 9 rats/group)
(C) Protein levels of pACCα and ACCα in the VMH (n= 7 rats/group)
of rats fed a SD stereotaxically treated within the VMH with GFP or AMPKα1-DN and AMPKα2-DN adenoviruses.
*P<0.05, **P<0.01, ***P<0.001 vs. SD GFP VMH. Statistical significance was determined by t-Student. Data expressed as mean±SEM.
SUPPLEMENTAL FIGURE 2. Effect of SR59230A treatment on mice lacking AMPKα1 in SF1 neurons
(A) Body weight change (n= 7 rats/group)
(B) Daily food intake (n= 7 rats/group)
(C) BAT temperature (n= 7 mice/group)
**P<0.01, ***P<0.001, vs. SF1-Cre AMPKα1<sup>flox/flox</sup> vehicle. Statistical significance was determined by t-Student. Data expressed as mean±SEM
SUPPLEMENTAL FIGURE 3. Effect of AMPKα1 deletion in SF1 neurons on thermogenic markers in WAT

(A) mRNA levels of thermogenic markers in the subcutaneous WAT of AMPKα1^{flox/flox} and SF1-Cre AMPKα1^{flox/flox} mice (n= 8 mice/group)

*P<0.05, **P<0.01, vs. AMPKα1^{flox/flox}. Statistical significance was determined by t-Student. Data expressed as mean±SEM
SUPPLEMENTAL FIGURE 4. Effect of deleting AMPKα1 in SF1 neurons on hepatic glucose metabolism in HFD-induced obese mice

(A) Protein levels of gluconeogenesis and glycolytic enzymes in the liver of AMPKα1\textsuperscript{floxFlo} and SF1-Cre AMPKα1\textsuperscript{floxFlo} mice fed a HFD (n=7 mice/group)

*P<0.05, **P<0.01 vs. HFD AMPKα1\textsuperscript{floxFlo}. Statistical significance was determined by t-Student. Data expressed as mean±SEM.