TNFα drives mitochondrial stress in POMC neurons in obesity

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Consuming a calorically dense diet stimulates microglial reactivity in the mediobasal hypothalamus (MBH) in association with decreased number of appetite-curbing pro-opiomelanocortin (POMC) neurons; whether the reduction in POMC neuronal function is secondary to the microglial activation is unclear. Here we show that in hypercaloric diet-induced obese mice, persistently activated microglia in the MBH hypersecrete TNFα that in turn stimulate mitochondrial ATP production in POMC neurons, promoting mitochondrial fusion in their neurites, and increasing POMC neuronal firing rates and excitability. Specific disruption of the gene expressions of TNFα downstream signals TNFSF11A or NDUFA1 in the MBH of diet-induced obese mice reverses mitochondrial elongation and reduces obesity. These data imply that in a hypercaloric environment, persistent elevation of microglial reactivity and consequent TNFα secretion induces mitochondrial stress in POMC neurons that contributes to the development of obesity.
Brain microglia maintain a healthy local environment for optimal neuronal functioning, thereby help ensure innate immune protection under physiological as well as pathophysiological conditions. In response to immune challenges, microglia become activated, causing them to synthesize and release cytokines that consequently trigger pro-inflammatory responses. Depending on the circumstances, microglia-secreted tumour-necrosis factor-α (TNFα) and its downstream signals can have beneficial effects on neuronal survival and protection, but in other circumstances may exert detrimental effects leading to neuronal dysfunction. Consuming a calorically dense (high-carbohydrate, high-fat) HCHF diet stimulates rapid microglial reactivity in the mediobasal hypothalamus (MBH) and is associated with increased TNFα production. When consumption of the HCHF diet becomes chronic and induces obesity (diet-induced obesity; DIO), the induced microglial reactivity persists, of the HCHF diet becomes chronic and induces obesity (diet-associated with increased TNFα production). Depending on the circumstances, microglia become activated, causing them to synthesize other cytokines, interleukin (IL)-1β and IL-6, did not have this daily-rhythmic pattern (Fig. 1g). The rhythmic pattern of microglial activity was also observed in MBH of rats on chow diet (Supplementary Fig. 1a–d). Fasting chow-fed lean mice for 24 h eliminated the daily-rhythmic pattern of hypothalamic microglial activity (Supplementary Fig. 1e,f,i,j), implying that the pattern is driven by food intake. Furthermore, in comparison to the ad libitum condition, 24 h fasting resulted in significantly lower TNFα gene expression, while 4 h refeeding following the 24 h fast caused a significant increase of TNFα gene expression (Supplementary Fig. 1k). We conclude that microglial activity in the MBH represents a physiological response to nutrient intake.

Microglial activity in MBH in DIO mice elevated persistently. Importantly, the daily-rhythmic pattern of hypothalamic microglial activity was absent in DIO mice chronically fed the HCHF diet (Fig. 1c–i). The gene expressions of TNFα, IL-1β and IL-6 were all higher in DIO mice at ZT4 (Fig. 1g), with no further elevation at ZT16. In 24 h fasted DIO mice, the number of iba1-ir microglia was significantly lower at ZT16 than at ZT4 (Supplementary Fig. 1g–j). Thus, in DIO mice fed the HCHF diet, microglial reactivity and TNFα production are persistently elevated throughout the day/night cycle in the MBH, and the local neurons are therefore continuously exposed to high, non-rhythmic levels of TNFα.

After 4 months on the HCHF diet, DIO mice had significantly higher numbers of and more reactive microglia in close proximity to POMC neurons than occurred in age-matched chow-fed lean mice (Fig. 1h–l). In fact, there was evidence of direct contact between the cell bodies of microglia and neurons (Supplementary Fig. 2a–d). This suggests that in DIO mice, increased inflammatory interactions likely occur directly between POMC neurons and the adjacent reactive microglia, perhaps analogous to the previous finding that toll-like receptor 2-mediated microglial activation also increases the contacts between reactive microglia and POMC neurons. Moreover, consistent with a previous study, there was significant POMC neuronal loss after 8 months on the HCHF diet (Fig. 1m).

TNFα stimulates mito-ATP production in hypothalamic neurons. Since the reduction of POMC neurons may have been induced by chronic exposure to microglial-derived TNFα, and since mitochondria are critical regulators of neurodegenerative pathogenesis, we next determined the impact of TNFα on the integrity of mitochondrial bioenergetics in mouse primary hypothalamic neurons, and compared it to the impact of IL-1β and IL-6. After 16 h of incubation with 5 nM, but not with 0.5 or 2.5 nM TNFα, there was a significant increase of non-mitochondria- and mitochondria-contributed (ATP-linked) cellular oxygen consumption rate (OCR; Supplementary Fig. 3a–d). In contrast, there was no increase of OCR from neurons incubated 16 h with comparable doses of IL-1β or IL-6 (Fig. 2a,b and Supplementary Fig. 3c,e). The TNFα-stimulated OCR was paralleled by increased mitochondrial copy number (Fig. 2c)—increased citrate synthase activity (the pacemaker enzyme in the Krebs cycle; Fig. 2d), and increased mitochondrial respiration chain complex I (Supplementary Fig. 3f). Mitochondrial dynamics and bioenergetics are tightly interconnected. Proper mito-fusion has a protective function in that it enables mitochondria to exchange contents for enhanced protein complementation, mitochondrial DNA repair and equal distribution of metabolites. TNFα-induced stimulation of complex I was also associated with increased cellular levels of the mito-fusion-regulating protein optic atrophy 1 (Opa1; Supplementary Fig. 4a,b). Therefore, the normal daily rise of TNFα presumably reflects the process of optimally synchronizing neuronal ATP production with hypothalamic cell matrix function, systemic metabolism status and environmental caloric availability.

TNFα induces mitochondrial elongation in neurites. To provide sufficient ATP for neural synaptic activity, mitochondria are assembled in soma and then transported along the neurites to reach the synapse. Given that TNFα stimulates Opa1 protein expression, we next determined the impact of TNFα on mitochondrial morphology in neurites of hypothalamic neurons in vivo. We generated a G-deleted rabies virus encoding for mitochondrial-targeted red fluorescence protein (RABVΔG-MitoRFP; Supplementary Fig. 4c). The RABVΔG-MitoRFP was injected into the paraventricular nuclei (PVN) unilaterally in lean mice to retrogradely label the MBH–PVN projecting neurons. No detectable reactive microglia or astrocytes were found close to the RABV-infected neurons (Supplementary Fig. 5), indicating no local immune response to RABV-infected neurons. Ten days after the RABVΔG-MitoRFP injection, 1 pmol of TNFα was infused into the MBH and mitochondria were assessed 16 h later. In MBH neurons, mitochondria in soma and neurites were labelled by MitoRFP, and the average length of mitochondria was significantly higher in the TNFα-infused group (Fig. 2e–i), demonstrating that TNFα had induced mitochondrial elongation in the neurites of the MBH neurons.

Results

Daily rhythm of microglial activity in MBH of lean mice. Previous studies on the response of MBH microglial reactivity to specific diets were mainly performed during the light phase when nocturnal mice and rats are resting and feeding very little. This is important because we have found that during the dark phase (ZT16, 4 h after lights off), the number of iba1-ir microglial cells and of the processes per cell in the MBH are increased relative to levels during the light phase (that is, at ZT4, 4 h after lights on) in lean, chow-fed mice (Fig. 1a,b,e,f). MBH microglial number and activity were associated with elevated gene expression, while 4 h refeeding following the 24 h fast caused a significant increase of TNFα gene expression, when consumption of the HCHF diet becomes chronic and induces obesity (diet-induced obesity; DIO), the induced microglial reactivity persists, and the number of appetite-curbing pro-opiomelanocortin (POMC) neurons decreases. We asked whether the reduction in POMC neuronal function is secondary to the persistent microglial activity in MBH in DIO mice elevated persistently. Depending on the circumstances, microglia become activated, causing them to synthesize other cytokines, interleukin (IL)-1β and IL-6, did not have this daily-rhythmic pattern (Fig. 1g). The rhythmic pattern of microglial activity was also observed in MBH of rats on ad libitum condition (Supplementary Fig. 1a–d). Fasting chow-fed lean mice for 24 h eliminated the daily-rhythmic pattern of hypothalamic microglial activity (Supplementary Fig. 1e,f,i,j), implying that the pattern is driven by food intake. Furthermore, in comparison to the ad libitum condition, 24 h fasting resulted in significantly lower TNFα gene expression, while 4 h refeeding following the 24 h fast caused a significant increase of TNFα gene expression (Supplementary Fig. 1k). We conclude that microglial activity in the MBH represents a physiological response to nutrient intake.

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Mitochondria elongate in neurites of POMC neurons in DIO. We next injected RABVAG-Mito<sup>repp</sup> into the PVN of lean and DIO mice to determine whether DIO mice, in which the TNF<sub>x</sub> level is persistently increased by the reactive microglia, also exhibit elongated mitochondria in POMC neurons. DIO mice had fewer short mitochondria, ranging around 0.5 μm (Fig. 2j–p), but more elongated mitochondria, ranging from 2 to 13 μm (three-dimensional animation of Fig. 2n in Supplementary Video 1). The comparable mitochondrial elongation in neurites of vehicle-treated obese mice and in TNF<sub>x</sub>-treated lean mice are consistent with TNF<sub>x</sub> being the cause of mitochondrial elongation.

TNF<sub>x</sub> increases POMC neuronal firing rate. Cellular energy production is driven by the demand for cellular energy consumption. Because neuronal firing is particularly energetically expensive<sup>3,13</sup>, we asked whether TNF<sub>x</sub>-stimulated neuronal mitochondrial activity is associated with altered neuronal firing rate and excitability. Patch-clamp electrophysiological recordings were made from identified POMCe<sup>repp</sup> neurons in acute brain slices containing the arcuate nucleus (Fig. 3a–c) and which were pre-incubated either in vehicle or in TNF<sub>x</sub>. There were no differences in resting membrane potential or input resistance between groups. When continuous firing activity was analysed in spontaneously active neurons (artificial cerebrospinal fluid [aCSF]: 3/10) or in neurons in which firing was evoked by slight membrane depolarization with d.c. current injection, TNF<sub>x</sub> caused a significantly higher ongoing firing rate (Fig. 3d–f). Moreover, applying depolarizing current steps of increasing duration resulted in a significantly higher number of evoked action potentials in POMC neurons pre-incubated in TNF<sub>x</sub> (Fig. 3g–i). Collectively, these results indicate that TNF<sub>x</sub> drives neuronal energy demands by increasing excitability and...
that the input/output function of POMC neurons is consequently coordinated with the mitochondrial activity.

**TNFα modulates mito-function in hypothalamic neurons.**

To determine the specific molecular underpinnings linking these processes, we next dissected the signalling pathways mediating the effects of TNFα on mitochondrial bioenergetics. Specifically, we profiled key genes involved in mitochondrial respiration by PCR array, and found *Ndufab1* (NADH dehydrogenase (ubiquinone) 1, alpha/beta, subcomplex 1) and *Atp6v1e2* (ATPase, H+ transporting, lysosomal V1 subunit E2) gene expression to be upregulated in response to TNFα (Supplementary Fig. 6a–e). We then used lentiviral particles encoding mouse short hairpin RNA (shRNA) to selectively knockdown receptors of the TNF superfamily (*Tnfrsf11a*) and their downstream signals (Supplementary Table 1), and subsequently measured OCR in hypothalamic neurons. *In vitro* knockdown of *Tnfrsf11a* and its downstream signalling targets identified the nuclear factor-kB survival pathway to be regulating mitochondrial capacity (Supplementary Fig. 7).

**Knockdown of TNFα downstream signals in MBH reduces DIO.** To further determine TNFα-mediated signalling events in mitochondria, we selectively applied *in vivo* neurotropic serotype 2 adeno-associated virus (AAV-2)-expressing shRNA to knockdown *Tnfrsf11a*, TNF receptor-associated protein1 (*Trap1*), a mitochondrial chaperone that regulates the metabolic switch between mitochondrial respiration and aerobic glycolysis [14], or *Ndufab1*. Knocking down the gene expression of *Tnfrsf11a*, *Trap1* or *Ndufab1* in the neurons of MBH of lean mice had no effect on food intake or body weight relative to what occurred in response in the AAV-scrambled shRNA control group (Supplementary Fig. 8), indicating that the remaining gene expression (there was 60% knocking-down efficiency) may be sufficient to maintain the functional role of these genes under physiological conditions. In DIO mice, however, targeted gene disruption of *Tnfrsf11a* or *Ndufab1* resulted in significantly reduced food intake and body weight gain (Fig. 4a,b).

**Reverse mito-elongation in POMC neurites in DIO.** RABVΔAG-Mito<i>\textsuperscript{RFP}</i> was then injected into the PVN in combination with
AAV-shRNA injection into the MBH to determine whether modulation of mitochondrial dynamics is a possible cause for these effects on systemic energy homeostasis. In comparison to the elongated mitochondria that were present in the control AAV-infused DIO mice, knockdown of Tnfrsf11a and Ndufab1 significantly increased the number of short mitochondria, ranging from 0.5 to 1 μm, and resulted in shorter mean mitochondrial length, respectively (Fig. 4c–j). Thus, in the DIO condition, Tnfrsf11a, and to lesser extent Ndufab1, but not Trap1, are important in mediating the effects of TNFα on mitochondrial dynamics in MBH neurons, as well as in the MBH control of food intake and body weight.

Discussion

The demand for cellular energy consumption determines cellular energy production. TNFα stimulates the mitochondrial fusion process as well as ATP production, implying that TNFα can drive neurons into the highly energy-demanding state. This is important when there is high synaptic activity and it is characterized by increased neuronal firing rate and excitability, each reflecting the neuronal stress response to TNFα (Supplementary Fig. 9). This concept is supported by the observation that there is adaptively increased maximal respiratory activity in association with increased mito-biogenesis, as well as mitochondrial fusion in the neurites. Under physiological conditions, the day/night cycle of TNFα is coordinated to the resting/feeding cycle, to control cellular energy homeostasis. Furthermore, mitochondrial oxygen consumption is tightly associated with the production of reactive oxygen species. Thus, our findings on TNFα-stimulating POMC neural firing rat are also consistent with the previous report that under physiological conditions, reactive oxygen species activates POMC neurons and reduces feeding.

However, in the DIO condition, TNFα levels become constantly elevated, driving persistent neuronal activation and energy demand, and consequently increased mitochondrial activity in neurons. The persistently induced mitochondrial stress ultimately impairs systemic energy homeostasis in key hypothalamic neurons. This increase in mitochondrial and cellular stress will in turn result in more neuronal debris, and thus stimulate microglial activity to achieve debris clearance. Thus, these processes might develop into a vicious circle driving pathological activation of microglia and damaging neuronal function and cellular integrity.

In the current study, the in vivo shRNA knockdown of targeted genes were mediated by AAV-2 that dominantly presents natural tropism towards neurons, but not to microglia or other cells in the brain, assurances the targeted genes in microglial cells were not affected, thus the effects from Tnfrsf11a and Ndufab1 shRNA on body weight and food intake were not caused by loss of function of the targeted genes in microglia and changes in their daily rhythmic activity.

In summary, we here demonstrat for the first time that microglial TNFα physiologically promotes cellular energy metabolism of key POMC neuron populations that sense and govern systemic metabolism to match nutrient availability. The daily rhythmic pattern of this activity is matched to the diurnal behaviour and food intake patterns of the mice. Overconsumption of calories disrupts the pattern by causing persistently elevated TNFα, which over time causes functional impairment of hypothalamic POMC neurons, thereby generating an additional pathogenic drive towards impaired energy homeostasis and obesity.

Methods

Animals. All rodent studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the Helmholtz Center Munich, Germany; the University of Cincinnati; or the Augusta University,
**Figure 4** | Knocking down TNFα downstream signals in the MBH of DIO mice reduces the DIO and reverses the mitochondrial elongation in POMC neurons. (a,b) Cumulative food intake and body weight gain at day 20 in DIO mice expressing Ndufab1 shRNA (n = 8) or Tnfrsf11a shRNA (n = 7), but not Trap1 shRNA (n = 10), were significantly lower than in DIO mice expressing scrambled shRNA (n = 9); for food intake, F(1,30) = 3.247, P = 0.036; t15 = 2.437, P = 0.028 for Ndufab1 versus control (Ctrl); t34 = 2.204, P = 0.045 for Tnfrsf11a versus Ctrl; for BW gain, F(1,30) = 2.985, P = 0.047, t15 = 2.342; P = 0.034 for Ndufab1 versus Ctrl; t34 = 2.222, P = 0.043 for Tnfrsf11a versus Ctrl). (c, d) Mitochondria in the neurites of POMC neurons (arrowheads in f, i) of DIO mice expressing Tnfrsf11a shRNA (n = 5 mice) or Ndufab1 shRNA (n = 5 mice), but not Trap1 shRNA (n = 4 mice), were shorter than those in DIO mice expressing scrambled shRNA (n = 5 mice). For each length, F(1,15) = 4.070, P = 0.027 for 2 μm, F(1,15) = 3.468, P = 0.04 for 5 μm; for average length, F(1,15) = 4.472, P = 0.020; t5 = 4.505, P = 0.002 for Tnfrsf11a versus Ctrl; t8 = 2.235, P = 0.056 for Ndufab1 versus Ctrl. Higher magnification of MitoTracker™-labelled mitochondria (arrows) are presented in g, j. Scale bars, 30 μm in e, h; 60 μm in f, i; 8.5 μm in g, j. *P < 0.05, **P < 0.01, ***P < 0.001. Data are presented as means ± s.e.m. P values were analysed by one-way ANOVA followed by post hoc t-test.

**USA.** Wild-type male mice (C57BL/6Jr) were purchased from Janvier (Le Genest-Saint-Irène, France); POMC^{GFP} (Stock No: 009593) and NPY^{GFP} (Stock No: 006417) male mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) with the C57BL/6J genetic background. Ten-week-old male Wistar rats were purchased from Charles River (Sulzfeld, Germany). All mice and rats were group housed on a 12 h light, 12 h dark cycle (light from 7:00 to 19:00) at 22 °C, with free access to standard chow diet (LM-485, Teklad) and water before experiments. DIO was induced by HCHF diet purchased from Research Diets (D12451, Research Diets Inc., New Brunswick, NJ, USA). HCHF diet feeding was started at week 8 of age. Body weight and food intake were monitored according to each experimental design. Mice were single-house after receiving stereotaxic infusions or brain surgeries.

**Primary hypothalamic neuronal culture.** For the primary hypothalamic neuronal culture, hypothalami were extracted from embryonic day 15 (E15) mouse embryos and dissociated to single cells after digestion with trypsin (Sigma-Aldrich, T4674) and DNase I (Invitrogen, 18068-015). Neurons were plated on XF96-PS plates (Seahorse Bioscience, USA) coated with poly-L-lysine (Sigma-Aldrich) at a density of 2,000 neurons per mm², and they were cultured in Neurobasal (Life Technologies, 12348-017) supplemented with B-27 and GlutaMAX I (Life Technologies, 3505-0061). After 7 days in culture, glial cells were efficiently removed, and neurons started to develop synaptic processes. On day 10, neuronal cells were used for extracellular flux measurement.

**Plate-based respirometry.** To compare the effects of TNFα, IL-1β, IL-6 and vehicle on primary hypothalamic neuronal extracellular flux, cultured primary hypothalamic neurons seeded on XF96-PS plates were treated with TNFα (5 nM, R&D systems, 410-MT, UK), IL-1β (5 nM, R&D systems, 410-ML), IL-6 (5 nM, R&D systems, 406-ML, UK) or 0.5% BSA (vehicle control, Thermo Fisher) for 16 h at 37 °C. Cells were then washed with XF assay medium containing 25 mM glucose (pH adjusted to 7.5) and incubated for 1 h in a 37 °C air incubator. The XF96 plate (Seahorse Bioscience) was then transferred to a temperature-controlled (37 °C) Seahorse (extracellular flux) analyser (Seahorse Bioscience) and subjected to an equilibration period. One assay cycle comprised a 1 min mix, 2 min wait and 3 min measure period. After four basal assay cycles, oligomycin (1 μg ml⁻¹) was added by automatic pneumatic injection to inhibit the ATP synthase and thus approximate the proportion of respiration used to drive ATP synthesis and proton leak. After three further assay cycles, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (0.5 μM) was added the same way to stimulate maximal respiration in mitochondria by chemical uncoupling. After another three assay cycles, rotenone (4 μM) plus antimycin A (2 μM) was added to inhibit the respiratory chain and determine the non-mitochondrial respiratory rate.

**Electrophysiology.** Hypothalamic brain slices were prepared according to the methods previously described28. Briefly, male POMC^{GFP} mice were anaesthetised with pentobarbital (50 mg kg⁻¹ intraperitoneal); brains dissected out and hypothalamic coronal slices (210 μm) containing the arcuate nucleus were cut in an
and antibiotics in 5% CO2. CLU177 cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (penicillin 100 IU/ml) in a CO2 incubator at 37°C, containing 5% CO2 at 37°C. Cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (penicillin 100 IU/ml, 250 U/ml streptomycin) in a CO2 incubator at 37°C.

Viral construction for lentiviral shRNA constructs was performed in vitro. Triplicate infections were performed with 10 pg of each lentiviral shRNA vector. The vectors were pseudotyped with the VSV-G envelope protein and the viral supernatants were used to transduce naive 293T cells. Three days after infection, the cells were harvested and the lentiviral vectors were amplified by transducing 293T cells with the harvest supernatant. The amplified vectors were then used to infect 293T cells to generate a stock of lentiviral shRNA constructs.

ShRNA-mediated gene silencing of TNFα downstream signals in vitro. CLU177 cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (penicillin 100 IU/ml, 250 U/ml streptomycin) in a CO2 incubator at 37°C. Cells were treated with 0.5% BSA or TNFα for 16 h. Cells were snap-frozen in liquid nitrogen for (1) gene expression analysis by Mouse Mitochondrial Energy Metabolism PCR Array (QIAGEN), (2) mitochondrial DNA copy number assay (Dartost R&D, MCNI) and (3) citrate synthase activity colorimetric assay (BioVision, K318-100), according to the manufacturer’s instructions.

For virus infections, the control groups were set at −0.2 mm posterior to bregma and −4.6 mm depth; for infection into MBH, stereotaxic coordinates were set at −0.2 mm posterior to bregma and −0.3 mm lateral to bregma, −1.5 mm depth. For infusion of TNFα or MitoRFP into the MBH, to determine the impact of AA-vshRNA on mitochondrial morphology.

Viral infections of TNFα or MitoRFP were performed in vitro. Triplicate infections were performed with 10 pg of each lentiviral shRNA vector. The vectors were pseudotyped with the VSV-G envelope protein and the viral supernatants were used to transduce naive 293T cells. Three days after infection, the cells were harvested and the lentiviral vectors were amplified by transducing 293T cells with the harvest supernatant. The amplified vectors were then used to infect 293T cells to generate a stock of lentiviral shRNA constructs.

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016-580-084, USA) for 1 h. To detect iba-ir with GFAFP in sections, sections were rinsed and incubated with biotinylated secondary horse anti-rabbit IgG for 1 h, and then rinsed and incubated with streptavidin-conjugated Alexa Fluor 488 for 1 h. All sections were then rinsed and mounted on gelatin-coated glass slides, dried, covered with polyvinyl alcohol mounting medium containing DABCO (Sigma-Aldrich, USA), observed and imaged by confocal microscopy (Leica SP5, Germany). For image analysis, microscopy: Leica TCS SP5 II; objective lenses: HCX PL APO, 63×, numerical aperture = 1.3; imaging medium: glycerol; camera: Leica TCS SP5 II; acquisition software: LAS AF were used. Images were taken z-stack with sequential scanning of the middle of sections, z-galvo range was 25 μm, z-step size was 0.1 μm. Images were processed by IMARIS7.6.4 (Bitplane, UK) for three-dimensional reconstruction. 2. For quantification of the number of microglia contacting neighbouring POMC neurons, the total number of eGFP cells and those eGFP cells that had close contact with iba-ir cells were counted, the percentage of contacting cells among the total eGFP cells was calculated in a blind manner. To quantify POMC neuron density in DIO mice fed 8 months of HCHF diet, an additional group of POMC-GFP mice kept under HCHF diet for 8 months was compared to a group of age-matched lean mice. Brain sections were processed with perfusion fixation, sectioning and imaging as described above (one section per mouse). EGFP cell number quantification was performed in a blind manner. Mitochondrial length labelled by RABYAG-MitoRFP was manually measured by NeuroLucida in a blind manner.

Western blotting. To detect mitochondrial bioenergetics and dynamics protein expressions in vehicle versus TNFα-treated hypothalamic neurons, CLU177 hypothalamic cells were cultured as described above in six-well plates. At 70–80% confluence, followed by serum starvation for 6 h, cells were treated with TNFα (5 nM) or vehicle (0.5% BSA) for 16 h. After thorough rinsing, cells were snap-frozen in liquid nitrogen. Protein was extracted using RIPA buffer containing protease and phosphatase inhibitor cocktail (Thermo Fisher) 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium butyrate (Sigma-Aldrich, St Louis, MO, USA). The lysates were then centrifuged at 10,000 r.p.m., resolved by SDS–PAGE and transferred to nitrocellulose membranes (45 mmol l–11,000, C2/C2 74,000 magnification for posterior analysis.) The data that support the findings of this study are available from the corresponding author on reasonable request.

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Additional information

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