Increased energy expenditure, lipolysis, and hyperinsulinemia confer resistance to central obesity and type 2 diabetes in mice lacking alpha$_{2A}$-adrenoceptors

Running title: Alpha2A-adrenoceptors and obesity in mice

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List of non-standard abbreviations:

AR = adrenoceptor

AgRP = agouti-related peptide

BAT = brown adipose tissue

CRH = corticotropin-releasing hormone

DIO = diet-induced obesity

Epi = epinephrine

FA = fatty acid

HFD = high fat diet

NE = norepinephrine

NPY = neuropeptide Y

PGC1-α = PPAR-γ co-activator-1α

POMC = pro-opiomelanocortin

RER = respiratory exchange ratio

T2D = Type 2 Diabetes

UCP-1 = uncoupling protein 1

WAT = white adipose tissue

WT = wild-type

Abstract

The α_{2A}-adrenoceptors (ARs) are Gi-coupled receptors, which prejunctionally inhibit the release of norepinephrine (NE) and epinephrine (Epi), and postjunctionally insulin secretion and lipolysis. We have earlier shown that $\alpha_{2A}^{-/-}$ mice display sympathetic hyperactivity, hyperinsulinemia and improved glucose tolerance. Here we employed $\alpha_{2A}^{-/-}$ mice and placed the mice on a high-fat diet (HFD) to test the hypothesis that lack of α_{2A}-ARs protects from diet-induced obesity (DIO) and type 2 diabetes (T2D). In addition, high caloric diet was combined with running wheel exercise to test the interaction of diet and exercise. HFD was obesogenic in both genotypes, but $\alpha_{2A}^{-/-}$ mice accumulated less visceral fat than their WT controls, were protected from T2D, and their insulin secretion was unaltered by the diet. Lack of α_{2A}-ARs associated with increased sympatho-adrenal tone, which resulted in increased energy expenditure and fat oxidation rate potentiated by HFD. Fittingly, $\alpha_{2A}^{-/-}$ mice displayed enhanced lipolytic responses to Epi, and increased fecal lipids suggesting altered fat mobilization and absorption. Subcutaneous white fat appeared to be thermogenically more active (measured as *Ucp1* mRNA expression) in $\alpha_{2A}^{-/-}$ mice, and brown fat showed an increased response to norepinephrine. Exercise was effective in reducing total body adiposity and increasing lean mass in both genotypes, but there was a significant diet-genotype interaction, as even modestly increased physical activity combined with lack of α_{2A}-AR signalling promoted weight loss more efficiently than exercise with normal α_{2A}-AR function. These results suggest that blockade of α_{2A}-ARs may be exploited to reduce visceral fat and to improve insulin secretion.

1. Introduction

The α_{2A}-adrenoceptor (α_{2A}-AR) is an important regulator of sympathoadrenal tone and blood glucose homeostasis, and it also plays a role in the control of fat storage. Norepinephrine (NE) and epinephrine (Epi) act on post-junctional α_{2A}-ARs to inhibit insulin secretion from pancreatic β-cells [1,2], and lipolysis in adipocytes [3], whereas pre-junctional α_{2A}-ARs in the central nervous system (CNS), on sympathetic nerves and on adrenomedullary chromaffin cells mediate feedback inhibition of neurotransmitter release [4]. An increased α_{2A}/β_3-AR ratio in adipocytes has been reported to promote diet-induced obesity (DIO), revealing an important interaction between these adrenoceptor subtypes and diet on body fat distribution [5].

A human *ADRA2A* variant (rs553668) results in overexpression of α_{2A}-ARs, and associates with earlier onset of type 2 diabetes (T2D) [6] and with male obesity [7]. In contrast, a 6.3-kb *DraI* restriction fragment length gene variant form [8] of the *ADRA2A* confers reduced expression of α_{2A}-ARs [9]. The 6.3-kb *DraI* restriction fragment variant associates with reduced abdominal fat in men [10], and also an association with improved insulin sensitivity was found, even after adjustment for visceral fat, suggesting that the variant may have protective effects against the metabolic complications of obesity [10].

Evidence from animal models also suggests α_{2A}-ARs to play an important role in regulating glucose metabolism. In addition to suppression of glucose-stimulated insulin release in response to catecholamines [11,12], α_{2A}-ARs also mediate suppression of perinatal β-cell replication leading to reduced adult β-cell numbers and impaired glucose homeostasis in mice [13]. Previous results indicate that yohimbine, a classical non-selective α_2-AR antagonist, is able to normalize blood glucose values in rats with T2D *via* promoting insulin secretion, but has no effect on glucose levels in normal rats [14]. We recently published a study where wild-type (WT) mice were treated either with the peripherally acting α_2-AR antagonist MK-467

(vatinoxan) alone or in combination with the sulphonylurea drug glibenclamide. MK-467 increased insulin secretion, and reduced blood glucose dose-dependently, and potentiated glibenclamide's hypoglycaemic actions [15]. Thus, inhibition of α_{2A}-ARs may be beneficial for blood glucose regulation, but more information is needed on the effects of loss of α_{2A}-AR activity on body composition, especially since blockade of α_{2A}-ARs leads to elevated insulin levels, which may promote weight gain.

Inhibition of α_{2A}-ARs also prevents autoregulation of catecholamine release from nerve terminals, and thus increases neurotransmitter release [16]. Alterations in sympathoadrenal activity have long been associated with the pathophysiology of obesity [17]. Evidence from rodent models gave rise to the Mona-Lisa hypothesis (Most Obesities kNown Are Low In Sympathetic Activity), which was based on an idea that lower sympathetic activity would lead to obesity via reduced thermogenesis [18]. Fittingly, high sympathoneural tone has been reported to favor success in diet-induced weight loss [19], blunted sympathetic responses to physiological stressors to precede obesity [20], and low adrenomedullary tone expressed as the release of Epi has been associated with obesity in humans [21–25]. However, increased spillover into plasma or urinary secretion of NE has also been shown in obese humans in several study populations [26–29]. Hence, it is still incompletely understood how sympathoadrenal activity correlates with the development of obesity.

Mice lacking α_{2A}-ARs ($\alpha_{2A}^{-/-}$ mice) display increased sympathoadrenal tone [30,31], hyperinsulinemia and improved glucose tolerance [31,32], but differences in body weight have not been reported between $\alpha_{2A}^{-/-}$ and WT control mice under standard diet conditions and without stressors. However, mice are generally resistant to weight gain on standard rodent chow, and the effects of reduced α_{2A}-AR signaling on obesogenic insults have not been comprehensively addressed. The objective of the present study was to determine the effects and mechanisms of depleted α_{2A}-AR signaling on metabolic health in $\alpha_{2A}^{-/-}$ male mice,

following the original finding of improved glucose tolerance and hyperinsulinemia [32]. Here, we show that $\alpha_{2A}^{-/-}$ mice display a number of beneficial metabolic qualities, including protection against central obesity, T2D and insulin resistance, and promotion of white adipose tissue (WAT) browning when exposed to a high fat diet (HFD). In addition, DIO was found to be further reduced when lack of α_{2A}-ARs was combined with increased physical activity.

2. Material and methods

2.1 Animals

The employed $\alpha_{2A}^{-/-}$ mouse line was generated as previously described [33], and it has been back-crossed to C57Bl/6J mice [34] for over 10 generations. A WT control line was obtained during the back-crossing. Age-matched male mice were housed with same-sex littermates on a 12 h light-dark cycle with *ad libitum* access to tap water and standard rodent chow (Special Diets Services Inc., Essex, UK). DIO was induced with a high-fat diet(HFD, 45 kcal% fat, 35 kcal% carbohydrates, 20 kcal% protein, Research Diets D12451, Research Diets Inc., New Brunswick, NJ, USA, in Studies 1 & 2) , or Western type diet (WD, 40 kcal% fat, 43 kcal% carbohydrates, 17 kcal% protein, Research Diets D12079B, in Study 3). Mice were fasted for 4 h prior to sacrifice (start at 6 am), and one cohort of HFD-fed (3 weeks) mice was fasted overnight (o/n). Animals were euthanized (2% isoflurane anesthesia, exsanguination and cervical dislocation), and tissues were collected and snap-frozen in liquid nitrogen and stored at -70° C. The experimental protocol was approved by the National Animal Experiment Board of Finland, and all experiments were performed according to ICLAS guidelines.

2.2 Study design

This study was composed of 3 experimental sub-studies. Numbers and ages of animals per group are given below in the description of the study protocols. In study 1 (S1), we investigated the $\alpha_{2A}^{-/-}$ phenotype during 9 weeks on HFD. Based on the weight gain curves derived from S1, study 2 (S2) focused on biological responses after a short diet intervention, *i.e.* before development of massive DIO, addressing mechanisms that protect $\alpha_{2A}^{-/-}$ mice from central obesity. In study 3 (S3), voluntary wheel running for 6 weeks was used as an intervention to include an additional sympathoadrenal stimulus in order to study its interaction with the lack of α_{2A}-AR signaling.

2.3 S1: Body weight, food intake, body composition and glucose tolerance test (GTT)

HFD was started at 12 weeks of age and implemented for 9 weeks (n=8-9/group), and in an additional single-housed cohort for 3 weeks (n=8/group). Body weight and food intake were measured weekly. Fat mass and fat-free mass were measured with EchoMRI-700 (Echo Medical Systems LLC, Houston, TX, USA) before the diet intervention, and after 4 and 9 weeks. GTT [35] was performed at week 6. Groups of age-matched chow-fed WT and $\alpha_{2A}^{-/-}$ animals were included in the GTT experiment. These animals were sacrificed at the same age as the HFD animals (31 weeks) to obtain control samples for the quantitative real-time PCR (qPCR) analyses. Serum insulin and lipid concentrations were analysed from submandibular blood samples collected after 4 h of fasting before the diet intervention and after 9 weeks. Subcutaneous WAT, brown adipose tissue (BAT), hypothalamus and liver were collected for biochemical analyses.

2.4 S1: Analysis of hepatosteatosis

Liver cryosections (10 µm) from 4-6 animals were post-fixed with 10% formalin on glass slides and lipids were visualised with oil-red-O (ORO) staining. Hepatic triglycerides were assayed from liver samples (n=3-4/group) according to a previously published method [36]. Expression of genes related to β-oxidation, and fatty acid (FA) storage and metabolism were studied with quantitative real time PCR (qPCR).

2.5 S2: Mechanisms for resistance to DIO in $\alpha_{2A}^{-/-}$ mice

2.5.1 S2.1: Catecholamine and neuropeptide Y (NPY) secretion

Thirty-six mice were used to investigate urinary catecholamine secretion (n = 9-10/group), whole body energy expenditure (n = 6/group) and locomotor activity (n = 7-10/group). Mice were 12 weeks of age at the start of the experiment. Urine samples were collected over 24 h during individual housing in metabolic cages (Tecniplast, Buguggiate, Italy) (before and after

2 weeks on HFD). Urine was collected into pre-weighed tubes containing acetic acid and sodium disulfide as preservatives, and mineral oil to prevent evaporation. Samples were kept at -70°C until analysis. Concentrations of NE and Epi and their metabolites normetanephrine and metanephrine were determined with a multiplex immunoassay [37]. Plasma samples were collected by terminal blood sampling after 3 weeks on HFD, and circulating NPY was measured with a commercial RIA kit [35].

2.5.2 S2.2: Whole body energy expenditure and locomotor activity

After urine collection, 6 mice of both genotypes were taken for whole-body energy metabolism assessment (before and after 3 weeks on HFD) with indirect calorimetry (Oxylet System, Panlab, Barcelona, Spain). Animals were acclimated in metabolic chambers for 24 h. CO_2 and O_2 levels were collected over 3-min epochs every 18 min for each mouse over a period of 24 h following acclimation. Average day- (6 am to 6 pm) and night time (6 pm to 6 am) values were calculated for the oxygen consumption measures and respiratory exchange ratio (RER). Lean body mass was then determined with EchoMRI. The association between energy expenditure (kcal/day) and lean mass was evaluated, and because the lean mass significantly contributed to the oxygen consumption, energy expenditure values were normalized to lean body mass [38]. RER was calculated as the ratio VCO_2/VO_2. Locomotor activity was assessed with a home cage photobeam activity system (SD Instruments, San Diego, CA, USA).

2.5.3 S2.3: In vivo lipolysis assay

Mice on standard chow diet (12 weeks of age, n = 6/group) were implanted with two venous catheters (jugular vein for sampling and femoral vein for dosing) under 2% isoflurane anesthesia and buprenorphine (0.1 mg/kg, Temgesic®, Schering Plough) analgesia. After a 3-day recovery, mice were dosed i.v. with 10 μg/kg of the β-AR agonist isoproterenol (Isuprel®, Hospira), or 1 mg/kg of Epi (Adrenalin®, Leiras). Blood sampling was automated

(AccuSampler®µ, VeruTech, Lund, Sweden). The mice were fasted for 3 h, a baseline blood sample (20 µl) was collected, and either isoproterenol or Epi was administered. Blood samples were collected at 5, 10 and 20 min after dosing. Serum glycerol values were measured (Free Glycerol Reagent, Sigma-Aldrich Inc, St. Louis, MO, USA) and normalised to baseline values to account for possible period and sequence effects.

2.5.4 S2.4: Brown adipocyte oxygen consumption

Interscapular brown adipocytes from 3-week HFD-fed mice (12 weeks of age, n = 5/group) were isolated as previously described [39], and cells were suspended in Krebs-Ringer bicarbonate buffer at 150,000 cells/ml. 300,000 cells were injected into the respirometer (Oxygraph-2K, Oroboros Instruments, Innsbruck, Austria), and basal and NE-stimulated (7.5 µM) oxygen consumption was measured. The results were averaged from quadruple measurements.

2.5.5 S2.5: Gastrointestinal transit and fat excretion

Mice (31 weeks of age, n = 6-8/group) were fasted for 6 h and then administered 0.25 ml of 10% charcoal in 5% *gummi arabicum* by gavage. The animals were euthanized after 15 min with CO_2 sedation and cervical dislocation. The distance travelled by the charcoal suspension in the small intestine was measured, and expressed as percentage of total small intestine length. For fecal analyses, mice (15 weeks of age, n = 7-10/group) were housed for 24 h in grid-floor cages without bedding material. Their food intake was measured, and feces were weighed and frozen in liquid nitrogen. 100 mg fecal samples were dried at 60°C and the lipid phase was isolated (Folch's method [40]) and weighed.

2.6 S3: Effect of voluntary physical activity on weight gain

16-Week-old mice fed with regular chow (n = 12-13/group) or WD-fed mice (6 weeks pre-feeding, n = 17-23/group) were placed in individual cages with wireless running wheels (Med

Associates Inc, St. Albans, VT, USA). After 1 week, the mice were divided into 2 groups with pair-matched activity levels, and the wheels of the inactive control group were locked, *i.e.* the wheels were left in the cages but they did not spin. Body weights, food intake and activity levels were followed weekly for 5 more weeks. Body composition after 6 weeks of exercise was analysed with EchoMRI, and serum samples were collected for insulin and adipokine analyses.

2.7 Biochemical assays

Serum insulin (Mouse Ultrasensitive Insulin ELISA kit, Mercodia, Uppsala, Sweden or MILLIPLEX Mouse Adipokine Panel (MADKMAG-71K), Merck Millipore, Billerica, MA, USA), triglycerides (Serum Triglyceride Determination kit, Sigma-Aldrich Inc, St. Louis, MO, USA), and non-esterified free fatty acids (NEFA-HR(2) kit, Wako Diagnostics, Richmond, VA, USA), plasma NPY (Euro-Diagnostica, Malmö, Sweden), and the serum adipokines leptin and resistin (MADKMAG-71K) were analysed with commercial kits.

2.8 *qPCR*

Total RNA from subcutaneous WAT, BAT, liver and the hypothalamus was extracted with Qiazol reagent according to manufacturer's instructions (Qiagen, Germantown, MD, USA). RNA was reverse-transcribed to cDNA (High Capacity RNA-to-cDNA Kit, Applied Biosystems Inc., Foster City, CA, USA) and quantified with the KAPA SYBR® FAST Master Mix kit (Kapa Biosystems Inc., Wilmington, MA, USA) in an Applied Biosystems 7300 Real-Time PCR System. Ribosomal S29 was used as control in the $^{\Delta\Delta}$Ct method. Primer sequences are listed in supplementary Table 1.

2.9 Statistical analyses

All values are presented as means ± SEM. Data were analysed with 1- or 2-way ANOVA, or repeated measures 2-way ANOVA followed by Tukey's or Bonferroni post-hoc tests.

GraphPad Prism 5.0 software was used. $P<0.05$ was considered statistically significant.

3. Results

3.1 Study 1: Susceptibility to DIO in $\alpha_{2A}^{-/-}$ mice

3.1.1 $\alpha_{2A}^{-/-}$ *Mice show reduced visceral fat accumulation under high-caloric conditions*

The rate of weight gain during HFD was similar for WT and $\alpha_{2A}^{-/-}$ mice during 9 weeks on HFD (Fig. 1A). However, group-housed $\alpha_{2A}^{-/-}$ mice consumed less HFD than WT mice (Fig. 1B). To investigate feeding patterns in more detail, separate cohorts of mice were housed individually, and after 3-week habituation on regular chow, they were placed on HFD for 3 weeks. No genotype differences were found in the feeding behaviour of single-housed mice (Fig. 1C), and energy intake calculations demonstrated that HFD only transiently increased caloric intake, suggesting efficient self-regulation of food intake based on its energy density (Fig. 1D).

Body composition analyses before and after 4 and 9 weeks of HFD revealed no significant differences in relative fat or lean body mass between $\alpha_{2A}^{-/-}$ and WT mice (Fig. 1E-F). HFD increased adiposity ($p<0.001$) and decreased lean mass ($p<0.001$) in both genotypes by week 4, but no major changes were observed between weeks 4 and 9 (Fig. 1E-F). Despite similar fat percentages, HFD-fed $\alpha_{2A}^{-/-}$ mice had significantly less mesenteric and retroperitoneal, *i.e.* visceral, WAT than WT controls (Fig. 1G). HFD significantly reduced serum triglycerides in all groups, and increased NEFAs in WT mice (Table 1). Diet-induced hepatosteatosis was verified by an expert pathologist from ORO-stained sections, and it was visually slightly more prominent in $\alpha_{2A}^{-/-}$ mice than in the WT animals (Supplementary Fig. S1), although biochemical determination of triglycerides showed almost similar values in both genotypes (liver triglycerides, WT: 15.0 ± 1.7 mg/g tissue; $\alpha_{2A}^{-/-}$ 18.6 ± 2.4 mg/g tissue: p=NS). Hepatic gene expression profiles for the most relevant genes involved in FA metabolism were compared between HFD-fed $\alpha_{2A}^{-/-}$ and WT mice by qPCR analysis. *Acox1* (encoding for oxidative

enzyme acyl-Coenzyme A oxidase 1), but not *Cpt1* (carnitine palmitoyltransferase 1), was significantly decreased (WT 1.0 ± 0.07; $\alpha_{2A}^{-/-}$ 0.7 ± 0.07 fold change: $p<0.01$) in $\alpha_{2A}^{-/-}$ mice pointing towards decreased β-oxidation. No difference was found in genes involved in lipogenesis *e.g. Fas* (encoding for fatty acid synthase), *Acc1* (acetyl Co-A carboxylase 1) or *Srebp1c* (sterol regulatory element binding protein 1c), nor in *Pck1* (phosphoenolpyruvate carboxykinase 1, cytosolic) regulating gluconeogenesis (data not shown). Thus, although $\alpha_{2A}^{-/-}$ mice had less visceral fat, they were more prone to develop fatty liver, and a possible pathway may be reduced β-oxidation.

3.1.2 Sustained improved glucose tolerance and insulin secretion in $\alpha_{2A}^{-/-}$ mice on HFD

Despite the obesogenic effect of the HFD and hepatosteatosis, blood glucose values were significantly lower in $\alpha_{2A}^{-/-}$ mice in comparison with WT controls (Fig. 2A-B). Glucose tolerance in HFD-fed $\alpha_{2A}^{-/-}$ mice was slightly impaired compared to chow-fed animals (Fig. 2A-B). WT mice showed severely impaired glucose tolerance on HFD, with an average fasting blood glucose level of 12.3 ± 0.7 mmol/l (Fig. 2A). HFD-induced hyperinsulinemia was evident in WT, but not in $\alpha_{2A}^{-/-}$ mice (Fig. 2C).

3.2 Study 2: Mechanisms for resistance to DIO in $\alpha_{2A}^{-/-}$ mice

3.2.1 $\alpha_{2A}^{-/-}$ Mice show reduced responses to HFD-induced changes in catecholamine secretion

$\alpha_{2A}^{-/-}$ Mice secreted significantly more Epi but not NE into urine compared to WT mice on chow diet (Fig. 3A-B). HFD increased NE output only in WT mice, but reduced Epi secretion in both genotypes (Fig. 3A-B). However, urinary Epi remained significantly higher in $\alpha_{2A}^{-/-}$ mice (Fig. 3B). Strong induction by HFD ($p<0.001$), but no genotype differences, was also observed in catecholamine metabolite excretion (data not shown). No differences between the genotypes were observed in plasma NPY after 3 weeks on HFD (data not shown).

3.2.2 Whole body energy expenditure and lipolysis are increased in $\alpha_{2A}^{-/-}$ mice

Energy expenditure (and thus, oxygen consumption) was significantly increased both in mice fed with regular chow and in HFD-fed (3 weeks) $\alpha_{2A}^{-/-}$ mice compared with WT animals ($p<0.001$) (Fig. 4A). HFD significantly increased energy expenditure in both groups, but the diet effect was more prominent in $\alpha_{2A}^{-/-}$ mice than in WT controls, especially at night (Fig. 4A). RER was not different between the genotypes on regular chow (Fig. 4B). HFD induced a reduction in night time RER in both genotypes, and RER was significantly smaller in $\alpha_{2A}^{-/-}$ mice compared to WT animals, suggesting improved fat oxidation efficiency in $\alpha_{2A}^{-/-}$ mice (Fig. 4B). Horizontal and vertical locomotor activity scores were significantly lower in $\alpha_{2A}^{-/-}$ mice than in the controls, and HFD promoted more sedentary behaviour in both genotypes (Fig. 4C-D).

Responses to lipolytic agents were investigated *in vivo* with i.v. administration of isoproterenol and Epi. Isoproterenol increased serum glycerol levels in both groups (Table 2). Epi was not a potent stimulator of lipolysis in WT mice, but a significant rise in circulating glycerol was observed in $\alpha_{2A}^{-/-}$ mice (Table 2).

3.2.3 Thermogenic activity of WAT is increased in $\alpha_{2A}^{-/-}$ mice

In chow-fed mice, no differences in *Ucp1* (encoding for uncoupling protein-1 (UCP-1)) expression were observed between the genotypes (Fig. 5A), but the expression of *Ppargc1a* (PPAR-γ co-activator-1α (PGC1-α)) was significantly increased, and *Adrb3* (encoding for β_3-AR) showed a strong tendency to be increased in $\alpha_{2A}^{-/-}$ BAT compared to WT animals (Fig. 5A). 9 Weeks of HFD strongly suppressed the expression of both *Ucp1* and *Ppargc1a* (Fig. 5A). In contrast, *Adrb3* expression was increased by HFD without significant genotype differences (Fig. 5A). In order to investigate BAT function, oxygen consumption was studied in isolated brown adipocytes from 3-week HFD-fed mice. Basal oxygen consumption was

slightly reduced in $\alpha_{2A}^{-/-}$ cells, but when the cells were activated with NE, $\alpha_{2A}^{-/-}$ brown adipocytes responded more vigorously than WT cells (Fig. 5B).

9-Week HFD exposure suppressed WAT *Ucp1* expression similar to BAT in both genotypes (Fig. 5C). Interestingly, *Ucp1* expression in both regular chow- and HFD-fed $\alpha_{2A}^{-/-}$ mice was significantly higher compared to WT animals (Fig. 5C), and thus, it appears that white adipocytes of $\alpha_{2A}^{-/-}$ mice have an increased propensity for browning. However, *Ppargc1a* and *Adrb3* expression in WAT were not influenced by HFD or genotype (Fig. 5C).

3.2.4 Normal gastrointestinal transit but increased fat excretion in $\alpha_{2A}^{-/-}$ mice

In addition to increased sympathetic tone and energy expenditure, reduced adiposity may be related to differences in entry of nutrients from the gut. $\alpha_{2A}^{-/-}$ Mice had significantly longer small intestines than WT controls ($p<0.001$; data not shown), albeit gastrointestinal transit was similar between the genotypes (WT 54 ± 4 %; $\alpha_{2A}^{-/-}$ 52 ± 7 % of small intestine length: p=NS). 24 h fat intake was calculated based on consumed food and its fat content reported by the manufacturer. Fat intake was smaller in $\alpha_{2A}^{-/-}$ mice compared to WT controls (WT 0.50 ± 0.03 g/24 h; $\alpha_{2A}^{-/-}$ 0.39 ± 0.04 g/24 h: $p<0.05$), but 24 h fecal mass did not significantly differ between the genotypes (WT 240 ± 4 mg; $\alpha_{2A}^{-/-}$ 200 ± 2 mg: p=NS). Despite lesser fat intake, fecal fat content was significantly increased in $\alpha_{2A}^{-/-}$ mice in comparison with WT controls (WT 23 ± 2 µg/mg; $\alpha_{2A}^{-/-}$ 37 ± 2 µg/mg feces: $p<0.05$).

3.2.5 Hypothalamic Npy and AgRP are regulated by α_{2A}-ARs under high caloric conditions

Although no differences in food intake were observed between the genotypes under basal conditions, the stress-induced reduced feeding encouraged us to look into the possible interactions of HFD and α_{2A}-ARs on neuropeptide circuitry in the hypothalamus. On regular chow, there were no differences in mRNA levels of appetite-related peptides (*Npy*, agouti-

related peptide (*Agrp*), pro-opiomelanocortin (*Pomc*) and corticotropin-releasing hormone (*Crh*)) between the genotypes (Fig. 5D). HFD exposure for 9 weeks clearly activated the expression of all investigated genes and HFD-fed $\alpha_{2A}^{-/-}$ mice showed significantly higher *Npy* and *Agrp* expression levels compared to WT controls (Fig. 5D). The gene expression patterns were further investigated after an o/n fast to find out whether the observed differences were state- or trait-related. After 3 weeks of HFD, o/n-fasted hypothalamic *Npy* levels were still significantly higher in $\alpha_{2A}^{-/-}$ mice in comparison with WT controls ($p<0.05$, Supplementary Fig. S2), with no significant genotype differences in the other investigated genes.

3.3 Study 3: Depletion of α_{2A}-ARs potentiates physical activity-induced weight loss in a DIO model

As $\alpha_{2A}^{-/-}$ mice display increased sympathetic tone, and showed resistance to DIO, we next tested whether lack of α_{2A}-AR signalling promotes exercise-induced weight loss. DIO was induced with WD, which is an isocaloric diet with HFD, and shown to be equally effective in promoting adiposity in male C57Bl/6 mice in our earlier study [41]. Activity levels were again significantly lower in $\alpha_{2A}^{-/-}$ mice both on regular chow and under WD conditions (Fig. 6A-B). Physical activity had significant effects on body weight gain and body composition in both genotypes with both diets (Fig. 6C-F). However, weight loss was greater in regular chow-fed WT mice, whereas $\alpha_{2A}^{-/-}$ mice lost more weight on WD (Fig. 6C-D). WD, rich in saturated fats and carbohydrates, was pre-fed for 6 weeks before introducing the running wheels, and it increased body weights in both genotypes, but more profoundly in the $\alpha_{2A}^{-/-}$ group (Fig. 6G). $\alpha_{2A}^{-/-}$ Mice consumed more WD than WT controls during the pre-running period (Fig. 6H), which explains their greater weight gain. After 5 weeks of running, there was no longer a significant difference in absolute body weights between the $\alpha_{2A}^{-/-}$ and WT mice (data not shown). Physically active $\alpha_{2A}^{-/-}$ mice fed WD consumed less food than their inactive controls,

whereas physically active WT mice ate more than their non-active controls (Fig. 6I). Serum concentrations of leptin, resistin and insulin were determined after 6 weeks of running on both diets. Insulin levels were significantly higher in $\alpha_{2A}^{-/-}$ mice, which was associated with decreased resistin levels, but physical activity had no significant effect (Fig. 6J-K). Chow-fed mice showed a significant effect of exercise, but no genotype effect on leptin levels, whereas WD + activity suppressed leptin levels only in WT mice, in line with the observed reduction in their adiposity (Fig. 6L). Physical activity had no effect on serum leptin in $\alpha_{2A}^{-/-}$ mice.

4. Discussion

The role of α_{2A}-ARs in the physiological regulation of energy metabolism is still largely unknown, although they have been recognized as key receptors in controlling the secretion of catecholamines and thus, sympatho-adrenal tone, fat deposition, and insulin secretion. In this study, we show that $\alpha_{2A}^{-/-}$ mice are more resistant to abdominal fat deposition than their WT controls under high caloric conditions. The reduction is noteworthy, because it was most prominent in visceral fat, a metabolically unfavourable fat depot [42], although no genotype differences in body weight or food intake were observed. In addition, $\alpha_{2A}^{-/-}$ mice were able to maintain good glucose tolerance due to lack of inhibitory pancreatic β-cell α_{2A}-AR function resulting in hyperinsulinemia, whereas HFD-fed WT mice developed impaired glucose tolerance and showed severe hyperinsulinemia, suggesting insulin resistance. Our results support the notion of an important role of pancreatic α_{2A}-ARs in regulating insulin release and glucose tolerance. Furthermore, against one of our hypotheses, chronic hyperinsulinemia did not worsen DIO in $\alpha_{2A}^{-/-}$ mice in comparison with WT controls. In fact, increased insulin secretion associated with improved insulin sensitivity may actually promote favourable redistribution within WAT compartments, that is, from visceral to subcutaneous locations, similar to insulin-sensitizing drugs that are used for the treatment of T2D [43,44]. However, hyperinsulinemia may also promote the development of hepatosteatosis as insulin is known to inhibit enzymes involved in FA β-oxidation [45], and some evidence of this was observed in HFD-fed $\alpha_{2A}^{-/-}$ mice.

Catecholamines and their metabolites were measured from urine, and we observed that $\alpha_{2A}^{-/-}$ mice secreted significantly more Epi on both diets than the WT animals, although secretion of Epi was suppressed after feeding HFD. Interestingly, urinary NE secretion was not significantly altered in $\alpha_{2A}^{-/-}$ mice, likely due to fast re-uptake or metabolism of this transmitter,

but the result does not rule out regional differences in sympathetic activity that cannot be determined with this method. Metabolic and fat oxidation rates were also found to be increased in $\alpha_{2A}^{-/-}$ mice, and they were increased by HFD in both genotypes, which reflects a sympathoneural activation by the diet. However, increases were more prominent in $\alpha_{2A}^{-/-}$ mice. When combining these findings with the results of the 9-week HFD experiment, it is evident that enhanced sympatho-adrenal activity and increased energy expenditure play important roles in the amelioration of DIO in $\alpha_{2A}^{-/-}$ mice. Fittingly, greater adrenomedullary responses during mental stress have been inversely associated with long-term changes in BMI and waist circumference in humans [22]. Furthermore, in WT control mice with normal sympatho-adrenal function, HFD was associated with increased secretion of NE and decreased secretion of Epi. Thus, increased sympathoneural and suppressed adrenomedullary activity are adaptations to rather than causes of increased body weight, which fits with previously reported findings in humans [25–28], and with results from obese animal models linking DIO with suppressed Epi responses [23,46].

Similarly to our $\alpha_{2A}^{-/-}$ mice, reduced α_{2A}-AR signaling has also been associated with decreased abdominal fat in men [10]. The mechanism in mice and men could be decreased fat deposition or enhanced fat mobilization, or both, especially in abdominal WAT depots. The former may be a direct result of the lack of antilipolytic post-junctional α_{2A}-ARs, which cannot be verified in our mouse model that lacks both pre- and post-junctional receptors. On the other hand, visceral adipocytes have a higher β/α_{2A}-AR ratio, and therefore higher sensitivity and responsiveness to catecholamine-induced lipolysis than *e.g.* subcutaneous fat cells [47]. Activation of lipolysis was measured *in vivo* in freely moving mice after stimulation with isoproterenol (a selective β-AR agonist) and Epi (a mixed α-AR and β-AR agonist). The response to isoproterenol was similar between the genotypes. However, Epi-induced lipolysis was negligible in WT mice, but observable in $\alpha_{2A}^{-/-}$ mice. This indicates that in the absence of

functional α_{2A}-ARs, Epi-induced β-adrenergic lipolysis is not counteracted *via* α_{2A}-ARs, and thus provides an additional mechanism how $\alpha_{2A}^{-/-}$ mice are protected from visceral obesity.

In rodents, sympatho-adrenal activation is a direct stimulus for BAT thermogenesis. NE-induced β_3-AR activation starts a cascade of intracellular events leading to activation of PGC1-α and UCP-1 [48,49]. Accordingly, *Ppargc1a*, but not *Ucp1*, mRNA levels were significantly increased in BAT from regular chow-fed $\alpha_{2A}^{-/-}$animals, and a tendency to increased *Adrb3* expression was observed. The functional assay performed after 3 weeks on HFD showed that brown adipocytes from $\alpha_{2A}^{-/-}$ mice were slightly less metabolically active under unstimulated conditions, but more responsive to exogenous NE-stimulation than cells from WT animals, which fits with the slightly higher baseline expression level of *Adrb3* and the *in vivo* lipolysis results. α_{2A}-ARs are also present in BAT, and may thus inhibit β-adrenergic activation [50]. After longer HFD exposure, thermogenic gene expression levels indicated reduced thermogenic activity and increased *Adrb3* expression irrespective of the α_{2A}-AR genotype. This increase in *Adrb3* likely reflects suppression of sympatho-adrenal activity by the diet, as *Adrb3* mRNA levels in BAT are regulated by physiological levels of sympathetic nerve activity [51].

Activation of UCP-1 can also take place in WAT upon adrenergic stimulation, and the most prominent depot for browning in rodents is the subcutaneous WAT [52]. We show here that the lack of α_{2A}-ARs leads to increased expression of *Ucp1* in subcutaneous WAT, even when combined with an energy-dense diet. This may be a direct effect mediated by depletion of post-junctional α_{2A}-ARs, since subcutaneous WAT is the only fat depot where α_{2A}-ARs dominate over β-ARs [53]. Thus, it is evident that increased sympatho-adrenal activity promotes thermogenesis in BAT and subcutaneous WAT, but more mechanistic studies are still required to detail the impact of α_{2A}-AR antagonism on weight loss by increased thermogenesis.

Stress and catecholamines inhibit gut motility and digestive processes *via* β–ARs [54,55]. $\alpha_{2A}^{-/-}$ Mice showed increased lipid output suggesting reduced fat absorption, and total small intestine length was also longer compared to WT animals. This phenotype combined to resistance to DIO resembles that of a mouse lacking leptin receptors in intestinal epithelial cells [56]. Whether the decreased fat absorption in $\alpha_{2A}^{-/-}$ mice results from impaired α_{2A}-AR or leptin signaling in the gut warrants more detailed studies. Another interesting finding was that $\alpha_{2A}^{-/-}$ mice consumed less food during this experiment performed on grid floors, which could have been an indirect stress response mediated by the neuronal circuits in the brain controlling feeding.

Interactions of HFD and α_{2A}-AR-mediated appetite regulation were investigated by analysing neuropeptides in the hypothalamus. High caloric diet increased the expression of all investigated peptides, likely reflecting the modulatory effects of NE that have been shown previously [57]. Although we did not detect differences in circulating NPY levels between the genotypes, we did observe increased hypothalamic *Npy* and *Agrp* mRNA levels in $\alpha_{2A}^{-/-}$ mice after 9 weeks of HFD. Similar increases in *Npy* were also seen after shorter HFD exposure combined with an overnight fast, suggesting that hypothalamic NPY neurons are under α_{2A}-AR regulation under high caloric conditions. However, increased *Npy* and *Agrp* expression was not associated with hyperphagia or decreased energy expenditure in our setting. Previous studies have shown that NPY's hyperphagic response could be prevented, and sibutramine's and bupropion's hypophagic effects potentiated by blocking pre-junctional α_2-ARs, pointing to an important role of these receptors in regulation of caloric intake [58–60]. Also earlier studies have demonstrated that activation of central α_2-ARs increases food intake [61]. Thus, ablation of central α_{2A}-ARs can directly induce satiety, but the effect may be counterbalanced by enhanced expression of *Npy* and *Agrp* in the hypothalamus to compensate the hypophagia. The net effect is thus no significant effect on food intake, which may be the underlying cause

why α_2-AR antagonists alone are ineffective in appetite control, but show additive effects when combined with e.g. sibutramine or bupropion [58,60].

Finally, we combined physical activity with regular or a high fat, high carbohydrate diet to test the interaction of sympathetic stress with α_{2A}-ARs. Exercise was effective in reducing whole body adiposity and increasing lean mass in both genotypes on both diets, but there was a significant interaction between the diets and genotypes. Physical activity was not as effective in promoting weight loss on regular chow as it was on WD in $\alpha_{2A}^{-/-}$ mice, whereas in the WT animals, the direction was the opposite. The activity levels were more than 50% smaller in $\alpha_{2A}^{-/-}$ mice compared to WT group, which accounts for at least part of the interaction. Physical immobility has been observed in $\alpha_{2A}^{-/-}$ mice before, and suspected to be linked to anxiety *via* hyperactivity in central noradrenergic systems [34]. Before exercise, WD-fed $\alpha_{2A}^{-/-}$ mice gained more weight than WT mice; an effect that was not seen on HFD. This discrepancy may be due to different macronutrient composition of the diets (low vs. high carbohydrates) leading to higher preference for WD in $\alpha_{2A}^{-/-}$ mice, as they consumed significantly more food than WT animals during pre-feeding. NE acting in the hypothalamic paraventricular nucleus has been shown to selectively promote carbohydrate intake without a clear effect on intake of fat in rats [62,63]. WT mice allowed to run compensated their increased energy expenditure by increasing food intake, whereas active $\alpha_{2A}^{-/-}$ mice consumed less food than their sedentary controls, providing further evidence that central α_{2A}-ARs regulate feeding under conditions with increased sympatho-adrenal activity. Physical activity had very little effects on serum levels of hormones reflecting glucose homeostasis, but genotype differences in insulin and resistin levels were evident. Resistin, which is a marker of insulin insensitivity [64], was decreased in $\alpha_{2A}^{-/-}$ mice, which fits with their improved glucose metabolism [31,32], and also provides additional support for the suggested improved insulin sensitivity of the model on HFD in this study. Leptin is associated with increased adiposity, and the activity-induced weight loss suppressed

leptin levels in active, regular chow-fed animals and WD-fed WT mice as expected. However, WD in $\alpha_{2A}^{-/-}$ mice did not alter leptin levels in proportion to adiposity, suggesting an interplay between leptin synthesis and release, and catecholamines in obesity, as previously hypothesized [65,66].

5. Conclusions

The current results, and our previous findings demonstrating the insulinotropic efficacy of genetic depletion of α_{2A}-ARs [31,32] and the peripherally acting α_2-AR antagonist MK-467 [15], underline an important role of α_{2A}-ARs in mediating regulation of metabolism and glucose homeostasis. In $\alpha_{2A}^{-/-}$ mice, HFD-induced obesity was ameliorated *via* increased energy expenditure, Epi-mediated lipolysis, WAT browning, and reduced fat absorption, and glucose homeostasis remained normal due to hyperinsulinemia. There is a possibility that increased insulin counteracted the maximum weight loss and promoted hepatosteatosis, but there is no evidence that insulin sensitivity was impaired. If anything, insulin sensitivity was improved and resistin levels were decreased in the $\alpha_{2A}^{-/-}$ mice. However, as the mice used in the current study were rather young, long-term effects of α_{2A}-AR depletion and complications of obesity remain to be determined. We also discovered a significant interaction between physical activity and α_{2A}-ARs, as even modestly increased physical activity levels combined with a lack of α_{2A}-AR signalling more efficiently promoted weight loss than exercise with normal α_{2A}-AR function. Our results from mice thus agree with the findings of human genetic studies associating reduced *ADRA2A* expression with decreased abdominal obesity, and increased *ADRA2A* expression with obesity and T2D. Our results may pave the way for the development of α_{2A}-AR-selective antagonists to constitute a strategy to treat obesity and T2D. However, it remains to be determined to which extent such effects may be achieved with merely blockade of peripheral α_{2A}-ARs, because the possible side effects from central α_{2A}-AR

antagonism (hypertension, anxiety, among others) may be intolerable. We also do not know how important the observed stress-induced hypophagia might be in the $\alpha_{2A}^{-/-}$ mice; this is an effect that is unlikely to be achieved with peripheral receptor antagonism. Peripheral α_{2A}-ARs are obviously directly involved in the regulation of glucose-induced insulin secretion, lipolysis, and regional sympathoadrenal tone, but is pharmacological modulation of this system sufficient to boost energy expenditure and glucose metabolism in order to achieve sustainable weight loss and satisfactory long-term blood glucose control? Perhaps such treatments should be targeted to genetically selected subjects that are most susceptible to such an intervention (rs553668 variant carriers), or they should be combined other forms of therapy, *e.g.* physical exercise.

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Figure legends

Figure 1. Effects of HFD (high fat diet) and α_{2A}-AR deficiency on body weight, food intake and adiposity.

(A) Body weight gain in wild-type (WT) and $\alpha_{2A}^{-/-}$ mice with access to one week of regular chow and 9 weeks of HFD.

(B) Weekly food intake of HFD in group-housed mice (consumption per cage divided by the number of animals). There was a significant main effect for genotype, $F(1, 7) = 11.08$, $p < 0.05$.

(C-D) Weekly food intake (C) or energy intake (D) of regular chow or HFD in single-housed mice. The start of HFD is marked with a dashed line.

(E) Average fat mass normalized to body weight, measured with EchoMRI-700 before HFD, and after 4 and 9 weeks on the diet. There was a significant main effect for genotype, $F(2, 30) = 122.5$, $p < 0.001$.

(F) Average lean body mass normalized to body weight, measured with EchoMRI-700 before HFD, and after 4 and 9 weeks on the diet. There was a significant main effect for genotype, F $F(2, 30) = 53.36$, $p < 0.001$.

(G) Dissected fat depot weights at five different sites after 9 weeks on HFD. Epididymal and subcutaneous weights represent unilateral depots.

□ WT and ■ $\alpha_{2A}^{-/-}$. Values are means ± SEM, n=8-9/group. Repeated measures 2-way ANOVA (A-F) or 1-way ANOVA (G): +++ = $p < 0.001$: overall effect of diet; * = $p < 0.05$ and ** = $p < 0.01$: overall effect of genotype.

Figure 2. $\alpha_{2A}^{-/-}$ Mice are protected from type 2 diabetes.

(A) Intraperitoneal glucose tolerance test (GTT, 1 g/kg, i.p.) in wild-type (WT) and $\alpha_{2A}^{-/-}$ mice on regular chow and HFD. The administration of glucose is marked with a dashed line. There was a significant main effect for genotype, $F(3, 27) = 200.6$, $p < 0.001$, and a significant interaction, $F(12, 108) = 8.561$, $p < 0.001$.

(B) Areas under the curve (AUC) for blood glucose values from GTT. There was a significant main effect for genotype, $F(1, 27) = 236.4$, $p < 0.001$, and a significant interaction, $F(1, 27) = 78.10$, $p < 0.001$.

(C) Fasted serum insulin concentrations in WT and $\alpha_{2A}^{-/-}$ mice before and after 9 weeks on HFD. There was a significant interaction, $F(1, 24) = 4.357$, $p < 0.05$.

Δ WT regular chow, ▲ $\alpha_{2A}^{-/-}$ regular chow, □ WT HFD, ■ $\alpha_{2A}^{-/-}$ HFD. White bars = regular chow; black bars = HFD. Values are means ± SEM, n=6-9/group. Repeated measures 2-way ANOVA (A, C) and regular 2-way ANOVA (B) with Bonferroni post-hoc comparisons: +++ = $p < 0.001$: overall effect of diet; *** = $p < 0.001$: overall effect of genotype; Post-hoc analyses: # = $p < 0.05$, ## = $p < 0.01$ and ### = $p < 0.001$: effect of diet; § = $p < 0.05$ compared with WT mice on the same diet.

Figure 3. Effects of HFD (high fat diet) and α_{2A}-AR deficiency on urinary catecholamine secretion.

(A) 24 h urinary norepinephrine (NE) secretion (normalised to creatinine) before and after 2 weeks on HFD in wild-type (WT) and $\alpha_{2A}^{-/-}$ mice. There was a significant interaction, $F(1, 35) = 10.23$, $p < 0.01$.

(B) 24 h urinary epinephrine secretion (normalised to creatinine) before and after 2 weeks on HFD in wild-type (WT) and $\alpha_{2A}^{-/-}$ mice. There was a significant main effect for genotype, $F(1, 17) = 24.47$, $p < 0.001$ and diet $F(1, 17) = 6.061$, $p < 0.05$.

White bars = regular chow; black bars = HFD. Values are means ± SEM, n=9-10 /group. Repeated measures 2-way ANOVA with Bonferroni post-hoc comparisons: + = $p < 0.05$: overall effect of diet; *** = $p < 0.001$: overall effect of genotype; Post-hoc analyses: ### = $p < 0.001$: effect of diet.

Figure 4. $\alpha_{2A}^{-/-}$ Mice show increased whole body energy consumption yet decreased activity levels.

(A) Average daytime (6 am – 6 pm) and night time (6 pm – 6 am) whole body energy expenditure levels normalised to lean body mass before and after 3 weeks on HFD in wild-type (WT) and $\alpha_{2A}^{-/-}$ mice. There was a significant main effect for genotype, $F(1, 40) = 1382$, $p < 0.001$, and a significant interaction, $F(3, 40) = 13.48$, $p < 0.001$.

(B) The respiratory exchange ratio (RER), a measure of metabolism substrate choice (carbohydrate/fat), expressed as a ratio of CO_2 production and O_2 consumption in WT and $\alpha_{2A}^{-/-}$ mice. There was a significant interaction, $F(3, 32) = 36.59$, $p < 0.001$.

(C) Average daytime (6 am – 6 pm) horizontal locomotor activity scores in WT and $\alpha_{2A}^{-/-}$ mice. There was a significant main effect for genotype, $F(1, 60) = 8.471$, $p < 0.001$, and diet, $F(1, 60) = 7.696$, $p < 0.01$.

(D) Average night time (6 pm – 6 am) vertical locomotor activity scores in WT and $\alpha_{2A}^{-/-}$ mice. There was a significant main effect for diet, $F(1, 60) = 11.07$, $p < 0.001$.

White bars = regular chow; black bars = HFD. Values are means ± SEM, n=4-6/group. Repeated measures 2-way ANOVA with Bonferroni post-hoc comparisons: ++ = $p < 0.01$: overall effect of diet; *** = $p < 0.001$: overall effect of genotype; Post-hoc analyses: # = $p < 0.05$, ## = $p < 0.01$ and ### = $p < 0.001$: effect of diet; §§§ = $p < 0.001$ compared with WT mice on the same diet.

Figure 5. α_{2A}-AR deficiency increases *Ucp1* expression in subcutaneous white adipose tissue (WAT), and causes changes in hypothalamic gene expression levels under high caloric conditions.

(A) Expression of *Ucp1*, *Ppargc1α* and *Adrb3* in the brown fat on regular chow or HFD (9 weeks) in wild-type (WT) and $\alpha_{2A}^{-/-}$ mice. *Ucp1*: a significant main effect for diet, $F(1, 27) = 32.33$, $p < 0.001$; *Ppargc1α*: a significant main effect for diet, $F(1, 26) = 36.7$, $p < 0.001$ and a significant interaction, $F(1, 26) = 4.667$, $p < 0.05$; *Adrb3*: a significant main effect for diet $F(1, 26) = 5.584$, $p < 0.05$.

(B) Unstimulated and 7.5 μM norepinephrine (NE)-stimulated brown adipocyte oxygen consumption, measured with a Clark electrode, after 3 weeks on HFD. There was a significant main effect for NE treatment, $F(1, 16) = 33.42$, $p < 0.001$, and a significant interaction, $F(1, 16) = 5.186$, $p < 0.05$.

(C) Expression of *Ucp1*, *Ppargc1α* and *Adrb3* in subcutaneous WAT on regular chow or HFD (9 weeks) in WT and $\alpha_{2A}^{-/-}$ mice. *Ucp1*: There was a significant main effect for genotype, $F(1, 19) = 5.625$, $p < 0.05$, and diet, $F(1, 19) = 6.715$, $p < 0.05$.

(D) Expression of *Npy*, *AgRP, Pomc* and *Crh* in the hypothalamus on regular chow or HFD (9 weeks) in WT and $\alpha_{2A}^{-/-}$ mice. *Npy*: a significant main effect for diet, $F(1, 25) = 62.56$, $p < 0.001$ and a significant interaction, $F(1, 25) = 4.323$, $p < 0.05$; *AgRP*: a significant main effect for diet, $F(1, 26) = 74.49$, $p < 0.001$ and a significant interaction, $F(1, 26) = 6.113$, $p < 0.05$; *Pomc*: a significant main effect for diet, $F(1, 25) = 96.11$, $p < 0.001$; *Crh*: a significant main effect for diet, $F(1, 26) = 42.45$, $p < 0.001$.

White bars = WT, black bars = $\alpha_{2A}^{-/-}$. Values are means ± SEM, n=5-8/group. Regular 2-way ANOVA (A, C, D) and repeated measures 2-way ANOVA (B) with Bonferroni post-hoc comparisons: + = $p < 0.05$ and +++ = $p < 0.001$: overall effect of diet (A, C, D) or NE-

treatment (B); * = $p < 0.05$: overall effect of genotype; Post-hoc analyses: § = $p < 0.05$ and §§ = $p < 0.01$: compared with WT mice on the same diet (A, D) or treatment (B).

Figure 6. Depletion of α_{2A}-ARs potentiates physical activity induced weight loss under high caloric conditions.

(A) Average weekly activity levels on regular chow in wild-type (WT) and $\alpha_{2A}^{-/-}$ mice. There was a significant interaction, $F(5, 60) = 3.098$, $p < 0.05$.

(B) Average weekly activity levels on Western diet (WD) in WT and $\alpha_{2A}^{-/-}$ mice. There was a significant main effect for genotype, $F(1, 19) = 18.48$, $p < 0.001$.

(C) Weight gain during 6-week running period on regular chow in WT and $\alpha_{2A}^{-/-}$ mice. During the first week, all animals had access to wheels (shaded area) after which the animals were divided into active and inactive groups. There was a significant interaction $F(18, 126) = 8.822$, $p < 0.001$.

(D) Weight gain during 6-week running period on WD in WT and $\alpha_{2A}^{-/-}$ mice with similar setting as in (C). There was a significant interaction $F(21, 238) = 12.54$, $p < 0.001$.

(E) Average fat mass normalized to body weight, measured with EchoMRI-700 in active and inactive control (ctrl) groups on regular chow or WD in WT and $\alpha_{2A}^{-/-}$ mice. Chow: a significant main effect for activity, $F(1, 22) = 22.45$, $p < 0.001$. WD: a significant main effect for activity, $F(1, 36) = 16.39$, $p < 0.001$, and genotype, $F(1, 36) = 10.88$, $p < 0.01$.

(F) Average lean body mass normalized to body weight, measured with EchoMRI-700 in active and inactive groups on regular chow or WD in WT and $\alpha_{2A}^{-/-}$ mice. Chow: a significant main effect for activity, $F(1, 22) = 16.6$, $p < 0.001$. WD: a significant main effect for activity, $F(1, 36) = 17.47$, $p < 0.001$, and genotype, $F(1, 36) = 12.53$, $p < 0.01$.

(G-H) WD-induced weight gain (G) and food consumption (H) before running in WT and $\alpha_{2A}^{-/-}$ mice. Mice were separated into individual cages after week 2 (dashed line), after which food consumption monitoring started. There was a significant interaction in weight gain: $F(6, 228) = 16.94$, $p < 0.001$, and in food consumption: $F(3, 114) = 8.422$, $p < 0.001$.

(I) Average weekly food consumption in active and inactive control groups on regular chow or WD in WT and $\alpha_{2A}^{-/-}$ mice. WD: a significant interaction, $F(1, 37) = 8.242$, $p < 0.01$.

(J-L) Serum insulin (J), resistin (K) and leptin (L) levels in active and inactive control groups on regular chow or WD in WT and $\alpha_{2A}^{-/-}$ mice. Insulin, chow: a significant main effect for genotype, $F(1, 20) = 6.619$, $p < 0.05$; Insulin, WD: a significant main effect for genotype, $F(1, 34) = 20.23$, $p < 0.001$; Resistin, chow: a significant main effect for activity, $F(1, 21) = 6.005$, $p < 0.05$, and genotype, $F(1, 21) = 11.61$, $p < 0.01$; Resistin, WD: a significant main effect for genotype, $F(1, 34) = 20.16$, $p < 0.001$; Leptin, chow: a significant main effect for activity, $F(1, 19) = 6.887$, $p < 0.05$; Leptin, WD: a significant interaction, $F(1, 34) = 4.488$, $p < 0.05$.

□ WT, active; ■ $\alpha_{2A}^{-/-}$, active (A-D, G-H); Δ WT, ctrl; ▲ $\alpha_{2A}^{-/-}$, ctrl (C-D); White bars = WT; black bars = $\alpha_{2A}^{-/-}$ (E-F, I-L). Values are means ± SEM, n=4-13/group. Repeated measures 2-way ANOVA (A-D, G-H) and regular 2-way ANOVA (E-F, I-L) with Bonferroni post-hoc comparisons: + = $p < 0.05$ and +++ = $p < 0.001$: overall effect of exercise; * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$: overall effect of genotype; Post-hoc analyses: # = $p < 0.05$, ## = $p < 0.01$ and ### = $p < 0.001$: effect of exercise; § = $p < 0.05$, §§ = $p < 0.01$ and §§§ = $p < 0.001$ compared with WT mice on the same diet.

Supplementary data

Figure S1. Diet-induced hepatosteatosis in wild-type (WT) and $\alpha_{2A}^{-/-}$ mice.

Representative images of WT (left) and $\alpha_{2A}^{-/-}$ (right) liver cryosections stained with oil-red-O for lipids after 9 weeks on HFD. Scale bar is 100 μm.

Figure S2. Overnight fasting is a strong inducer of hypothalamic *Npy* expression in $\alpha_{2A}^{-/-}$ mice under high caloric conditions.

Expression of *Npy*, *AgRP, Pomc* and *Crh* in the hypothalamus after 4h or 16h fast in WT and $\alpha_{2A}^{-/-}$ mice. *Npy*: a significant main effect for genotype, $F(1, 24) = 12.99$, $p < 0.01$, and fast, $F(1, 24) = 19.38$, $p < 0.001$; *AgRP*: a significant main effect for fast, $F(1, 24) = 142$, $p < 0.001$ and a significant interaction, $F(1, 24) = 7.328$, $p < 0.05$; *Pomc*: a significant main effect for fast, $F(1, 23) = 102.8$, $p < 0.001$ and a significant interaction, $F(1, 23) = 4.686$, $p < 0.05$; *Crh*: a significant main effect for fast, $F(1, 24) = 21.11$, $p < 0.001$.

White bars = WT, black bars = $\alpha_{2A}^{-/-}$. Values are means ± SEM, n=3-9/group. Regular 2-way ANOVA with Bonferroni post-hoc comparisons: +++ = $p < 0.001$: overall effect of fast; ** = $p < 0.01$: overall effect of genotype; Post-hoc analyses: §§ = $p < 0.01$ and §§§ = $p < 0.001$: compared with WT mice with the same length of fast.

Table 1. Concentrations of non-esterified fatty acids (NEFA) and triglycerides in serum.

	WT Control chow	WT HFD	$\alpha_{2A}^{-/-}$ Control chow	$\alpha_{2A}^{-/-}$ HFD
NEFAs (mmol/l)	0.39 ± 0.02	0.67 ± 0.05 ***	0.82 ± 0.10§§§	0.63 ± 0.03*
Triglycerides (mg/ml)	1.06 ± 0.10	0.38 ± 0.06	1.01 ± 0.10	0.54 ± 0.06

WT = wild-type; HFD = high fat diet. Values are means ± SEM, n=8/group. In NEFA, there was a significant diet x genotype interaction, $F(1, 14) = 27.53$, $p < 0.0001$. Post-hoc analyses of NEFA: * = $p < 0.05$ and *** = $p < 0.001$: effect of diet within genotype; §§§ = $p < 0.001$ compared to WT mice on the same diet. In triglycerides, there was a significant main effect for diet, $F(1, 14) = 62.78$, $p < 0.0001$.

Table 2. Isoproterenol- and epinephrine-stimulated lipolysis measured as glycerol release *in vivo*.

	Glycerol % of baseline		
Isoproterenol (10 μg/kg i.v.)	5 min	10 min	20 min
WT	83 ± 4	119 ± 9	116 ± 9
$\alpha_{2A}^{-/-}$	86 ± 13	125 ± 14	96 ± 12
Epinephrine (1 mg/kg i.v.)	5 min	10 min	20 min
WT	83 ± 14	116 ± 14	92 ± 12
$\alpha_{2A}^{-/-}$	142 ± 9	137 ± 21	120 ± 27

WT = wild-type. Values are means ± SEM, n=5-6/group. There was a significant main effect for time with isoproterenol $F(2, 20) = 21.17$, $p < 0.0001$ with no significant time x genotype interaction. In Epi, there was a significant main effect for genotype $F(2, 20) = 6.731$, $p < 0.05$ with no significant interactions.

Supplementary Table 1. Primer pairs used for quantitative real-time PCR (qPCR).

Target		Sequence (5' – 3')	Genebank Accession no.
Acox1	for	gcccaactgtgacttccatc	NM_001271898.1
	rev	gccaggactatcgcatgatt	
Cpt1	for	gctgtcaaagataccgtgagc	NM_013495.2
	rev	tctccctccttcatcagtgg	
Fas	for	gctgctgttggaagtcagc	NM_007987.2
	rev	agtgttcgttcctcggagtg	
Acc1	for	gcgtcgggtagatccagtt	NM_133360.2
	rev	ctcagtggggcttagctctg	
Srebp1c	for	gatgtgcgaactggacacag	NP_035610
	rev	catagggggcgtcaaacag	
Pck1	for	atgtgtgggcgatgacatt	NM_011044.3
	rev	aacccgttttctgggttgat	
Ucp1	for	ggcctctacgactcagtcca	NM_009463.3
	rev	taagccggctgagatcttgt	
Ppargc1a	for	tcgcaacatgctcaagccaaacca	NM_008904.2
	rev	agccggagactgggccgttt	
Adrb3	for	ggccctctctagttcccag	NM_013462.3
	rev	tagccatcaaacctgttgagc	
Npy	for	ccgctctgcgacactacat	NM_023456.3
	rev	tgtctcagggctggatctct	
Agrp	for	ctttcgcggaggtgctagat	NM_001271806.1
	rev	aggactcgtgcagccttacac	
Pomc	for	caagccggtgggcaagaaacg	NM_001278581.1
	rev	ctaatggccgctcgccttccag	
Crh	for	aagaatacttcctccgcctgg	NM_205769.3
	rev	gttgctgtgagcttgctgagc	

Figure 1

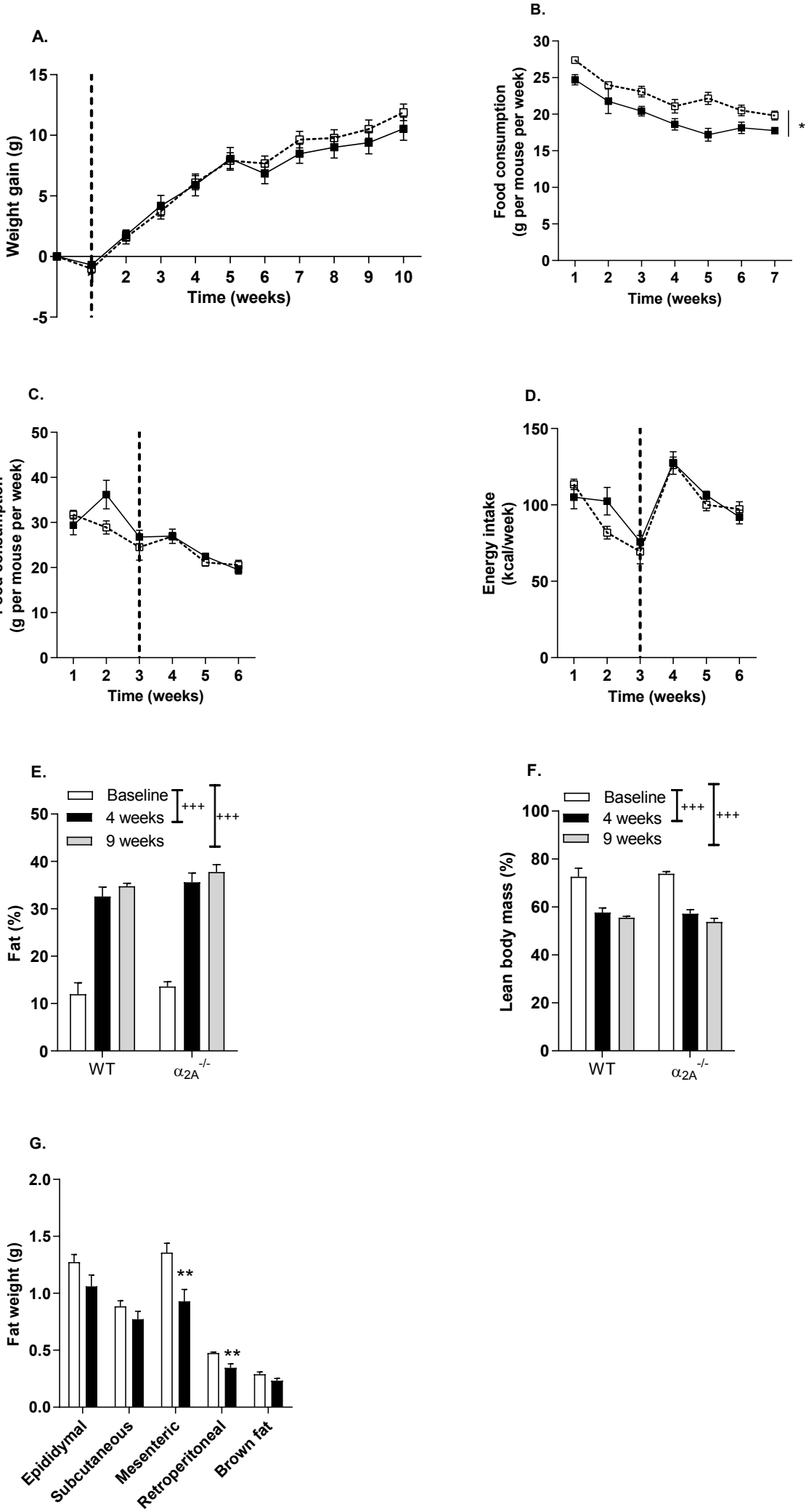
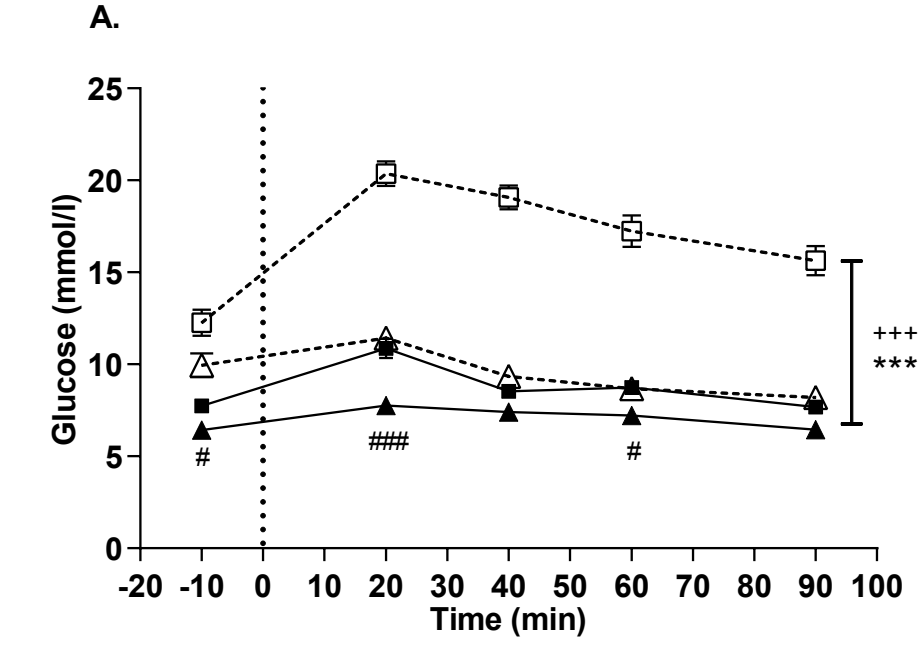
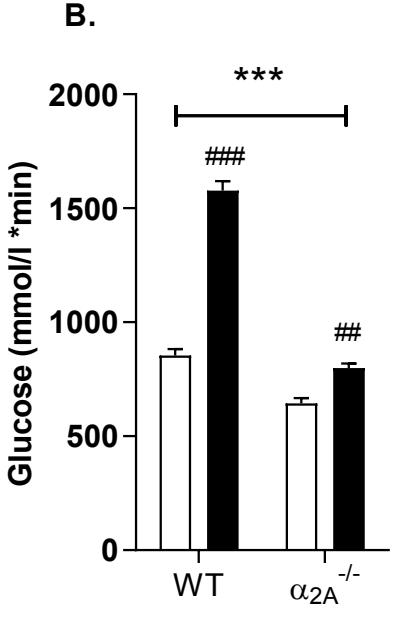

A.
Weight gain (g)
Time (weeks)
B.
Food consumption (g per mouse per week)
Time (weeks)
*
C.
Food consumption (g per mouse per week)
Time (weeks)
D.
Energy intake (kcal/week)
Time (weeks)
E.
Baseline
4 weeks
9 weeks
+++
+++
Fat (%)
WT
α2A-/-
F.
Baseline
4 weeks
9 weeks
+++
+++
Lean body mass (%)
WT
α2A-/-
G.
Fat weight (g)
**
**
Epididymal
Subcutaneous
Mesenteric
Retroperitoneal
Brown fat

Figure 2


A.
Glucose (mmol/l)
25
20
15
10
5
0
-20 -10 0 10 20 30 40 50 60 70 80 90 100
Time (min)
#
###
#
+++
***


B.
Glucose (mmol/l *min)
2000
1500
1000
500
0
***
###
##
WT
α2A-/-

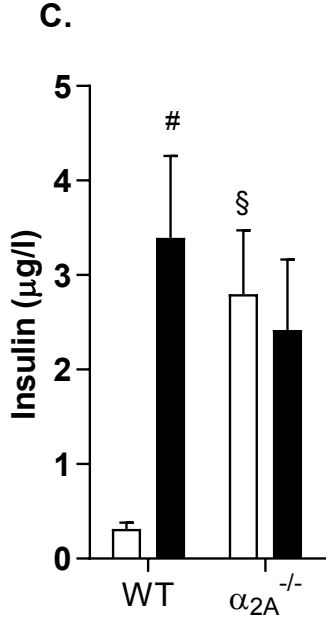

C.
Insulin (µg/l)
5
4
3
2
1
0
#
§
WT
α2A-/-

Figure 3

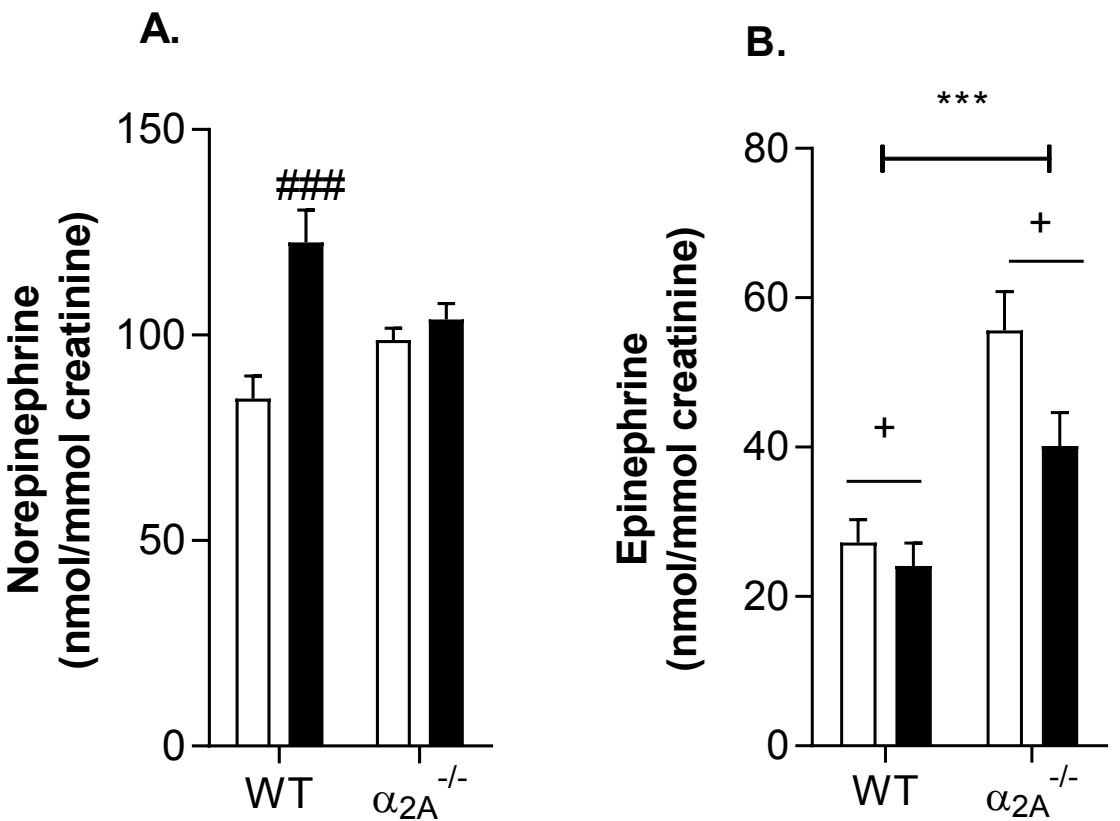

A.
150
100
50
0
###
Norepinephrine
(nmol/mmol creatinine)
WT
α2A -/-
B.
***
80
60
40
20
0
+
+
Epinephrine
(nmol/mmol creatinine)
WT
α2A -/-

Figure 4

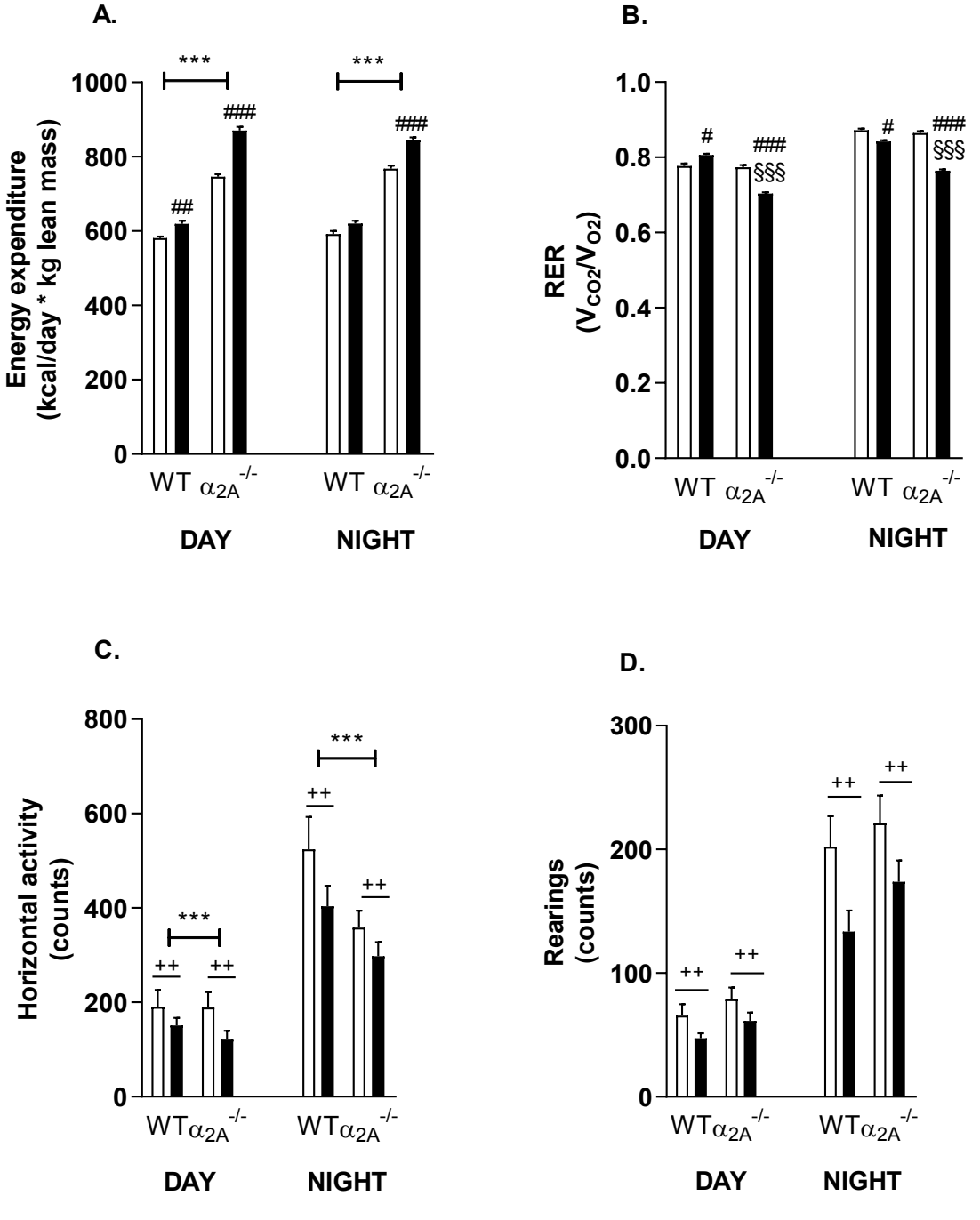
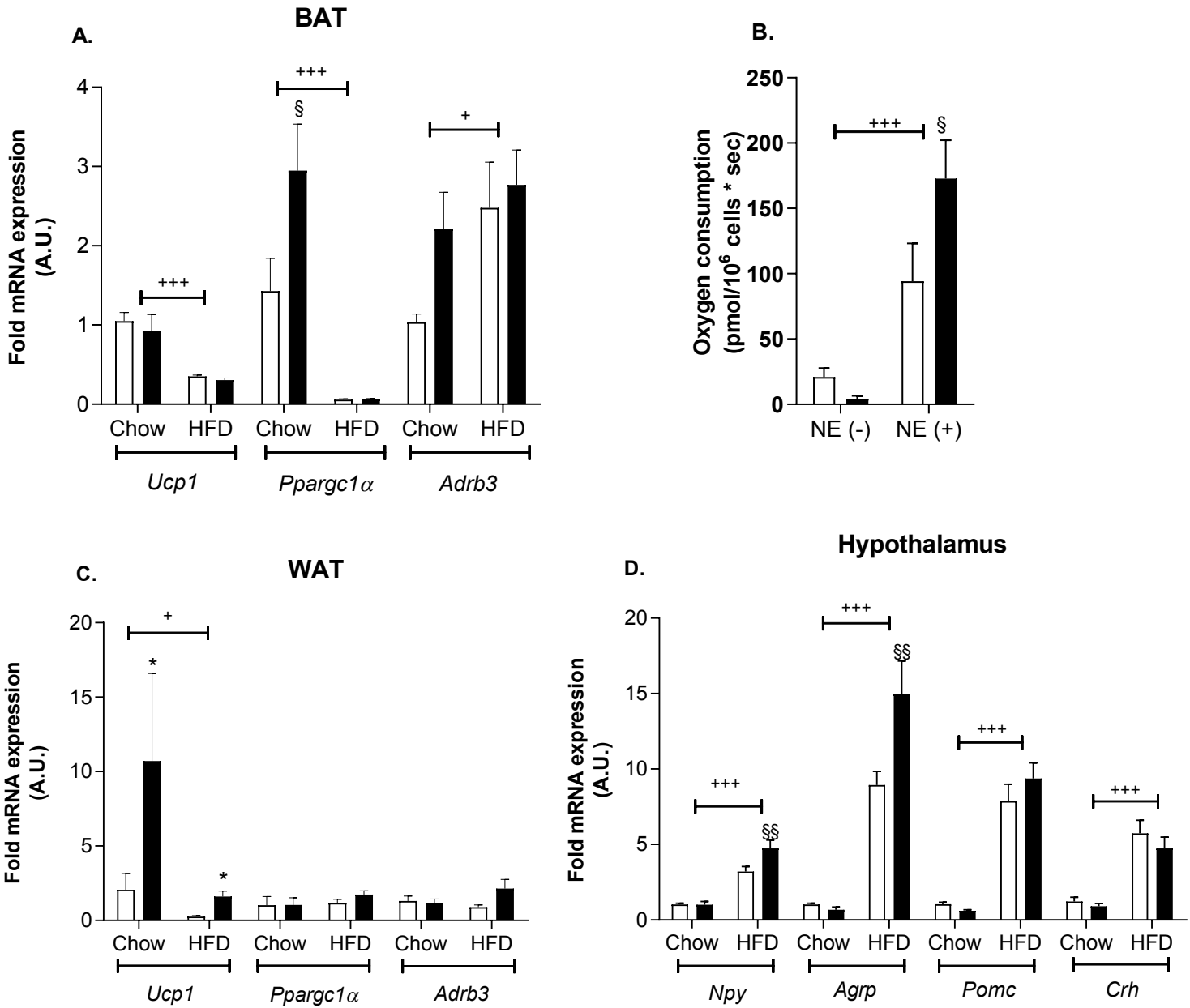

A.
Energy expenditure (kcal/day * kg lean mass)
1000
800
600
400
200
0
***
***
##
###
###
WT
α2A-/-
WT
α2A-/-
DAY
NIGHT
B.
RER (VCO2/VO2)
1.0
0.8
0.6
0.4
0.2
0.0
#
###
§§§
#
###
§§§
WT
α2A-/-
WT
α2A-/-
DAY
NIGHT
C.
Horizontal activity (counts)
800
600
400
200
0
***
***
++
++
++
++
WTα2A-/-
WTα2A-/-
DAY
NIGHT
D.
Rearings (counts)
300
200
100
0
++
++
++
++
WTα2A-/-
WTα2A-/-
DAY
NIGHT

Figure 5


BAT
A.
Fold mRNA expression (A.U.)
+++
§
+
+++
Chow HFD Chow HFD Chow HFD
Ucp1
Ppargc1α
Adrb3
B.
Oxygen consumption (pmol/10^6 cells * sec)
+++
§
NE (-) NE (+)
WAT
C.
Fold mRNA expression (A.U.)
+
*
*
Chow HFD Chow HFD Chow HFD
Ucp1
Ppargc1α
Adrb3
Hypothalamus
D.
Fold mRNA expression (A.U.)
+++
§§
+++
+++
§§
+++
Chow HFD Chow HFD Chow HFD Chow HFD
Npy
Agrp
Pomc
Crh

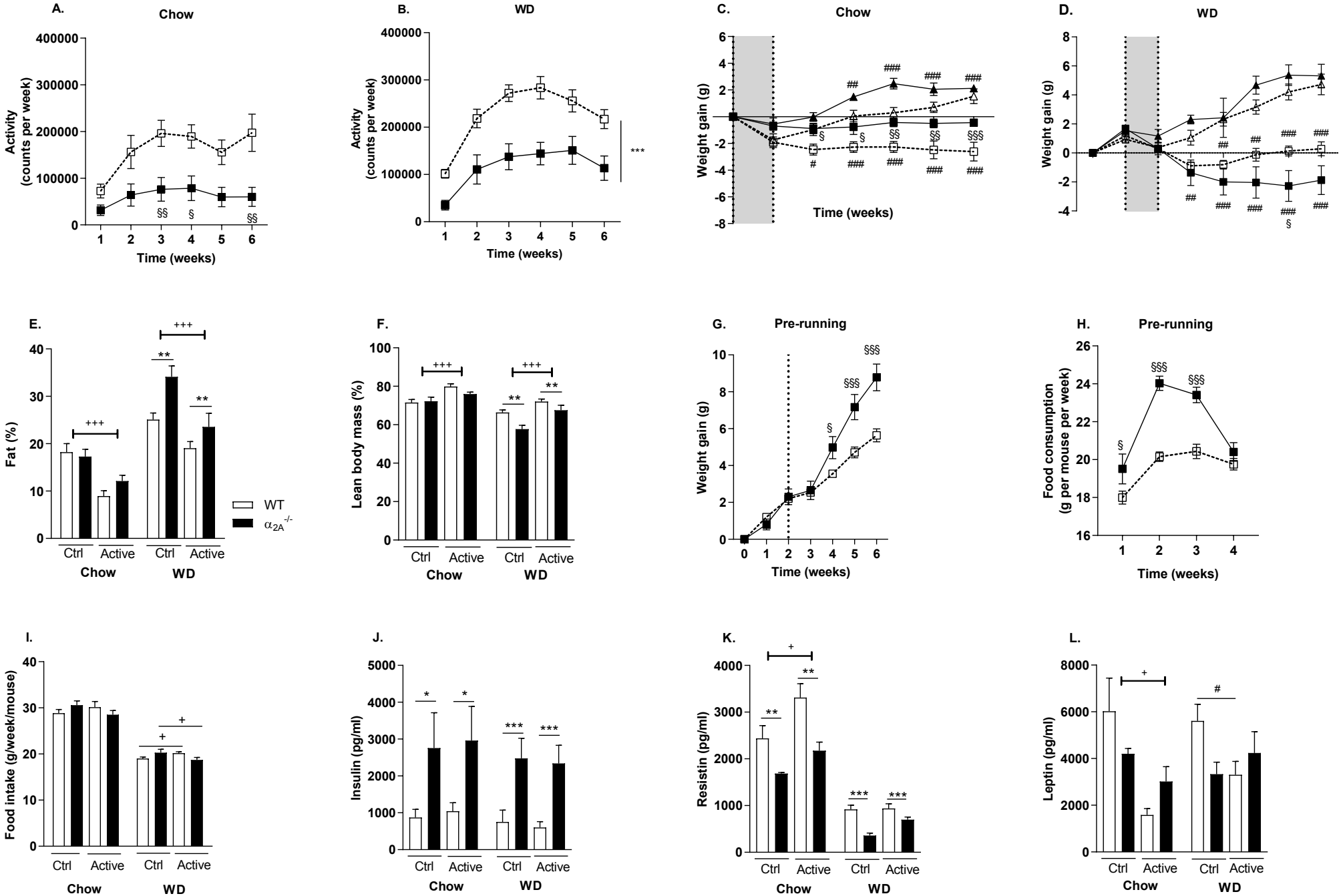
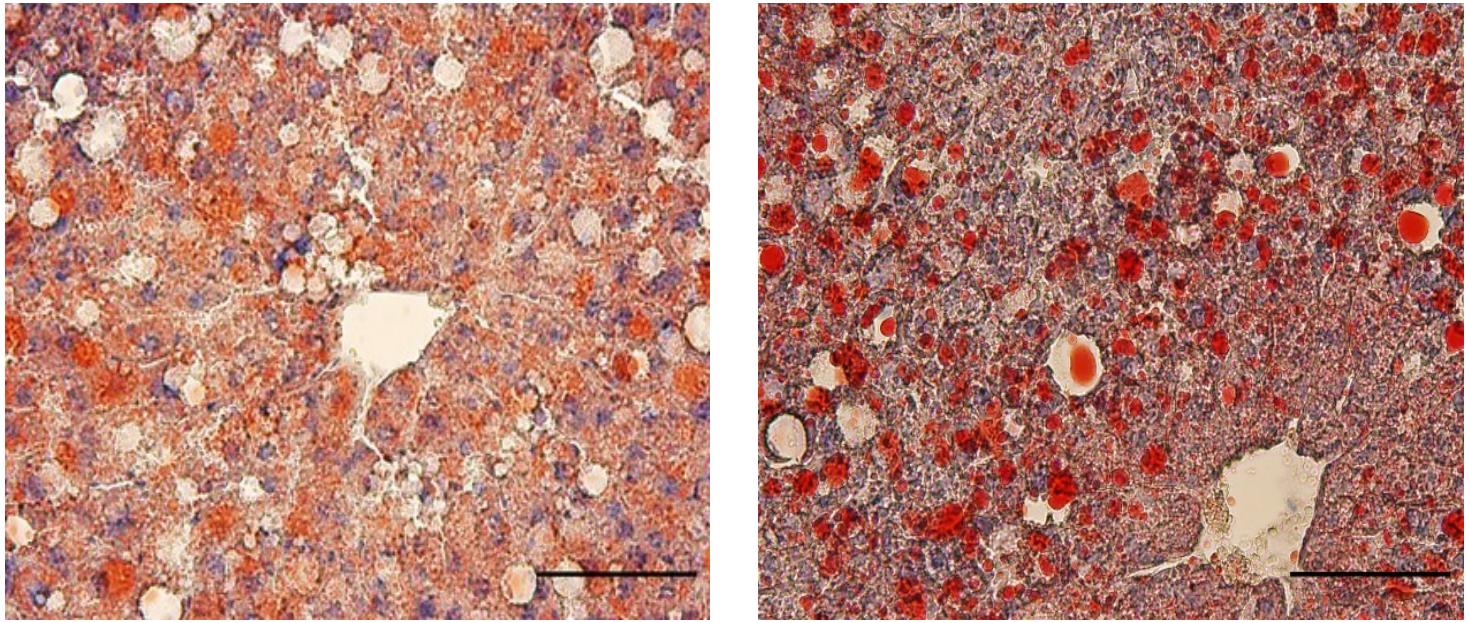

A.
Chow
Activity (counts per week)
Time (weeks)
B.
WD
Activity (counts per week)
Time (weeks)
C.
Chow
Weight gain (g)
Time (weeks)
D.
WD
Weight gain (g)
E.
Fat (%)
WT
α2A-/-
Ctrl
Active
Chow
WD
F.
Lean body mass (%)
G.
Pre-running
Weight gain (g)
Time (weeks)
H.
Pre-running
Food consumption (g per mouse per week)
Time (weeks)
I.
Food intake (g/week/mouse)
J.
Insulin (pg/ml)
K.
Resistin (pg/ml)
L.
Leptin (pg/ml)

Figure S1.



Supplementary Figure 2