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Supplemental Information

Selective Survival of Sim1/MC4R Neurons in Diet-Induced Obesity

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Supplementary Figures

Fig. S1

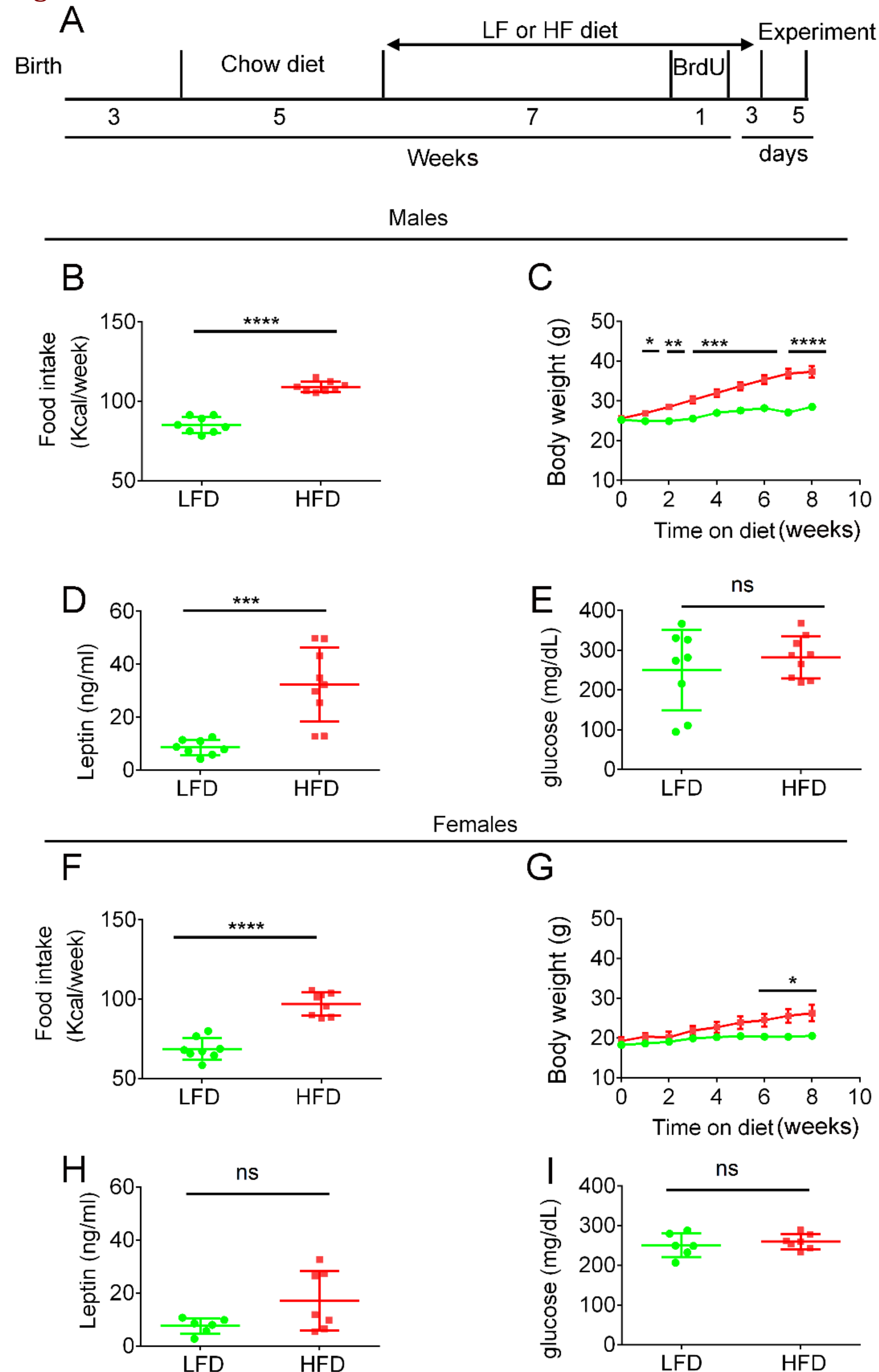

Figure S1. Related to Fig. 1. HF diet induces obesity in male and female Sapphire mice. (A) male and female 8-week-old mice were fed LF diet and high fat diet HF diet, respectively, for the following eight weeks. In the last week of diet treatment, all mice received one IP injection of BrdU, 50 mg/kg per day for seven days. (B-E) Weekly food intake per week, body weight, serum leptin, and blood glucose of male Sapphire mice treated with LFD, n = 8 and HFD, n = 9 mice. (F-I) Weekly food intake per week, weight, serum leptin, and blood glucose of female Sapphire mice treated with LFD, n = 6 and HFD diet, n = 7. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ****, $p < 0.0001$. B, D, E, F, H and I, data are represented as mean values +/- SD. C and G, data are represented as mean values +/- standard error of the mean (SEM).

Supplementary Figures
Fig. S2

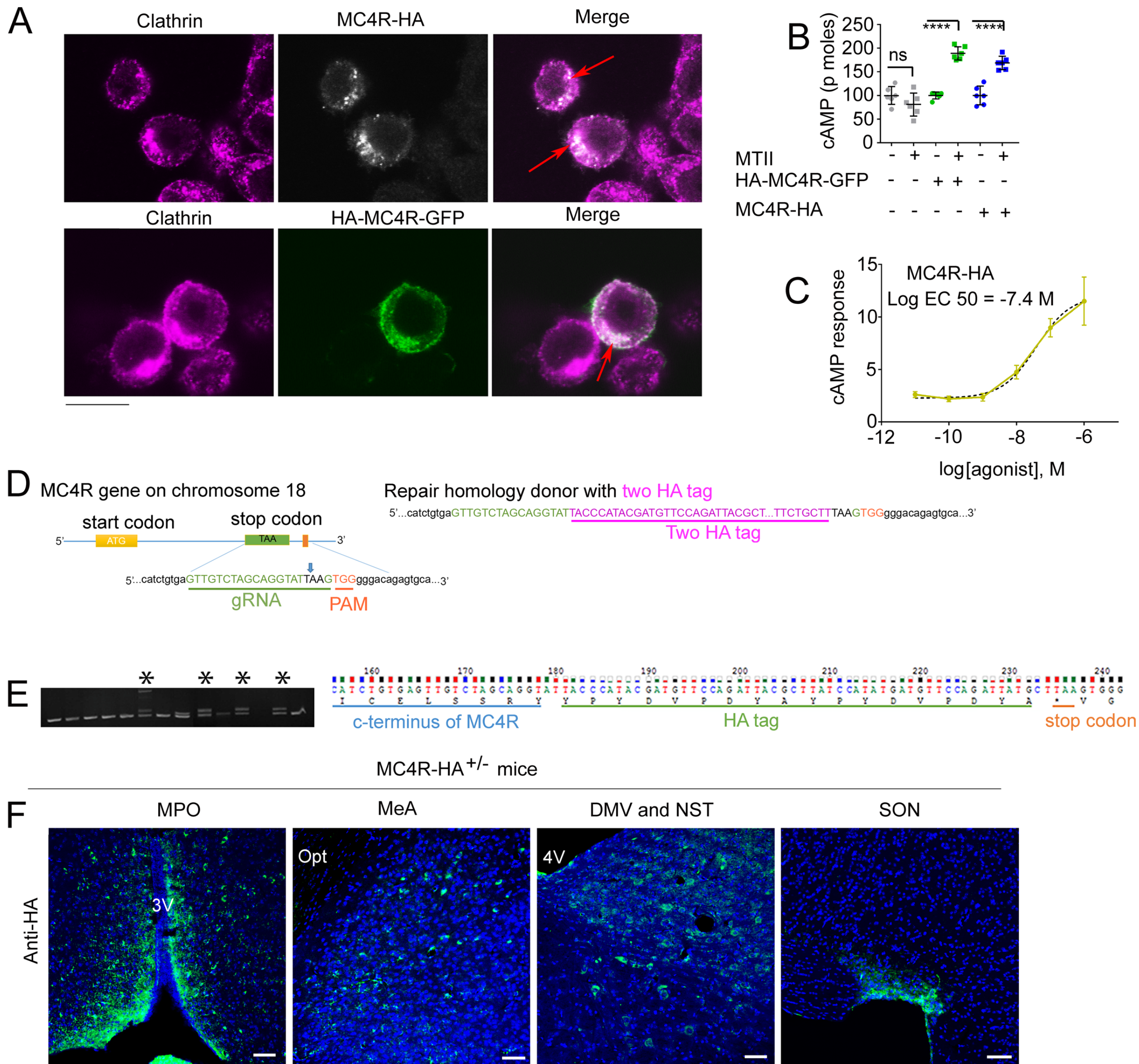

Figure S2. Related to Fig. 4. Knock-in MC4R-HA$^{+/-}$ mice express HA-tagged MC4R protein. (A) Confocal microscopy of Neuro2A cells expressing MC4R-HA and HA-MC4R-GFP immunostained with antibodies against HA and Clathrin. Red arrows indicate sites of MC4R-HA and HA-MC4R-GFP colocalization with clathrin. Scale bar, 10 μm. (B) Synthetic MC4R agonist, MTII, increases cAMP in Neuro2A cells expressing HA-MC4R-GFP and MC4R-HA, respectively. (C) Dose-response of MC4R-HA in Neuro2A cells. (D) Schematic of MC4R DNA coding region on chromosome 18 with guide RNA (gRNA) to target the 3' end of the MC4R gene and the repair homology donor with 2XHA tag (magenta). (E) DNA agarose gel analysis of MC4R PCR products. DNA from founder knock-in MC4R-HA mice has a higher molecular weight band due to insertion of 54 bp DNA encoding the 2XHA sequence (asterisks). Sequencing of the PCR products from founder mice indicates successful insertion of 2X HA sequence. (F) MC4R-HA is detectable in the medial preoptic area, MPO; Medial Amygdala, MeA; Dorsal Motor Nucleus of the Vagus, DMV; Nucleus of the Solitary Tract, NST; and Supraoptic Nucleus (SON) SON of the MC4R-HA$^{+/-}$ mouse. Scale bar, 60 μm.

Supplementary Figures
Fig. S3

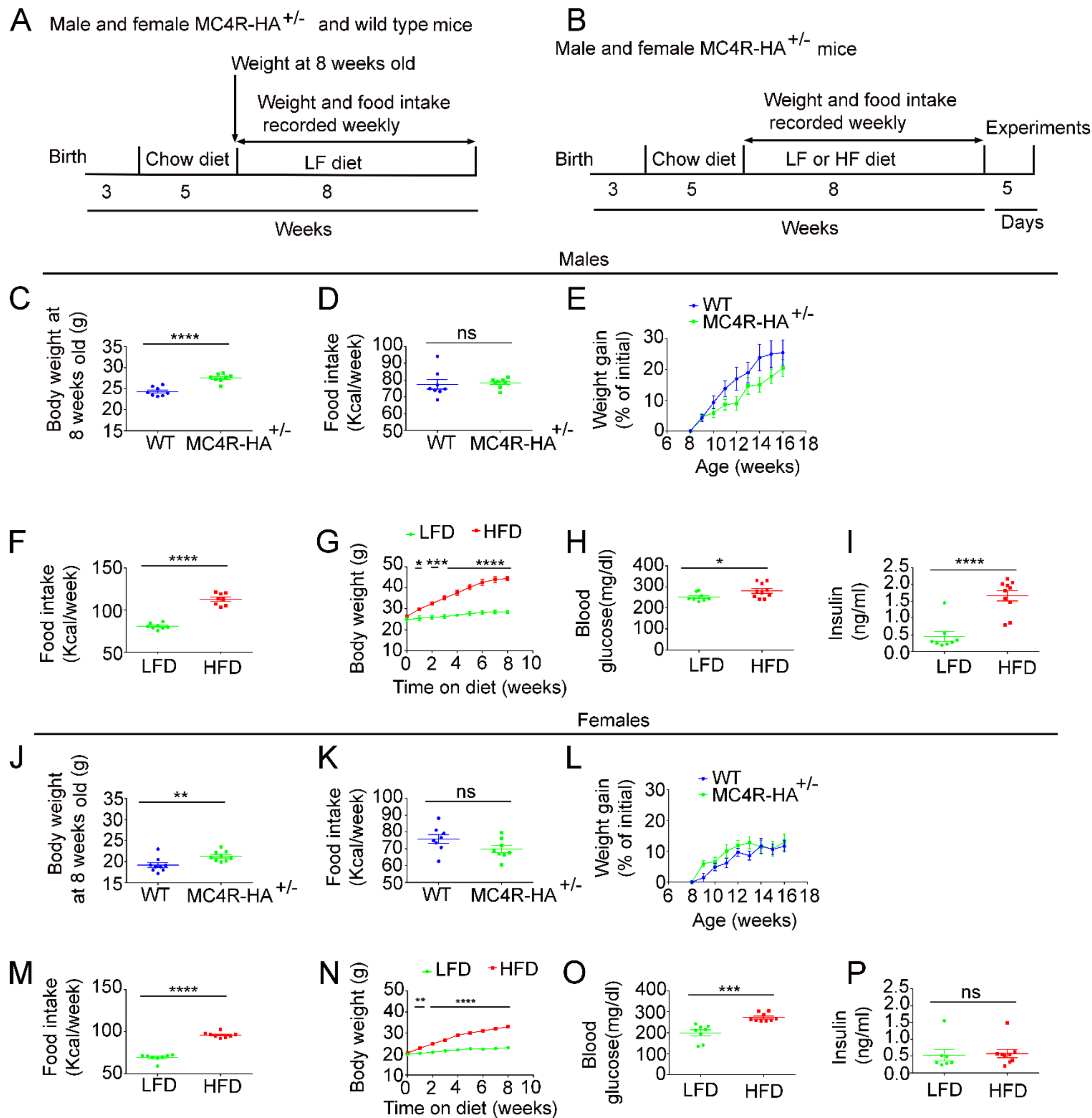

Figure S3. Related to Fig. 5. MC4R-HA$^{+/-}$ mice have similar weight gain and food intake as WT mice when fed a LF diet and develop obesity when exposed to HF diet. (A) Male and female 8 week-old wt and MC4R-HA$^{+/-}$ mice treated with LFD for 8 weeks. (B) Male and female MC4R-HA$^{+/-}$ mice treated with LFD and HFD diet, respectively for 8 weeks. (C-E) Body weight, food intake and weight gain of male WT mice (n= 8) and MC4R-HA$^{+/-}$ mice (n = 8) treated as in (A). (F-I) Food intake, bodyweight, blood glucose, and serum insulin of male MC4R-HA$^{+/-}$ mice treated as in B (LFD, n = 8 and HFD, n = 10). (J-L) Body weight, food intake and weight gain of female WT mice (n = 9) and MC4R-HA$^{+/-}$ mice (n = 9) treated as in (A). (M-P) Food intake, bodyweight, blood glucose, and serum insulin of female MC4R-HA$^{+/-}$ mice diet treated as in B (LFD, n = 7-9 and HFD, n = 9-10). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ****, $p < 0.0001$. C, D, F ,H , I , J , K , M, O and P, data are represented as mean values +/- SD. E, G, L and N, data are represented as mean values +/- standard error of the mean (SEM).

Transparent Methods

Reagents

Capillary tubes for retro-orbital bleeding (Cat. # 22-260943), Optimal Cutting Temperature Compound (OTC, Cat. # 4585), formaldehyde (Cat. # BP531-500), Corning Penicillin/Streptomycin 50X (Cat. # MT30001CI), Standard High-Profile Disposable Blades (Cat. # 10-015-93) Corning® 100-1000 µL Universal Fit Racked Pipet Tips (Cat. # 07-200-304), AccuTec Blades™ Personna Single-edge Prep Razor Blades (Cat. # 12-640-18), Corning Falcon™ Cell Strainers (Cat. # 08-771-2) and Fisherbrand™ Superfrost™ Plus Microscope Slides (Cat. # 22-037-246) were purchased from Fisher Scientific. Neurobasal™-A Medium, no D-glucose, no sodium pyruvate, Cat. # A2477501, dithiothreitol (DTT, Cat. # R0861) and B-27™ Supplement (50X) serum free (Cat. # 17504044) MitoTracker™ Red CMXRos (MitoTracker, Cat. # M7512), and Hank's Balanced Salt Solution (HBSS, Cat. # 14170112) were purchased from ThermoFisher Scientific. Seahorse XF 100 mM pyruvate solution (Cat. # 103578-100) Seahorse XF 1.0 M glucose solution (Cat. # 103577-100) were from Agilent. 0.01% Poly-D-Lysine solution (Cat # 3439-100-01) was from R&D Systems. Fetal Bovine Serum (FBS) (Cat. # F-0500-A) was purchased from Atlas Biologicals. Papain (Cat. # LS0031191) and Earle's Balanced Salt Solution (EBSS, Cat. # LK003188) were purchased from Worthington Biochemical Corporation. Glass Bottom Culture Dishes/Plates was from Nest Scientific USA Inc. (Cat. # 801002). Ultra-Sensitive Mouse Insulin Enzyme-Linked Immunosorbent Assay (ELISA) Kit (Cat. # 90080) and Leptin ELISA Kit (Cat. # 90030) were from Crystal Chem. Nile Red (Cat. # N3013), bovine serum albumin (BSA) (Cat. # A7511-10G) and DNase I (Cat. #11284932001) were from Sigma-Aldrich. Heparin (Cat. # P87721) was purchased from Braun Medical. Click-it Terminal deoxynucleotidyl transferase Nick End Labeling (TUNEL) Assay kit (Cat. # C10619), Tyramide SuperBoost Kit with Alexa Fluor 488 or Alexa Fluor 647 conjugated to Tyramide (Cat. # B40936), and ProLong Gold anti-fade reagent with 4, 6-diamidino-2-phenylindole (DAPI) (Cat. # P36935) were purchased from Molecular Probes. Donkey normal serum (Cat. # Ab7475), goat normal serum (Cat. # ab156046), recombinant mouse FGF2 protein (Cat. # ab50235) and 5-Bromo-2'-deoxyuridine (BrdU, Cat. # ab142567) were purchased from Abcam. Streptavidin/Biotin Blocking Kit (Cat. # SP2002) was purchased from Vector Laboratories. Surveyor nuclease was purchased from Integrated DNA Technologies (Cat. # 706020, Coralville, IA). QIAquick Gel Extraction Kit and DNeasy DNA extraction kit were from QIAGEN. Isofluorane vaporizer was purchased from VetEquip Inc. (Livermore, CA, and USA). Bayer Contour Glucose Test Strip (Cat. # 56-7080) and glucose meter (Cat. # 567189) were bought from Save Rite Medical.

Table 1. Antibodies

Antibody	Vendor, Catalog #	Host, clonality	Dilution
Anti-α-MSH	Abcam, Cat. # ab123811	Rabbit, polyclonal	1/1000
Anti-NeuN	Abcam, Cat. # Ab128886	Rabbit, polyclonal	1/1000
Anti-rabbit (Cy5)	Jackson Immunoresearch, Cat. # 711-175-152	Donkey, polyclonal	1/500
Biotin-SP-Affinity Pure Anti-Rat	Jackson Immunoresearch, Cat. # 112-065-143	Goat, polyclonal	1/200
Anti-GFP	Abcam, Cat. # Ab13970	Chicken, polyclonal	1/1000
Anti-Chicken (Alexa Fluor® 488)	Abcam, Cat. # ab150169	Goat, polyclonal	1/1000

Anti-GRP78	Abcam, Cat. # ab21685	Rabbit, polyclonal	1/300
Anti-PSD95	Abcam, Cat. #: ab18258	Rabbit, polyclonal	1/1000
Anti-HA	Roche, Cat. # 11867431001	Rat, monoclonal clone 3F10	1/500
Anti-BrdU	Rockland, Cat # 600-401-C29	Rabbit, polyclonal	1/1000
Anti-doublecortin	Abcam, Cat # Ab153668	Chicken, polyclonal	1/500
Anti-Cox IV	Abcam, COXIV Cat #ab16056	Rabbit polyclonal	1/1000
Anti-KDEL	Enzo, Cat# ADI-SPA-827	Mouse, monoclonal	1/1000

Animals

MC4R-HA+/- mice. CRISPR/Cas9 gene-editing technology was used to insert two copies of the human influenza hemagglutinin peptide (2XHA) at the C-terminus of the endogenous MC4R. The sequence of the DNA encoding a 20 nucleotides guide RNA (gRNA) targeting the 3'end of murine MC4R DNA coding region on chromosome 18 was designed using the C57BL/6 mouse MC4R DNA sequence obtained from https://www.ncbi.nlm.nih.gov/nuccore/NC_000084.6?report=genbank&from=66857705&to=66860487&strand=true. The DNA oligonucleotide encoding the gRNA was synthesized with added BbsI restriction site at the 5' overhang and subcloned into the BbsI site of pX-330 vector, which contains the scaffold RNA and the Cas9 nuclease according to the published protocol (Cong et al., 2013). To insert the 2XHA sequence, a single-stranded ultramer (CT*G*AGGAAAACTTTCAAAGAGATCATCTGTTTCTATCCTCTGGGAGGCATCTGTGAGTTGTCTAGCAGGTATTACCCATACGATGTTCCAGATTACGCTTATCCATATGATGTTCCAGATTATGCTTAAGTGGGGGACAGAGTGCAAACTAGGTAGATACCTGCAGACTTTGTCACTCTGGCCCGATCTGAGCAGTG*T*AC, where *denotes the phosphorothioate) containing the DNA encoding 2XHA (head to tail) flanked by 72 nucleotides homologous to the 3' region of MC4R locus was designed and ordered from Integrated DNA Technology (IDT). The ultramer and pX-330 plasmid containing the DNA encoding the gRNA injected into C57BL/6 zygotes by the UAMS Genetic Models Core. To genotype the founder mice, genomic DNA was extracted from tail snips by using the DNeasy DNA extraction kit following the manufacturer's instructions. The region corresponding to the 3' of MC4R genomic DNA was amplified by PCR using 5'- CTTTCTCCATTTACTGTTCTACATCTCTTGCC-3' as the forward primer and 5'-GTGACAAAGTCTGCAGGTATCTACCTAG-3' as the reverse primer. The PCR products were run on a 2% agarose gel. The insertion of 2XHA was indicated by the presence of a higher molecular weight band corresponding to the insertion of 54 bp encoding 2XHA. The gel containing the upper molecular weight band was excised, and the DNA was purified from the gel by using the QIAquick Gel Extraction Kit following the manufacturer's instructions. The purified PCR product was sequenced at the UAMS DNA sequencing facility. One male founder mouse harboring the desired sequence was crossed with C57BL/6J female mice to obtain a colony of MC4R-HA+/- mice and then a colony of MC4R-HA+/+ mice. MC4R-HA+/- mice used for the experiments are derived from crossing of mice from the MC4R-HA+/+ colony to mice from the C57BL/6J mice (Cat. # 000664, The Jackson Laboratory), which are kept as colony at UAMS vivarium.

Sapphire mice: Mice that express GFP under the MC4R promoter (MC4R-GFP transgenic mice, Cat. # JAX 008323) were purchased from The Jackson Laboratory (Liu et al., 2003).

Sim1-Cre $^{+/-}$: Rosa-mEGFP$^{+/+}$ mice: the mice were generated as previously described by breeding Sim1-Cre$^{+/-}$ mice (Tg(Sim1-Cre) 1 Low L/J mice Cat. # 006451, The Jackson Laboratory) expressing Cre-recombinase under the Sim1 promoter REF with Rosa-mEGFP$^{+/+}$ mice (Gt(ROSA-MEGFP)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J (Cat. # 007676, The Jackson Laboratory) (Muzumdar et al., 2007), to obtain a colony of Sim1-Cre $^{+/-}$: Rosa-mEGFP$^{+/+}$ mice.

Sim1-Cre $^{+/-}$: Rosa-mEGFP$^{+/+}$: MC4R$^{+/-}$ mice

Sim1-Cre $^{+/-}$: Rosa-mEGFP$^{+/+}$: MC4R$^{+/+}$ mice were obtained by first crossing MC4R-HA$^{+/+}$ mice with Rosa-mEGFP$^{+/-}$ to obtain Rosa-mEGFP$^{+/+}$: MC4R$^{+/+}$ and then by crossing these mice with with Sim1-Cre $^{+/-}$ mice.

Animal care and diet

All mice were bred, housed, and conditioned with low fat (LF) and HF diets in the University of Arkansas for Medical Sciences (UAMS) vivarium. Mice were housed in a temperature-controlled environment with 12h: 12h light-dark cycle, "lights on" at 0600 h, and "lights off" at 1800 h and given *ad libitum* access to food and water. Each shoebox cage housed 2-5 mice and had the addition of one "mouse hut" per cage for enrichment. UAMS veterinarians checked on the mice daily to monitor their health. Mice pups were genotyped at day 19-21 of age. The DNA used for genotyping was extracted from a small piece of tail (<2 mm). Mice were weaned at 21 days of age. No randomization or blinding methods were used to assign mice to different experimental groups. Eight-week-old mice were fed in parallel either with D12450H LF diet (3.82 kcal/g, fat = 10 kcal %) from Research Diets, Inc. or with the custom HF diet D15012001 (4.70 kcal/g, fat = 45 kcal %) from Research Diets, Inc. as described previously (Nyamugenda et al., 2019) and Table 2. Bodyweight and food intake were recorded every week. The UAMS Institutional Animal Care and Use Committee (IACUC) approved the protocol used for the animal studies (AUP FILE approval #: 3788).

BrdU injection

For experiments involving BrdU injections, each mouse received an intraperitoneal injection of BrdU solution (50 mg/kg body weight) by using a 500 µl BD insulin syringe equipped with the BD Micro-Fine IV Needle (U-100, 0.35 mm (28G) x 12.7 mm) (1/2)). Each mouse was injected once per day at 3 PM for seven days.

Food intake

Male and female mice on LF and HF diets were housed 2-5 mice per cage with *ad libitum* access to food and water. Three to four cages were used for each group. Food intake per cage was measured every week by subtracting the grams of the food remaining in the cage from the grams of food left in the cage the previous week. On average, 25 g of food per mouse was left in the cage every week to ensure that mice do not run out of food. The average weekly food intake per mouse was calculated by dividing the amount of food eaten per cage (in grams) by the number of mice in the cage. To calculate caloric intake per mouse, the amount of food in grams was multiplied by the amount of kcal/g of food

Table 2. Low fat diet (LFD) and high fat diet (HFD)diet.

Mouse diet	D12450H	D15012001
	10 % LF diet	45% HF diet
Caloric Information		
Protein:	20% kcal	20% kcal
Carbohydrate:	70% kcal	35% kcal
Fat:	10% kcal	45% kcal
Energy Density:	3.82 kcal/g	4.7 kcal/g

Diet ingredients		
Casein	200.00 g	200.00 g
L-Cystine,	3.00 g	3.00 g
Corn Starch	452.20 g	72.8 g
Sucrose	176.80 g	172.80 g
Maltodextrin	75.00 g	100 g
Cellulose	50.00 g	50.00 g
Soybean Oil, USP	25.00 g	25.00 g
Lard	20.00 g	20.00 g
Palm Oil	0.00 g	157.5 g
Fatty acid profile		
Saturated (g)	10.1 g	80.2 g
Monounsaturated (g)	12.8 g	79.1 g
Polyunsaturated (g)	20.2 g	41 g
Total fat	43.1 g	200.3 g

Blood glucose

One end of the Bayer contour glucose test strip was inserted into the glucose meter. A drop of blood was collected on the other end of the strip, and the reading of the glucose meter was recorded. The process was repeated for a total of three readings per mouse. The data are reported as the average of the three readings.

Collection of blood from the retro-orbital sinus to measure leptin and insulin

Mice were brought to the laboratory from the UAMS vivarium for fasting and euthanasia. Mice were deprived of food for three hours before being euthanized. Each mouse was deeply anesthetized in the induction chamber connected to the isofluorane vaporizer. The oxygen flow meter was set to 1.5 liters/min, and the isofluorane vaporizer dial was set at the maximal level of 5%. When the mouse was laying on its side and breathing rhythmically, it was removed from the chamber. Deep anesthesia was monitored by paw pinch, tail pinch, and eye blink tests. Approximately 0.2 ml of blood was collected from the retro-orbital sinus using a capillary inserted at the medial canthus of the eye under the nictitating membrane, and the animal was immediately returned to the induction chamber connected to the isofluorane vaporizer.

Mouse fixation

Each deeply anesthesized mouse was removed from the induction chamber and placed on a tray with its face in the nose cone of a circuit to maintain the isoflurane/oxygen administration. Deep anesthesia was again monitored by paw pinch, tail pinch, and eye blink tests. The heart was exposed following an established protocol (Gage et al., 2012), and the mouse was perfused through the heart's left ventricle with heparinized saline (0.9% NaCl containing two units of heparin/ ml at a rate of 3-4 ml/min) for 30 min, and then with 4% formaldehyde in PBS, pH 7.4 for another 30 minutes. The brain for each mouse was harvested and post-fixed in PBS containing 4% formaldehyde for 48 hours at room temperature. The brain was washed with PBS and stored in a 20 ml scintillation vial containing PBS, 30% sucrose, and 0.01% sodium azide. The brain was kept at 4° C until sank at the bottom before slicing it into 30 µm coronal sections.

Brain Sections

When the brain was at the bottom of the vial containing 30% sucrose solution, it was sliced into 30 µm coronal sections using a cryostat (Microm HM 550, Thermo Scientific). First, the cerebellum and the prefrontal cortex were removed using a razor blade. The brain was then embedded in the clear Optimal Cutting Temperature Compound (OTC) on the cutting block and put in the cryostat chamber (-20°C) until the OTC medium turns

solid white (about 15-20 minutes). A Standard High-Profile Disposable Blade was inserted in the cryostat's blade holder, and the cryostat was set to 30 µm "fine cutting." The frozen brain on the cutting block was inserted in the cryostat's sample holder for cutting. The brain sections were collected in serial order and stored in a 24 well plate containing PBS and 0.01% Sodium azide at 4°C.

Selection of the brain sections used in the study

Serial adjacent brain sections, including the PVN, the nucleus of the lateral olfactory tract (LOT), Supraoptic nucleus (SON), (Bregma -0.70 to -1.06 mm, and the arcuate nucleus (Bregma -1.22 to -1.94 mm) were used in the study. Sections of male mice treated with LF and HF diets were immunostained at the same time. The same protocol of staining was used for female mice. Male and female mice experiments were not carried out in parallel.

Immunostaining

For all the staining steps, sections were floating in a 24-well plate shaking on a plate shaker set at 200 rounds per minute (rpm) unless noted otherwise. Each well contained one section. From each step to the next, the section was transferred from one well to another using a small paintbrush. Each section was incubated with 1 ml of 0.5% Triton x-100 in PBS for 1h at room temperature (permeabilization step). Each section was then incubated for 1h at room temperature in 500 µl of PBS containing 0.1% Triton x-100 (PBST) and 10% of normal serum from the species where the secondary antibody was raised. Each sample was incubated for 48 hours at 4°C with 300 µl of the primary antibody diluted in PBST containing 1% BSA. Each section was washed four times for 10 minutes with 1 ml of PBS at room temperature and then incubated with 300 µl of the secondary antibody diluted in PBST and 1% BSA (PBST/BSA) overnight at 4°C. Each section was washed four times with PBS for 10 minutes each at room temperature and counterstained using 1 ml of 300 nM DAPI for 15 minutes at room temperature in the dark. Each tissue was washed three times for 5 minutes each and transferred to gelatin-coated microscope slides. Tissues on microscope slides were dried in the dark for 15 minutes before adding 20 µl of 1,4-diazabicyclo [2.2.2]octane (DABCO) mounting medium (40 ml containing 100 mg DABCO dissolved in 10 ml PBS with the addition of 30 ml glycerol). The coverslip was sealed using nail polish and left to dry in the dark.

Detection of MC4R-HA in brain sections

Each brain section was incubated with 1 ml of 0.5% Triton X-100 in PBS for 1 hour at room temperature on a 24-well plate on a plate shaker set at 200 rpm. Then sections were transferred to a well containing three drops of 2% hydrogen peroxide (using the dispensing bottle from the Tyramide SuperBoost™ Kit. The plate was tilted on one side to make sure that sections were covered entirely with the solution and incubated without shaking for 1 hour at room temperature. Each section was then washed three times with 1 ml PBS for 10 minutes at room temperature on the plate shaker set at 200 rpm. Nonspecific binding was blocked by incubating each section for 1 hour at room temperature in blocking buffer (500 µl of 10% normal goat serum in PBST) on the plate shaker set to 200 rpm. To block endogenous biotin each section was transferred to a well containing the "Streptavidin Solution" from the Streptavidin/Biotin Blocking Kit (3 drops using the drop bottle). Sections were incubated for 30 minutes at room temperature without shaking. Sections were washed 3 times with PBS. Sections were transferred to a well containing and the "Biotin Solution" from the Streptavidin/Biotin Blocking Kit (3 drops using the drop bottle) for 30 minutes at room temperature without shaking. Each section was washed three times with 1 ml of PBS for 10 minutes each on the plate shaker set at 200 rpm. Each section was then incubated with 300 µl of rat monoclonal anti-HA High-Affinity primary antibody diluted 1/500 in PBST/BSA) for 48 hours at 4° on the plate shaker set at 200 rpm. Each section was washed four times with 1 ml of PBS for 10 minutes each on the plate shaker on the plate shaker set at 200 rpm at room temperature. Each section was incubated overnight at 4° with 300 µl of Biotin-SP-Affinity Pure Goat Anti-Rat IgG secondary antibody diluted in PBST/BSA on the plate shaker set at 200 rpm. Each section was

washed four times for 10 minutes each with 1 ml of PBS at room temperature on the plate shaker set at 200 rpm. Sections were individually transferred to a well containing three drops of streptavidin conjugated to HRP from the Tyramide SuperBoost™ Kit. Sections were incubated for 1 hour at room temperature without shaking. Each section was washed four times with 1ml PBS for 10 minutes each at room temperature on the plate shaker set at 200 rpm. During the last wash, the following solutions were prepared following the Tyramide SuperBoost protocol: 1) 100 X H_2O_2 solution, by adding one drop of 3% H_2O_2 to 1 ml of distilled water; 2) 1 X Reaction buffer, by adding one drop of 20X Reaction buffer to 1 ml dH_2O. The 100 x Tyramide stock solution using Tyramide SuperBoost™ Kits with Alexa Fluor 488 (or with Alexa Fluor 647) was prepared in DMSO from Tyramide SuperBoost™ Kit following manufacturer instruction. Then, the tyramide working solution was prepared according to the following table.

Tyramide working solution Components	Number of wells (1 section per well)				
	2	4	6	8	10
100X Tyramide stock solution	2.5 µl	5 µl	7.5 µl	10 µl	12.5 µl
100X H_2O_2 solution	5 µl	10 µl	15 µl	20 µl	25 µl
1X Reaction buffer: from 2	500 µl	1000 µl	1500 µl	2000 µl	2500 µl

The Reaction stop reagent provided in the Tyramide SuperBoost™ Kit was prepared by diluting the stock solution 1:11 in PBS (100 µl of the reagent in 1ml of PBS). Each brain section was individually transferred to a well containing 250 µl tyramide working solution. Care was taken to make sure that the section lays flat and is covered with the solution. The section is incubated individually for 6 minutes without shaking. Then 250 µl of the diluted Reaction stop reagent was added to the well to stop the reaction. The procedure was repeated for every section. Sections were then washed four times with 1 ml PBS for 5 minutes, each while shaking on the plate shaker at 200 pm. Tissues were transferred to gelatin-coated microscope slides and dried in the dark before adding 20 µl DABCO to each section. The coverslip was mounted and sealed with nail polish.

BrdU staining of brain sections.

Each brain section was incubated with 1 ml of 0.5% TritonX-100 in 1X PBS for an hour at room temperature. Each section was then incubated in 1 ml of 2 N HCl at 37 °C for 30 min. The section was incubated with 1 ml of 0.1 M boric acid (pH 8.5) at room temperature for 20, followed by three washes with 1 ml PBS for 5 minutes each at room temperature. Each section was blocked in 500 µl of 10% donkey normal serum for an hour at room temperature followed by incubation with 300 µl of rabbit anti-BrdU primary antibody diluted 1:1000 in PBS containing 0.1 mg/ml ovalbumin, 0.1% TritonX-100, and 0.01% Na azide for 48 hours at 4°C. The section was washed four times with 1 ml PBS for 10 minutes each and incubated with 300 µl of donkey anti-rabbit secondary antibody conjugated to Cy5 overnight at 4°C and then washed four times with 1 ml PBS for 10 minutes each at room temperature. Each section was counterstained with 1 ml of 300 nM DAPI for 15 minutes and then washed three times with 1 ml of PBS at room temperature. Each section was transferred to a gelatin-coated microscope slide and dried in the dark before adding the mounting medium and coverslip. All incubations were carried out on a plate shaker set at 200 rpm. Sections were imaged using Olympus Fluoview FV1000 microscope.

Nile red staining of liver sections

Livers were harvested from the same animals immediately after the brain. After 48 hours post-fixation in PBS containing 4% formaldehyde, each liver was washed 3 times with PBS and stored in PBS containing 30% sucrose and 0.01% sodium azide in a 50 ml conical tube and kept at 4° C for the following 48 hours, when the tissue sank to the bottom of the tube (some livers obtained from mice exposed to HF diet do not sink in 30%

sucrose solution). The livers were sectioned into 30 μm sections using the same protocol used for sectioning the brain. Each free-floating liver section was permeabilized using 1ml of 0.5% TritonX-100 in PBS for an hour at room temperature on a plate shaker set at 200 rpm. Each liver section was then incubated with 1 ml of 0.5 μM Nile Red for an hour at room temperature on a plate shaker set at 200 rpm followed by four washes with 1 ml of PBS for 10 minutes each. Each liver section was incubated with 1 ml of 300 nM DAPI for 15 minutes washed three times with 1 ml PBS for 5 minutes each. Each liver section was then transferred to a gelatin-coated microscope slide dried in the dark before adding the DABCO mounting medium and the coverslip. Sections were imaged using Olympus Fluoview FV1000 microscope.

Confocal microscopy

Images of the brain and liver sections were taken using a confocal microscope (Olympus Fluoview FV1000) equipped with 20 X/0.85 N.A Plan Apochromatic oil objective. Images were collected as a Z-stack of 8 optical slices of 3.0 μm thickness. Each Z-stack was converted into a two-dimensional maximum intensity projection image (MIP) by using the Olympus software. Higher magnification images were taken with the 60X/1.42 N.A Plan Apochromatic oil objective. All images of the same experiment were obtained with identical acquisition parameters.

Super-resolution microscopy

Images for analyzing the size of mitochondria and mitochondrial coverage area were taken using an inverted Zeiss ELYRA PS1 super-resolution microscope. Images were collected as a series of Z-stack of 12 optical slices using an alpha Plan-Apochromatic 100X/1.46 oil objective. The GFP in Sim1/MC4R neurons was detected by using a 488 nm laser, and COXIV was detected using a 642 nm laser. The 12 slice Z-stack was collected in Zeiss Zen Black software set to 3 grating rotations. Each optical slice was 2 μm resulting in z depth of 24 μm. For experiments that required imaging on different days, acquisition parameters were reloaded to ensure consistency. Images were processed using a proprietary structured illumination in Zen Black software. Structured illumination images were converted into 2D MIP images in XY projection and exported as TIFF files using Zeiss Zen Blue software.

Preparation of primary hypothalamic neurons and treatment with palmitate.

Primary cultures of hypothalamic neurons were prepared from 4 to 6-week old C57 and Sim1:Rosa mice fed ad libitum prior to sacrifice. Male and female mice (n = 6-8) were killed by CO_2 asphyxiation. All solutions, sterilized by filtration through 0.22 were equilibrated with 95% O2, 5% CO2. All glasswares, plasticwares and instruments are sterile. Brains were extracted and individually placed into 0.5 ml per well ice-cold Neurobasal Medium containing Neurobasal, pyruvate 0.23 mM, glucose 5 mM, and Penicillin, 100 IU, Streptomycin 100 μg/mL. For each brain, 1 brain section of 2 mm was cut using a Stoelting stainless steel brain matrix, with one single-edge blade placed anterior to the optic chiasm and the other blade placed anterior to the pons. From the coronal slice, a tissue rectangle of 2 mm width and 3 mm height centered around the entire 3rd ventricle to include the mediobasal hypothalamus and the PVN was dissected, the section was further cut by using single-edge blades into cubes of approximately 0.2 mm sides. The minced tissue was placed in an individual tissue culture well containing 0.5 ml EBSS with papain 20 U/ml, 1 mM EDTA, and 1mM DTT. The mixture was incubated in a tissue culture cabinet equilibrated with 95% air and 5% CO_2 for 15 min at 37 °C under agitation (OrbiShaker™ MP Orbital Microplate Shaker, 500 rpm), then DNAase was added from 1% w/v stock solution in EBSS to reach a final concentration of 0.005% w/v, and the mixture was further incubated in the tissue culture cabinet for other 15 min at 37 °C under continuous agitation. After digestion, the minced brain tissue in each well was triturated pipetting five times through a 1 ml pipet tip, pooled together, and filtered though a Corning Falcon™ Cell Strainer with 70 μm pores. The filtered cells were diluted to 6-8 ml by adding Neurobasal Medium and 6-8 aliquots of 1 ml were centrifuged in Eppendorf tubes placed on empty 10ml Falcon tunes to fit holders of an IEC clinical centrifuge and sample are spun for 2 min at 1000 RPM. Each pellet

was gently resuspended in 0.5 ml of Neurobasal Medium by pipetting five times through a 1ml pipet tip and transferred to another Eppendorf test tube containing 0.5 ml Neurobasal Medium with 10% FBS. Samples are centrifuged in a IEC clinical centrifuge for 2 min at 1000 RPM and each pellet was gently re-suspended in 0.3 ml of Hypothalamic Neuron Culture Medium containing Neurobasal Medium, 10% FBS, B27 diluted 1:50, 5 nM FGF2, and 0.5 µg/ml Vitamin D (from stock solution of 25 mg/ ml in DMSO) by pipetting five times through a 1ml pipet tip. The cell suspension in 0.3 ml volume is pipetted onto glass bottom culture dishes plates treated for 2h with 0.01% poly-lysine. The plates are transferred to the cell culture cabinet without agitation to allow for the cells to adhere to the glass bottom for 2h. Cells are washed by adding to the medium 1ml of HBSS, after gently swirling the medium for three times, the HBSS is replaced and the wash procedure is repeated once. Cells were cultured in 1 ml Hypothalamic Neuron Culture Medium for 10-14 days by replacing 0.5 ml of the medium with 0.5ml fresh medium every two days. On day 10-12 after plating, cells are incubated with and without 0.25 mM palmitate, prepared as described previously (Cragle and Baldini, 2014). To monitor mitochondrial membrane potential, MitoTracker™ Red is added to the medium to a final concentration of 200 nM and cells are further incubated for 30 min at 37 °C in the tissue culture cabinet. Live cells are washed twice with HBSS. Then cell medium is replaced with HBSS without phenol and containing pyruvate 0.23 mM and glucose 5 mM and cells are transferred to the stage of the Olympus microscope at room temperature to monitor fluorescence intensity of MitoTracker™ Red. Immediately after live cell microscopy, cells are fixed in formaldehyde and immunostained with antibodies against Cox. For some experiments, cultured neurons were derived from Sim1-Cre$^{+/-}$: Rosa-mEGFP$^{+/+}$ mice and immediately fixed to be immunostained with antibodies against PSD95.

Data analysis

Fluorescence intensity

Matching coronal brain sections from mice exposed to LF diet and HF diet were used for the analysis. MIP images were exported from the Olympus Fluoview FV10-ASW software (RRID: SCR_014215) as multi-tiff image files. The multi-tiff image files were opened with the ImageJ software (NIH, RRID: SCR_003070) as 16 bits RGB stack. The regions of interest (ROIs) with the same areas were drawn in matching sections of the PVN or arcuate nucleus of mice treated with LF or HF diet. The background for each image was subtracted by using the "subtract background" application under the "Image Processing" menu of the ImageJ. The raw integrated fluorescence intensity was measured within the ROIs. The results were exported from ImageJ as Excel files.

Number of NeuN, BrdU, and GFP-positive cells

Images were takes with the 20 X/0.85 N.A Plan Apochromatic oil objective. To count the number of NeuN and BrdU positive cells within the ROIs, multi-tiff images were opened with ImageJ software as 16 bits RGB stack. The ROI of the same area was drawn to outline the PVN. Using the "Analyze Particles" application within the Analyze menu of ImageJ, a table containing the number of particles (NeuN positive or BrdU positive cells) counts in the ROI was generated. The particle counts numbers were exported as Excel files and compared between the LF diet and HF diet. GFP positive cell counts were performed using the cell counter feature on ImageJ.

Number of cells expressing doublecortin and BrdU

Images were taken by using 60X/1.42 N.A Plan Apochromatic oil objective as a single XY scan. Four no-overlapping images were taken at each side of the 3rd ventricle, giving a total of 8 images per mouse (from two sides of the 3rd ventricle). Images were opened with the Olympus Fluoview FV10-ASW software and cells with doublecortin around BrdU positive nuclei were counted by using a manual cell counter. The data are reported as the sum of cell counts from 8 images per mouse.

Analysis of lipid abundance in the liver

To analyze lipid droplets in livers, MIP images were exported from Olympus Fluoview software, as indicated above. Multi-tiff images were opened in ImageJ as 16 bits RGB stack and adjusted by setting the lower threshold to 1000 pixels, and the upper threshold was automatically set to 3109 pixels. ROI was established to exclude holes at the central vein and destroyed areas of the tissues from the analysis. Using the “Analyze Particles” application within the analyze menu of ImageJ, the program generated a summary table containing the number of lipid droplets, and % area covered by lipid droplet. The table was exported as an Excel file to compare the average size of lipid droplets between mice exposed to LF diet and HF diet.

Abundance of mitochondria in Sim1/MC4R neurons

For measuring mitochondria abundance, confocal mages were exported from the Olympus Fluoview FV10-ASW software as a multi-tiff image format. The multi-tiff images were opened with ImageJ as 16 bits RGB stacks. ROIs were established around Sim1/MC4R neurons in the GFP channel, and the raw fluorescence integrated density within the ROI was measured in the COXIV (red) channel. The average raw integrated density of mitochondria per MC4R neuron, of at least 20 cells per mouse, was calculated and normalized.

Mitochondria size and mitochondria network

TIFF images were opened using ImageJ software and converted to 8 bits RGB stack. The ROIs were established by selecting areas around each cell in the GFP channel and adding the COXIV channel to the ROI manager application within the analyze menu. After selecting all cells, the lower threshold was set to 103, and the upper threshold was automatically set to 255 for all images. The area of each mitochondria particle was measured by using the “Analyze Particles” function to measure the area of the mitochondria in the COXIV channel. ImageJ generated a table containing the number of mitochondria particles and the area covered by each particle. The data were exported from ImageJ as an Excel file.

Statistical Analysis

The predefined criterion of the analysis has been to present all data, including outliers. All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Prism, RRID: SCR_002798). Statistical significance was calculated by a two-tailed unpaired t-test with Welch correction on two groups or, where indicated, by two-way ANOVA with Holm-Sidak multiple comparison test on multiple groups. A value of $P < 0.05$ was considered statistically significant. Data were expressed as mean ± S.D unless noted otherwise.

Supplemental references

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