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Fibroblast growth factor 21 deficiency aggravates obesity-induced hypothalamic inflammation and impairs thermogenic response

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Abstract

Objective and design Hypothalamic inflammation is closely associated with metabolic dysregulation. Fibroblast growth factor 21 (FGF21) is known to be an important metabolic regulator with anti-inflammatory properties. In this study, we investigated the effects of FGF21 deficiency on obesity-induced hypothalamic inflammation and thermogenic responses.
Materials and methods FGF21-deficient mice and/or wild-type (WT) mice were fed a high-fat diet (HFD) for 12 weeks.
Results FGF21-deficient mice fed an HFD showed increased levels of inflammatory cytokines compared with WT obese control, and this was accompanied by upregulation of gliosis markers in the hypothalamus. Expression of heat-shock protein 72, a marker of neuronal damage, was increased in the FGF21-deficient obese mice, and the expression of hypothalamic neuronal markers involved in anti-thermogenic or thermogenic responses was altered. Moreover, the protein level of uncoupling protein 1 and other thermogenic genes were markedly reduced in the brown adipose tissue of the FGF21-deficient obese mice.
Conclusions These findings suggest that FGF21 deficiency aggravates obesity-induced hypothalamic inflammation and neuronal injury, leading to alterations in hypothalamic neural circuits accompanied by a reduction of the thermogenic response.

Keywords FGF21 · Hypothalamic inflammation · Metabolism · Obesity

Introduction

Obesity is a major public health problem and is strongly associated with metabolic disorders such as insulin resistance and type 2 diabetes. Growing evidence shows that obesity-induced inflammation in both the peripheral and central system plays a crucial role in the development of metabolic dysregulation. In particular, the hypothalamus in the central system is responsible for the regulation of many fundamental physiological functions related to metabolism and maintenance of energy homeostasis [1]. Obesity-related prolonged hypothalamic inflammation increases neuronal injury [2–4], and is considered to cause impairment and dysregulation of the hypothalamic neuronal network, leading to disruption of energy homeostasis and whole-body thermoregulation in the peripheral system [5, 6]. However, the molecule linking hypothalamic neuronal response and thermogenic regulation in obese conditions has not been fully elucidated.

Fibroblast growth factor 21 (FGF21) is a member of the FGF family that is secreted by multiple organs, predominantly liver, muscle, and adipose tissue [7], and is considered a potent metabolic regulator [8, 9]. Administration of FGF21 has been shown to improve insulin sensitivity, glucose uptake, and lipid metabolism under obese conditions [8, 10–12]. FGF21 is presented in human cerebrospinal fluid and brain tissues of rodents [13] and circulating FGF21 can cross the blood–brain barrier (BBB) via simple diffusion [14, 15]. FGF21 receptors (FGF receptor 1/FGFR1 and β-Klotho) are found in several brain areas, including the hypothalamus [15, 16]. The functional effects of FGF21 occur after it binds

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to its receptor (FGFR1) and co-receptor (β-Klotho) in each designated tissue and on the surface of cells [17]. A study of the central nervous system (CNS) showed that FGF21 protects against high-fat diet (HFD)-induced cognitive impairment through metabolic regulation and inhibition of neuroinflammation and neurogenesis deficits in the hippocampus of obese mice [18]. Based on the metabolic functions and anti-inflammatory properties of FGF21 in both the peripheral and central system, we hypothesized that FGF21 is an important molecule linking hypothalamic inflammation to metabolic dysregulation in obese conditions.

In this study, we demonstrate that FGF21 deficiency aggravates obesity-induced hypothalamic inflammation and reduces thermogenic responses through alteration of the hypothalamic neural circuits.

Materials and methods

Animals

Six-week-old male C57BL/6 wild-type (WT) mice were purchased from Orient Bio Inc. (Busan, Korea) and whole-body FGF21-deficient (KO) mice on a C57BL/6 background (The Jackson Laboratory, US) were housed in plastic cages within a specific pathogen-free barrier animal facility that was maintained under a 12-h light/12-h dark cycle at 22 ± 2 °C. After 1 week of acclimatization, animals were randomly assigned to feeding with either a regular diet (RD; 13% of calories from fat; Harlan Teklad, Madison, WI, USA) or an HFD (60% calories from fat; Research Diets Inc., New Brunswick, NJ, USA) for 12 weeks, during which time they were given free access to food and water ($n = 12$ for each group). Body weight and food intake were recorded every week. At the end of the feeding period, animals were deprived of food for 4 h and then euthanized by CO_2 asphyxiation. The entire hypothalamus, hypothalamic-specific nuclei regions (arcuate nucleus/ARC, paraventricular nucleus/PVN, and lateral hypothalamus/LH), and brown adipose tissue (BAT) were subsequently collected and stored at − 75 °C. All animal cares and procedures were conducted according to protocols and guidelines approved by the animal care committee of the University of Ulsan, South Korea (LNY-16-020).

Quantitative real-time PCR (qRT-PCR)

One RNA sample of hypothalamic-specific nuclei was extracted from two combined animal tissues with Trizol reagent (Life Technologies, Carlsbad, CA, USA) and 2 μg aliquots of total RNA were reverse transcribed to cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Next, qRT-PCR amplification of the cDNA was performed using SYBR premix Ex Taq (TaKaRa Bio Inc., Foster, CA, USA) in a Thermal Cycler Dice (TaKaRa Bio Inc., Otsu, Shiga, Japan). All reactions were performed according to the following conditions: initial denaturation at 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Additionally, a BAT RNA sample was extracted from one animal tissue sample as described above. The results were analyzed with the Real-Time System TP800 software (TaKaRa Bio Inc.) and all values were normalized to the levels of the housekeeping gene, β-actin. The primers used in this analysis are listed in Table 1.

Western blot analysis

Tissues were lysed in lysis buffer (150 mM NaCl, 50 mM Tris–HCl, 50 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM EDTA, 1% IGEPAL) supplemented with 0.25% protease inhibitor cocktail (Sigma, St. Louis, MO, USA) and 1% phosphatase inhibitor cocktail (Sigma). Protein concentrations of the lysates were determined using BCA protein assay reagents (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of protein (10–15 μg) were subjected to western blot analysis using polyclonal antibodies to uncoupling protein 1 (UCP1, Abcam, ab10983, Cambridge, USA), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α, Abcam, ab54481), and alpha-tubulin (Abcam, ab7291). Protein bands were detected using an enhanced chemiluminescence western blotting detection kit (PerkinElmer, Waltham, MA, USA). Band intensities were quantified using the Image J program.

Immunohistochemistry analysis

For the tissue section, mice were deeply anesthetized and perfused transcardially with phosphate-buffered saline (PBS) followed by freshly prepared 4% paraformaldehyde (PFA) solution. Whole brains were collected, fixed with 4% PFA for 24 h, and dehydrated in 30% sucrose solution until it sank to the bottom of the tube. After dehydration, coronal brains including hypothalamus were sectioned (30 μm thick) using cryostat (Leica, Wetzlar, Germany) and stored at − 70 °C. Markers for microglia and astrocytes were immunostained as follows: hypothalamic slices were permeabilized in 0.5% PBS for 5 min, blocked with 5% normal donkey serum at room temperature (RT) for 1 h and incubated with primary antibodies for ionized calcium-binding adapter molecule-1 (Iba-1, 1:400, Abcam) or glial fibrillary acidic protein (GFAP, 1:400, Merck, Darmstadt, Germany) at 4 °C for 16 h and then at RT for 1 h. After washing, slides were incubated with Alexa-Fluor 488- or 555-conjugated secondary antibody (1:1000, Thermo Fisher Scientific Inc., Waltham, MA, USA) at RT for 1 h. For nuclear staining, slides were treated with DAPI (1:10,000, Thermo Fisher Scientific Inc.) for 10 min before mounting. Immunofluorescence was imaged

Table 1 Mouse primers used for qRT-PCR

Primer name	Forward primer sequence	Reverse primer sequence
Cpt-1β	GAGACAGGACACTGTGTGGGTGA	AGTGCCTTGGCTACTTGGTACGAG
Fgfr1	TCTCTGTTACCCAGTTGGGTCTGTC	GCAGAATTGAGTTGCCAAGTTGA
Fgf21	ACACTGAAGCCCACCTGGAGA	CTGCAGGCCTCAGGATCAAAG
Gfap	AGCTAGCCCTGGACATCGAGA	GGTGAGCCTGTATTGGGACAAC
Hsp72	CAGAGGCCAGGGCTGGATTA	ACACATGCTGGTGCTGTCACTTC
Iba-1	AGCTGCCTGTCTTAACCTGCATC	TTCTGGGACCGTTCTCACACTTC
Il-1β	TCCAGGATGAGGACATGAGCAC	GAACGTCACACACCAGCAGGTTA
Mch	GATTCCAGACATGACTTCTCAAATCATGGT	TCAGTGTCAGCTGGAAAAGCAATGG
Npy	CAGAAAACGCCCCCAGAA	AAAAGTCGGGAGAACAAGTTTCATT
Pparα	ACGCTCCCGACCCATCTTTAG	TCCATAAATCGGCACCAGGAA
Prdm16	CCTAGCCCTGAGCGATACTGTGA	ACAGACAATGGCTGGAATGGTG
Tnfα	AAGCCTGTAGCCCACGTCGTA	GGCACCACTAGTTGGTTGTCTTTG
Trh	AGCATCTTTTGGAGACATTCAG	CAGCTCCAGGTAGTTGACAAGGT
Ucp1	TACCAAGCTGTGCGATGTCCA	GCACACAAACATGATGACGTTCC
β-Klotho	GCATCGATGACCTGGCTCT	CAGTTTGAATGCATAGTAGCCTTTG
β-Actin	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA

Cpt-1β carnitine palmitoyltransferase-1 beta, *Gfap* glial fibrillary acidic protein, *Hsp72* heat-shock protein 72, *Iba-1* ionized calcium-binding adapter molecule-1, *Il-1β* interleukin-1 beta, *Mch* melanin-concentrating hormone, *Npy* neuropeptide Y, *Pparα* peroxisome proliferator-activated receptor alpha, *Prdm16* PR domain containing 16, *Tnfα* tumor necrosis factor alpha, *Trh* thyrotropin-releasing hormone, *Ucp1* uncoupling protein 1

using confocal microscopy (Carl Zeiss 780, Germany). Fluorescence quantitation and cell counting were performed throughout the entire rostro-caudal axis of the ARC (about 8 brain sections per animal). Fluorescence intensity was measured using the Image J program.

Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM). Statistical analyses were assessed by the Student's *t* test using GraphPad Prism 5 (San Diego, CA, USA). Differences were considered to be significant at $p < 0.05$.

Results

FGF21 and its receptors are expressed in the hypothalamus

Metabolic actions of FGF21 require its primary receptor FGFR1 in complex with β-Klotho, an essential cofactor for FGF21, for activation of its signaling pathway [17]. FGF21 and its receptors have been discovered in brain areas such as midbrain, hippocampus, and hypothalamus [15]. To examine whether obesity affects the expression of FGF21 and its receptors (FGFR1 and β-Klotho) in the hypothalamus, we measured mRNA expression levels in the hypothalamus of obese mice by RT-PCR. As shown in Fig. 1, the expression level of *Fgf21, Fgfr1*, and *β-Klotho* mRNAs in the hypothalamus was significantly increased in the HFD-fed obese mice compared with RD-fed control, indicating that FGF21 may compensate for the harmful effect of obesity-induced hypothalamic inflammation.

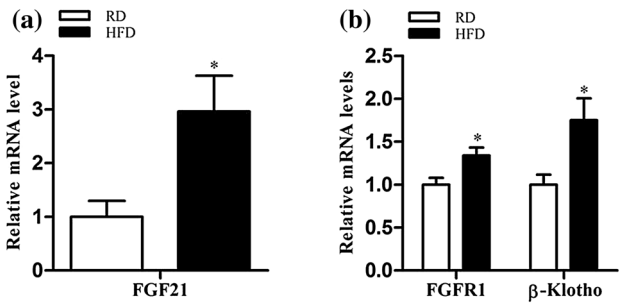

Fig. 1 Expression of FGF21 and its receptors (FGFR1 and β-Klotho) in the hypothalamus of HFD-fed obese mice. Expression of **a** *Fgf21*; **b** FGF21 receptors (*Fgfr1* and *β-Klotho*) mRNA levels in the hypothalamus of C57BL/6 WT mice fed a RD or HFD for 12 weeks, as determined by RT-PCR analysis ($n = 4$–5 for each group). All data are presented as mean ± SEM. *$p < 0.05$ significantly different compared with RD control

FGF21 deficiency induces brain atrophy in obese mice

Obesity is associated with a small brain volume, specifically hippocampus and ventromedial nucleus of the hypothalamus (VMH) [19]. In this study, we found that the brain of

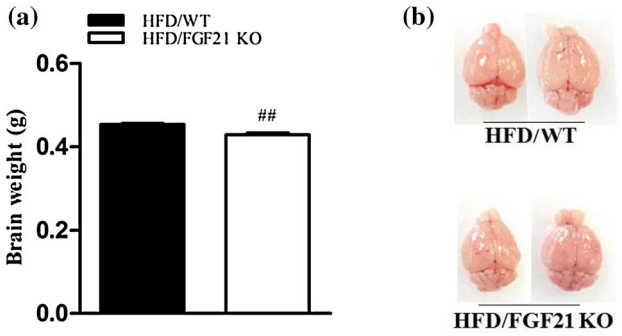

Fig. 2 Brain phenotype in FGF21-deficient HFD-fed obese mice. Changes in **a** brain weight; **b** brain morphology of C57BL/6 WT and FGF21-deficient mice fed a RD or HFD for 12 weeks ($n=10$ for each group). All data are presented as mean ± SEM. $^{\#\#}p<0.001$ significantly different compared with WT control

FGF21-deficient obese mice was smaller and weighed less than that of WT obese mice (Fig. 2), indicating that FGF21 deficiency exaggerates the extent of brain atrophy in obese condition.

FGF21 deficiency aggravates HFD-induced hypothalamic inflammation

Obesity and HFD feeding are closely associated with low-grade inflammation in both the peripheral and central system. We investigated the effects of FGF21 deficiency on obesity-induced hypothalamic inflammation by measuring the expression of the inflammatory cytokines (tumor necrosis factor alpha/*Tnfα* and interleukin-1 beta/*Il-1β*). mRNA expression of inflammatory cytokine levels was significantly higher in the whole hypothalamus of FGF21-deficient obese mice compared with WT obese control mice (Fig. 3a). Subsequently, we found that mRNA levels of *Iba-1* (a marker of microglia activation) and *Gfap* (a marker of astrocytes activation) were markedly upregulated in the whole hypothalamus of the FGF21-deficient obese mice compared with

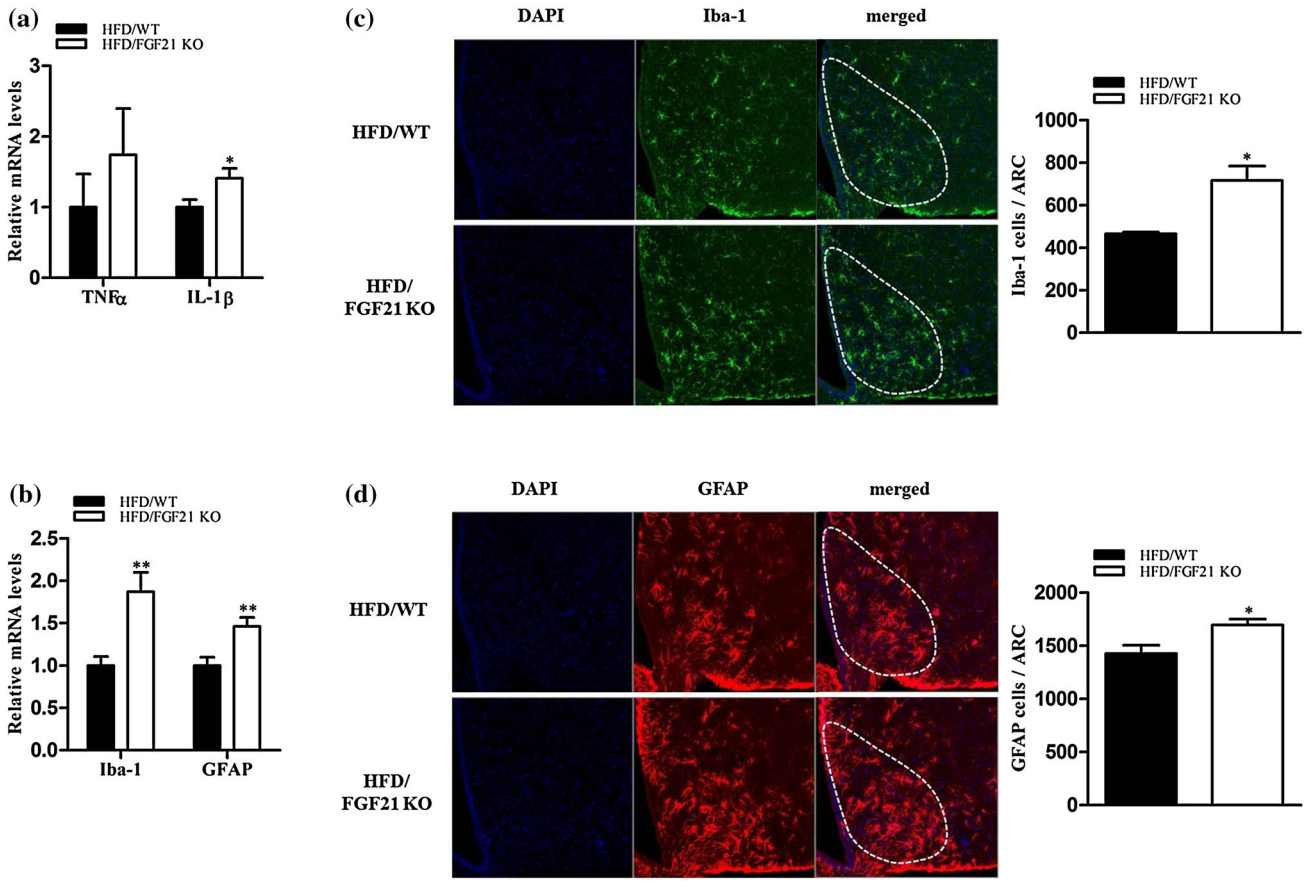

Fig. 3 Effect of FGF21 deficiency on inflammatory response in the hypothalamus of HFD-fed obese mice. Expression of **a** pro-inflammatory cytokines (*Tnfα* and *Il-1β*); **b** gliosis markers (*Iba-1* and *Gfap*) mRNA levels in the whole hypothalamus as determined by RT-PCR analysis ($n=6$ for each group). And immunohistological detection and quantification of **c** IBA-1 (microglia activation); **d** GFAP (astrocytes activation) protein levels in coronal sections of hypothalamic ARC ($n=3$ for each group) of C57BL/6 WT and FGF21-deficient mice fed an HFD for 12 weeks. All data are presented as mean ± SEM. $^{*}p<0.05$, $^{**}p<0.01$ significantly different compared with WT control

WT obese control mice (Fig. 3b). In addition, using immunohistological analysis, we confirmed that FGF21 deficiency markedly enhanced the expression of the proteins (IBA-1 and GFAP) in the ARC of HFD-fed obese mice (Fig. 3c, d). These findings suggested that FGF21 deficiency enhances the inflammatory response and this is attributed to the activation of glial cells in the hypothalamus of HFD-fed obese mice.

FGF21 deficiency alters hypothalamic neuronal response

Hypothalamic inflammation promotes neuronal injury and is, therefore, implicated as an initial cause of the dysregulation of neuronal activity and functions. We first examined the effects of FGF21 deficiency on the gene expression of heat-shock protein 72 (*Hsp72*), a marker of neuronal damage, in the hypothalamic regions of HFD-fed obese mice and found that *Hsp72* was significantly upregulated in the specific area of the hypothalamus (ARC, PVN, and LH) of FGF21-deficient obese mice relative to WT control obese mice (Fig. 4d). Next, we found that the hypothalamic ARC derived neuropeptide Y (*Npy*), the first-order neurons, controls sympathetic output involved in thermogenic regulation [20] significantly increased in FGF21-deficient obese mice (Fig. 4a). Importantly, we also confirmed that FGF21 deficiency resulted in a significant reduction of the thermogenic neurotransmitter thyrotropin-releasing hormone (*Trh*) mRNA in the hypothalamic PVN of obese mice (Fig. 4b). In contrast, expression of the anti-thermogenic neurotransmitter, melanin-concentrating hormone (*Mch*) significantly increase in the LH of FGF21-deficient obese mice (Fig. 4c). These results show that FGF21 deficiency causes a neuronal injury that may disrupt neuronal functions in the hypothalamus of HFD-fed obese mice.

FGF21 deficiency abolishes BAT thermogenesis function in obese mice

Hypothalamic neurons project to autonomic sites through the SNS, which is crucial for the regulation of BAT thermogenesis as one mechanism to maintain energy homeostasis [20, 21]. To investigate whether the alteration in hypothalamic neuronal circuits affects thermogenic activity in BAT, we measured protein levels of UCP1 and PGC-1α by western blotting. Compared to WT obese control mice,

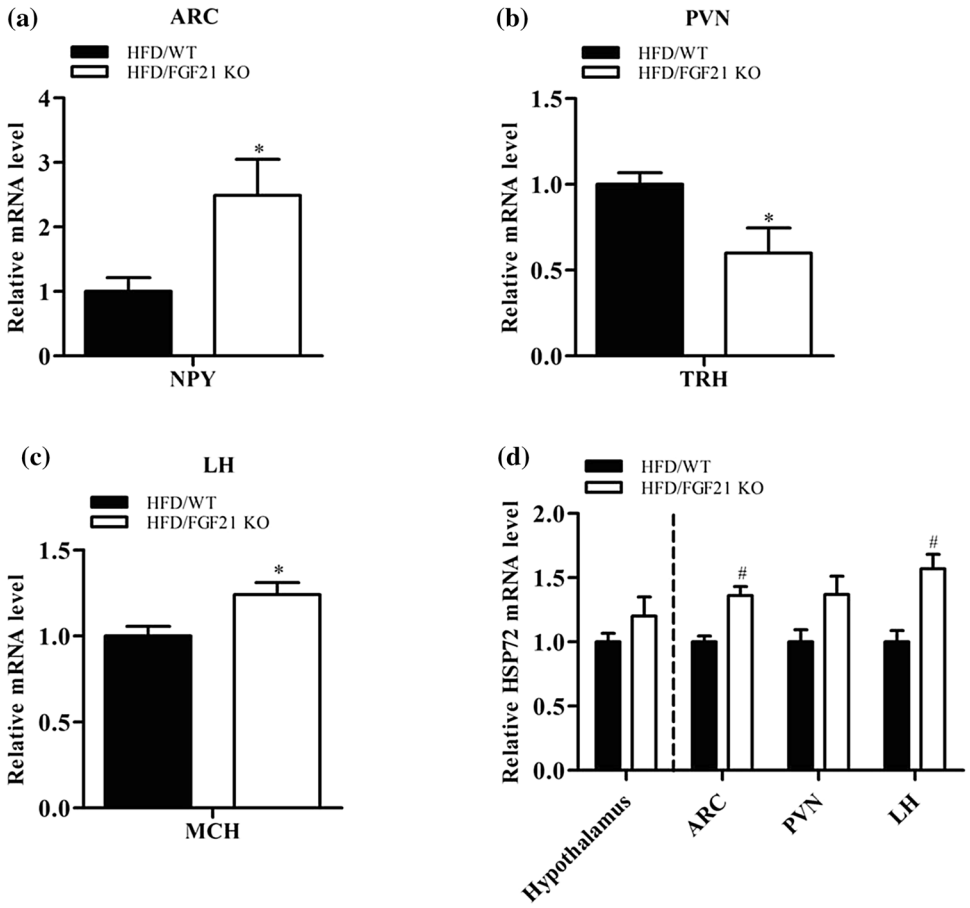

Fig. 4 Effect of FGF21 deficiency on hypothalamic neuronal circuits in HFD-fed obese mice. Expression of **a** *Npy* in the ARC; **b** thermogenic marker (*Trh*) in the PVN; **c** anti-thermogenic marker (*Mch*) in the LH; **d** neuronal damage marker (*Hsp72*) mRNA levels in the whole hypothalamus (include ARC, PVN, and LH) of C57BL/6 WT and FGF21-deficient mice fed an HFD for 12 weeks, as determined by RT-PCR ($n = 12$ for each group). All data are presented as mean ± SEM. $^{*}p < 0.05$, $^{\#}p < 0.005$ significantly different compared with WT control

the protein levels of UCP1 and PGC-1α in BAT were significantly reduced in FGF21-deficient obese mice (Fig. 5a, b). Moreover, this was accompanied by suppression of transcript levels of thermogenic genes (*Ucp1*, carnitine palmitoyltransferase-1 beta/*Cpt-1β*, peroxisome proliferator-activated receptor alpha/*Pparα*, and PR domain containing 16/*Prdm16*) (Fig. 5c) in the BAT of FGF21-deficient obese mice. These findings indicate that FGF21 deficiency suppressed BAT thermogenesis in the obese mice.

Discussion

Obesity promotes hypothalamic inflammation, characterized by activation of glial cells such as microglia and astrocytes with increased production of inflammatory cytokines such as *Tnfα* and *Il-1β* [6, 22]. In this study, we, for the first time, demonstrate that expression levels of hypothalamic inflammatory cytokines (*Tnfα* and *Il-1β*) are markedly upregulated in the whole hypothalamus of HFD-fed FGF21-deficient obese mice compared to control obese mice. It has been shown that the initiation and/or escalation of hypothalamic inflammation accompanied by the release of inflammatory mediators is closely associated with activation of glial cells such as astrocytes and microglia, a process termed gliosis [23]. Indeed, we observed upregulation of gliosis markers for microglia (*Iba-1*) and astrocytes (*Gfap*) mRNAs in the whole hypothalamus and the specific regions (particularly in ARC) of FGF21-deficient obese mice compared with the control mice. These findings indicate that FGF21 deficiency aggravates obesity-induced hypothalamic inflammatory responses accompanied by activation of glial cells.

Inflammation is well known to impair neurogenesis, synaptic plasticity, and neural function [2, 24]. Moreover, prolonged inflammation mediated by activated glial cells has been shown to drive neurotoxicity, resulting in neuronal damage [25]. Neuronal cells express HSP72, a chaperone protein that protects against oxidative stress and is, therefore, used as a neuronal injury marker, as a compensatory response after stress to promote cell survival and further prevent or limit injury [26]. Our previous study showed that under obese conditions hypothalamic lipid-containing astrocytes and/or microglia elicit inflammatory phenotypes and induce *Hsp72* in hypothalamic neurons [3, 27], indicating that the activated glial cells cause neuronal damage. It is likely that FGF21 functions to protect against neuronal injury [28]. A recent study demonstrated that FGF21 inhibits neuroinflammation and abrogates deficiencies in neurogenesis, leading to attenuation of HFD-induced cognitive impairment [18]. Of note, we found that expression of *Hsp72* was increased in the hypothalamic nuclei (ARC, PVN, and LH) of FGF21-deficient obese mice compared with those of WT control obese mice, indicating that FGF21 deficiency augmented obesity-induced hypothalamic neuronal injury. More importantly, we found that the brains of FGF21-deficient obese mice were smaller and lighter than those of the WT obese mice, which may reflect the increased neuronal injury. Given that brain volume is correlated with body mass index, numbers of inter-neuronal circuits, and/or brain structural deficits [29–31], our findings suggest that hypothalamic neuronal circuit involved energy metabolism may not properly function in the FGF21-deficient obese mice.

The hypothalamic neuronal signals are closely interlinked in the ARC, PVH, LH, VMH, and the integrated neuronal circuits are crucial for modulating food intake and thermogenic responses in the BAT [32]. The first-order neurons in the ARC participate in thermogenic regulation by projecting the signals to other nearby

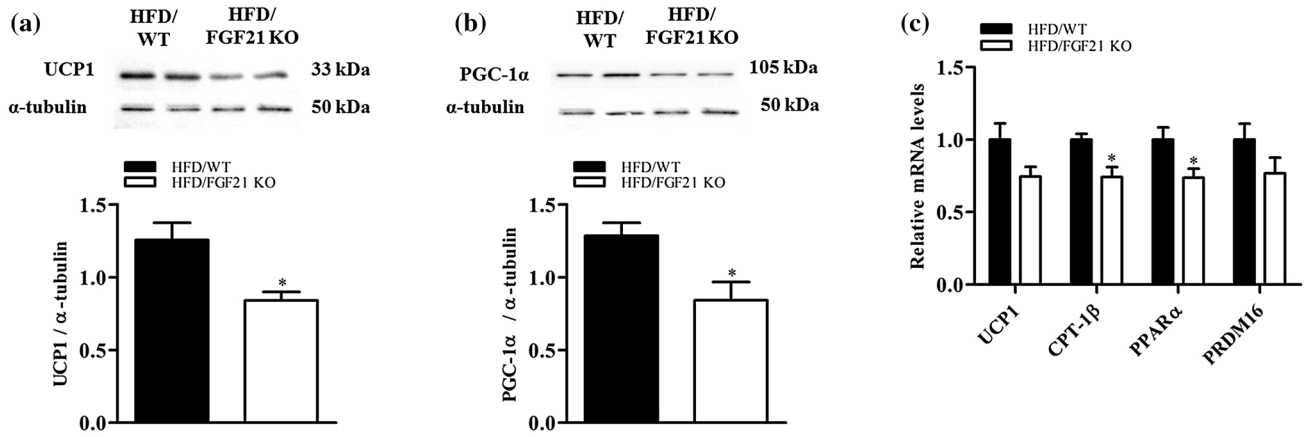

Fig. 5 Effect of FGF21 deficiency on BAT thermogenesis response in HFD-fed obese mice. Expression of **a** UCP1; **b** PGC-1α protein levels; **c** thermogenic markers (*Ucp1, Pparα*, and *Prdm16*); mitochondrial β-oxidation marker (*Cpt-1β*) mRNA levels in the BAT of C57BL/6 WT and FGF21-deficient mice fed an HFD for 12 weeks, as determined by western blotting and RT-PCR, respectively ($n = 4$–6 for each group). All data are presented as mean ± SEM. *$p < 0.05$ significantly different compared with WT control

hypothalamic neurons such as PVN and LH [21, 33]. Increased release of *Npy* mRNA in the ARC reduces BAT thermogenic activity by suppressing SNS outflow in the PVN [20]. Moreover, the second-order neurons such as TRH in PVN and MCH in LH also play essential roles in controlling metabolism and energy expenditure: MCH-expressing neurons in the LH regulate BAT thermogenesis via the SNS from LH to rostral raphe pallidus (rRPa), which inhibits the excitability of BAT sympathetic premotor neurons, and TRH-expressing neurons in the PVN increase SNS to BAT by stimulating the projection to rRPa [34–36]. Of note, obesity-induced hypothalamic inflammation is known to interfere with neuron-integrated thermogenic regulation [2, 37]. In this study, we observed the upregulation of *Npy* mRNA in the ARC of FGF21-deficient obese mice, and this was accompanied by increased expression of the anti-thermogenic neurotransmitter *Mch* in the LH and decreased expression of the thermogenic neurotransmitter *Trh* in the PVN of the HFD-fed obese mice, indicating that neuron-integrated thermogenic regulation could be interfered with FGF21 deficiency in the HFD-fed obese mice. Indeed, we found that a reduction in the mRNA and/or protein levels of UCP1 in the BAT of the FGF21-deficient obese mice, and this was accompanied by a significant downregulation in the BAT transcription factors (*Pparα* and *Prdm16*) and other thermogenic marker *Cpt-1β*, indicating that BAT-mediated thermogenic response was reduced in the FGF21-deficient obese mice. Given that the inflammatory responses increased in the whole hypothalamus and hypothalamic ARC of the FGF21-deficient obese mice, FGF21 may directly affect hypothalamic neuron-integrated thermogenic regulation and/or sympathetic outflow [38], which regulates energy expenditure, and the FGF21 action may be disrupted in the FGF21-deficient obese mice. Additionally, it has been shown that cold stress-induced thermogenic response of adipose tissue is impaired in FGF21-deficient mice [39]. Our findings together with this suggest that FGF21 plays an important role in the adaptive thermogenesis under cold and/or HFD-feeding conditions. Further study will be needed to overcome the limitation of the lack of littermate control.

In conclusion, FGF21 deficiency aggravates obesity-induced hypothalamic inflammation and neuronal injury, leading to alterations in hypothalamic neural circuits accompanied by a reduction of the thermogenic response. Thus, FGF21 may be an important molecule linking obesity-induced hypothalamic inflammation to thermogenic derangement in obese condition.

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Compliance with ethical standards

Conflict of interest Authors declare that there is no conflict of interest associated with this publication that could have influenced its outcome.

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