


FGF21 Attenuates High-Fat Diet-Induced Cognitive Impairment via Metabolic Regulation and Anti-inflammation of Obese Mice

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Abstract Accumulating studies suggest that overnutrition-associated obesity may lead to development of type 2 diabetes mellitus and metabolic syndromes (MetS). MetS and its components are important risk factors of mild cognitive impairment, age-related cognitive decline, vascular dementia, and Alzheimer's disease. It has been recently proposed that development of a disease-course modification strategy toward early and effective risk factor management would be clinically significant in reducing the risk of metabolic disorder-initiated cognitive decline. In the present study, we propose that fibroblast growth factor 21 (FGF21) is a novel candidate for the disease-course modification approach. Using a high-fat diet (HFD) consumption-induced obese mouse model, we tested our hypothesis that recombinant human FGF21 (rFGF21) administration is effective for improving obesity-induced

cognitive dysfunction and anxiety-like behavior, by its multiple metabolic modulation and anti-pro-inflammation actions. Our experimental findings support our hypothesis that rFGF21 is protective to HFD-induced cognitive impairment, at least in part by metabolic regulation in glucose tolerance impairment, insulin resistance, and hyperlipidemia; potent systemic pro-inflammation inhibition; and improvement of hippocampal dysfunction, particularly by inhibiting pro-neuroinflammation and neurogenesis deficit. This study suggests that FGF21 might be a novel molecular target of the disease-course-modifying strategy for early intervention of MetS-associated cognitive decline.

Keywords High-fat diet · Obese mice · Metabolic disorders · Cognitive impairment · Inflammation · Fibroblast growth factor 21

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Abbreviations

Akt	Protein kinase B
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
DCX	Doublecortin
DG	Dentate gyrus
FGF21	Fibroblast growth factor 21
FGFR1	Fibroblast growth factor receptor 1
GSK-3 β	Glycogen synthase kinase 3 beta
GTT	Glucose tolerance test
HbA1c	Glycated hemoglobin
HDL	High-density lipoproteins
HFD	High-fat diet
Iba1	Ionized calcium binding adaptor molecule 1
IGF-1	Insulin-like growth factor-1
IL-1 β	Interleukin 1 beta
IL-6	Interleukin 6

i.p.	Intraperitoneal injection
ITT	Insulin tolerance test
LDL	Low-density lipoproteins
MetS	Metabolic syndrome
MHCII	Major histocompatibility complex class II
PCR	Polymerase chain reaction
PSD95	Postsynaptic density protein 95
rFGF21	Recombinant human fibroblast growth factor 21
RI	Recognition index
SD	Standard diet
SEM	Standard error of the mean
TC	Total cholesterol
TG	Triglycerides
TNF α	Tumor necrosis factor alpha
WAT	White adipose tissue

Introduction

The prevalence of obesity has increased globally in the last three decades due to genetic, metabolic, behavioral, and environmental factors [1, 2]. Accumulating studies suggest that overnutrition-associated obesity may lead to development of metabolic syndrome and type 2 diabetes mellitus [3]. It has been suggested that metabolic syndrome (MetS) and its components are important risk factors of mild cognitive impairment, age-related cognitive decline, vascular dementia, and Alzheimer's disease [4, 5]. Although some potential mechanisms have been proposed, there is still a big knowledge gap at identifying roles and molecular mechanisms that underlie obesity and other individual components of the MetS in developing predementia and dementia syndromes [6, 7].

From the metabolic-neurocognitive dysfunction perspective, alterations of metabolic factors such as obesity itself and obesity-induced metabolic disorders can impact cognition and play an early and critical role [8]. Furthermore, the low degree of systemic pro-inflammation in adipose tissue circulation and neuroinflammation in the brain hippocampus may be one of the connections between obesity/diabetes and neurocognitive dysfunction [2, 9, 10]; thus, new therapeutic approaches have been proposed to modulate metabolic dysfunction and inflammation in the context of obesity and metabolic syndrome [2, 11]. Additionally, the elevated hippocampal pro-inflammation may lead to synaptic plasticity and neurogenesis deficits in the brain hippocampus that may result in synergistic exacerbation of cognitive deficits [2, 9, 10, 12].

However, it would also be very important to develop a newly proposed disease-course modification strategy toward early and effective risk factor management that could be clinically significant in reducing the risk of metabolic and cognitive decline [7]. Since individual risk factors or MetS components may independently impair metabolic and cognitive function, one ideal intervention approach is to simultaneously

control multiple obesity-related metabolic dysfunction and pro-inflammatory pathways [7, 13]. In this study, we aimed to test one of the disease-course control strategies that target multiple metabolic dysfunctions for early prevention and/or mitigation of the neurocognitive complications associated with obesity and diabetes. It would not only provide insight into the pathological causes of obesity-associated cognitive impairment but also inspire this novel strategy of multiple metabolic and inflammation targeting for preventing and even treating metabolic dysfunction-associated development of cognitive disorders [14].

One disease-course modification approach candidate is fibroblast growth factor 21 (FGF21). Fibroblast growth factors (FGFs) are a family that secreted proteins as signaling molecules with wide ranging functions in development, cell proliferation, and wound healing [15]. Emerging experimental investigations have demonstrated that FGFs may act as autocrine, paracrine, and/or endocrine hormones by binding to FGF receptors (FGFRs) [16]. Very interestingly, several FGFs have been linked to metabolism regulation [17]. Currently, three members have been discovered in function for regulation of energy metabolism: FGF1, FGF15/19, and FGF21 [18]. FGF21 is an endocrine member of the FGF family, primarily expressed in the liver, but it is also expressed in adipose tissue, skeletal muscle, and many other organs. It has a potent and central role in glucose and lipid metabolism, as well as in energy balance [19]. Human FGF21 (MW 19.5 kDa) is highly homologous to mouse FGF21 (~75% identity) [20]. The very low heparin-binding affinity makes FGF21 capable of crossing the blood-brain barrier (BBB) by simple diffusion [21]. FGF21 exerts potent and multiple metabolic actions [22, 23], and these benefits have been translated from rodents to obese humans with type 2 diabetes without causing side effects such as mitogenicity and hypoglycemia [24, 25]. Growing experimental findings have demonstrated that FGF21 is also a mediator of adaptive responses to tissue injury and repair in a variety of pathological conditions [23, 26, 27]. Although the FGF21-mediated signaling pathways remain unclear, the mechanisms of rFGF21 therapeutic effects may involve beneficial metabolic regulation [28], anti-inflammation [29, 30], antioxidative stress [31–33], inhibition of advanced glycation end products formation [31], and promotion of tissue repair [27, 34, 35].

In the present study, we propose FGF21 as a novel disease-course modification approach candidate. We believe that the multiple metabolic and anti-inflammation properties make rFGF21 an optimal therapeutic candidate for obesity-induced cognitive impairment. Using high-fat diet (HFD)-induced obese mouse model, we tested our hypothesis that rFGF21 administration is effective for improving obesity-induced cognitive dysfunction and anxiety-like behavior, by its multiple metabolic modulation and anti-inflammation actions.

Materials and Methods

Animals and rFGF21 Treatment

Male C57BL/6J mice (5 weeks old) were purchased from Jackson Laboratories. The mice were housed four mice per cage and maintained on high-fat diet chow (HFD; Research Diets 12492) or standard diet chow (SD). Recombinant human FGF21 (rFGF21) was expressed and purified from *Escherichia coli*, following the procedures described previously [36]. The amino acid sequence of the recombinant human FGF21 has been verified by Mass Spec sequencing and the final rFGF21 purity is greater than 95%. Since the half-life of recombinant FGF21 in rodents and primates has been determined to be approximately 1–2 h [37], daily or twice-daily administration is required in preclinical studies to achieve desired pharmacological effects [38]. “According to most experimental studies, feeding time with high fat diet to induce to obesity and cognitive impairment in mice varies from 14 to 40 weeks, thus we selected a prolonged HDF feeding time for 30 weeks” [39, 40]. After 30 weeks, HFD-induced obese mice were randomly divided into three groups. One HFD group was injected subcutaneously with rFGF21 (0.5 mg/kg) twice a day at intervals of about 12 h for 3 weeks, based on established dose regimen of rFGF21 in rodents [38]. One HFD group (HFD consumption for 30 + 3 weeks) and one age-matching SD group received a saline injection as non-treatment controls. Mice were maintained on their respective diets during the rFGF21 treatment period. All experiments were performed following protocols approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Measurements of Body Weight, Blood Glucose, and Glycated Hemoglobin Levels

During the experiment period, body weight and blood glucose were examined every 2 days. Blood glucose was measured with an Accu-Chek glucose meter (Roche Diagnostics, Germany). Blood glycated hemoglobin (HbA1c) level was measured with an A1cNow+ meter (PTS Diagnostics, USA), at 21 days after FGF21 treatment.

Glucose Tolerance Test and Insulin Tolerance Test

Mice were tested for glucose tolerance and insulin tolerance after 1 week of rFGF21 treatment. For the glucose tolerance test (GTT), mice were fasted overnight (16 h) and then intraperitoneally injected (i.p.) with glucose (2 g/kg, Sigma-Aldrich, UK). For the insulin tolerance test (ITT), mice were fasted for 4 h and then injected intraperitoneally with insulin (0.75 U/kg, Sigma-Aldrich, UK). Blood glucose

concentration was measured before and at 15, 30, 60, 90, 120, and 150 min after the glucose or insulin injection with an Accu-Chek glucose meter. The GTT or ITT that corresponds to the area under the curve (AUC) throughout the test period was calculated as previously described [41].

Assessments of Cognitive Function and Anxiety-like Behaviors

After 3 weeks of rFGF21 treatment, animal behavioral tests were assessed between 07 p.m. and 07 a.m. Cognition function was assessed by Y-maze and novel objective recognition tests, and anxiety-like behavior was assessed by elevated plus maze test. In the Y-maze test, mice were transferred from the home cage to the center of the maze and were allowed to explore the three arms freely for 5 min. Each alternation trial was captured by a digital video and the video clips were analyzed offline, in a blind manner as previously described [42]. An entry was recorded when all four limbs were within an arm. The number of arm entries and the number of alternations were recorded in order to calculate alternations, where % alternations = [(number of alternations)/(total arm entries – 2)] × 100. In the novel object recognition test, we followed the standard protocol as previously described [43]. It contains two phases: acquisition phase and trial phase. For the acquisition phase, mice were transferred to the arena in the presence of two identical objects located in two corners. After 10 min of object exploration, the mice were returned back to the home cage. For the trial phase, 24 h after initial exposure, one of the objects was replaced with a novel object, after which the mice were transferred again to the arena for 5 min of object exploration in the presence of one familiar (A) and one novel (B) object. Time spent exploring the novel object (B) was expressed as the recognition index (RI), where $RI = tB/(tA + tB) \times 100$ normalizing all data for statistical analysis. In the elevated plus maze test, we followed the standard protocol as previously described [44]. Briefly, mice were placed on the central platform of an opaque plexiglass maze that is 1 m high with two open arms and two closed arms (Med Associates, St. Albans, VT). Throughout the course of the 5-min trial, entries and time spent in the open and closed arms were monitored and analyzed by an Any-maze computer-operated video tracking system (Stoelting Co., Wood Dale, USA).

Measurement of Serum Insulin, Cytokine, and Lipid Levels of Mice

At 21 days after rFGF21 treatments, blood samples from the three groups of mice were collected via cardiac puncture. After clotting at room temperature for 1 h, blood samples were centrifuged at 3000 rpm/min for 15 min. Serum samples were transferred to new tubes and stored at –80 °C. Serum insulin,

IL-1 β , and TNF α concentrations were measured using a mouse insulin ELISA kit (Crystal Chem, USA), a mouse IL-1 β ELISA kit (Thermo Fisher Scientific, USA), and a mouse TNF α ELISA kit (Life Technologies, USA), respectively, according to the manufacturers' protocols. Serum total cholesterol (TC), triacylglycerol (TG), and high-density lipoprotein (HDL) concentrations were measured using an enzymatic kit (Pointe Scientific, USA) according to the manufacturers' protocols. Serum low-density lipoprotein (LDL) concentration was calculated using the formula $LDL-c = TC - c - HDL-c - (TG-c/5)$ as previously described [45].

Measurements of FGF21 and Receptor Protein Levels in Cerebrospinal Fluid and Hippocampus of Mice

To determine whether rFGF can cross the BBB, at 4 h after the first injection of rFGF21, mice were deeply anesthetized with isoflurane, and cerebrospinal fluid (CSF) was collected with a glass capillary tube from the cisterna magna as described previously [46]. Baseline serum level of FGF21 and FGF21 level in CSF after rFGF21 injection were measured using a mouse FGF21 ELISA kit (Biovendor, USA) according to the manufacturers' protocols. At 4 h after the first injection of rFGF21, mice were transcardially perfused with 0.01 M phosphate buffered saline (PBS) at pH 7.4, and brain tissues were collected for protein extraction. Brain protein levels of FGF21, FGF receptor 1 (FGFR1), phospho-FGFR1 (p-FGFR1), and β -Klotho were examined by western blots.

Reverse Transcription and Real-Time Polymerase Chain Reaction Assay

Following transcardial perfusion with 0.01 M phosphate buffered saline at pH 7.4, white adipose tissue (WAT), cerebral cortex, hippocampus, and hypothalamus tissues of mice were collected and stored at -80°C until processing. Total RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's instructions and reverse-transcribed as previously described [44]. Real-time polymerase chain reaction (PCR) was performed on an ABI 7500 Fast Real-Time PCR system using Taqman gene expression assays for IL-1 β (Mm00434228_m1), IL-6 (Mm00446190_m1), TNF α (Mm00443258_m1), brain-derived neurotrophic factor (BDNF) (Mm04230607_s1), insulin-like growth factor-1 (IGF-1) (Mm00439560_m1), CD206 (Mm01329362_m1), postsynaptic density protein 95 (PSD95) (Mm00492193_m1), synaptophysin (Mm01289818_g1), and for the housekeeping gene B2M (Mm00437762_m1) (Applied Biosystems, USA). Reactions were performed in duplicate according to the manufacturer's instructions. Relative expression levels were measured with the $2^{-\Delta\Delta C_t}$ method.

Western Blot Analysis

Western blot analysis was followed by the standard protocol as we previously described [47]. Protein samples were extracted from the stored hippocampus tissues, and they were homogenized in lysis buffer (Cell Signaling Technology) with protease inhibitor cocktail (Life Technologies) and phosphatase inhibitor cocktail (Sigma). Proteins were separated by 10% SDS-PAGE electrophoresis and transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% nonfat milk for 1 h and incubated overnight at 4°C with the primary antibodies: anti-FGF21 (1:1000, Abcam), anti-FGFR1 (1:200, Santa Cruz), anti-phospho-FGFR1 (1:1000, Abcam), anti- β -Klotho (1:1000, Abcam), anti-Akt (1:200, Santa Cruz), anti-phospho-Akt (1:100, Santa Cruz), anti-GSK-3 β (1:1000, Cell Signaling), anti-phospho-GSK-3 β (1:1000, Cell Signaling), anti-IL-1 β (1:1000, Abcam), anti-TNF α (1:1000, Abcam), anti-IL-6 (1:500, Novus), anti-PSD95 (1:1000, Abcam), anti-synaptophysin (1:1000, Cell Signaling), and anti-actin (1:10,000, Sigma). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized with enhanced chemiluminescence (GE Healthcare). Quantitative densitometry was performed on the protein bands by using ImageJ software.

Immunohistochemistry Analysis

Immunohistochemistry was conducted using the standard protocol as we described previously [47]. For the tissue section, mice were deeply anesthetized with isoflurane and perfused transcardially with PBS followed by 4% paraformaldehyde perfusion. The brain was removed and fixed in 4% paraformaldehyde at 4°C for 24 h. The brains were then transferred to 30% sucrose solution at 4°C until it sank to the bottom of the tube. After dehydration, 20- μm sections of the hippocampus were collected using a freezing microtome (Leica) and stored at -80°C . For double-labeling immunofluorescence staining, sections were incubated in PBS containing 0.1% Triton X-100 for 1 h followed by incubation in 5% fetal bovine serum for 1 h. After that, the sections were incubated overnight in PBS containing antibodies anti-Iba1 (1:1000, Wako Diagnostics) and anti-major histocompatibility complex class II (MHCII) (1:500, BioRad) at 4°C . On the following day, sections were washed with PBS and then incubated with secondary antibody conjugated to fluorescein (1:200, Invitrogen) for 1 h at room temperature. Sections were washed with PBS and then mounted with Vectashield mounting medium containing DAPI after drying. Fluorescence images were obtained using an Olympus inverted fluorescence microscope. The proportion of Iba1/MHCII double-positive cells was expressed relative to the total number of Iba1-positive

cells. Similarly, for doublecortin (DCX) labeling immunofluorescence staining, the sections were incubated overnight in PBS containing antibodies anti-DCX (1:200, Santa Cruz) at 4 °C. On the following day, sections were incubated with secondary antibody conjugated to fluorescein (1:200, Invitrogen) for 1 h after being washed in PBS. Two serial brain sections from each animal were used, and quantification was performed in the dentate gyrus (DG) areas of the hippocampus. The mean cell number in four DG areas was calculated for each animal. All counts were performed in a blind manner.

Statistical Analysis

All data are expressed as means \pm SEM and were analyzed using one-way ANOVA with post hoc Bonferroni-corrected *t* tests or one-way repeated measures ANOVA or unpaired *t* tests. Analyses were performed in GraphPad Prism version 5.0, and *P* < 0.05 was considered statistically significant.

Results

rFGF21 Corrects Cognitive Impairment and Anxiety-like Behavior in HFD Obese Mice

At 3 weeks after rFGF21 treatment, we examined and compared HFD-induced cognitive impairment and anxiety-like behavior between SD-, HFD-, and FGF21-treated HFD mice. In the Y-maze test, compared to SD control mice, HFD mice showed impaired spatial recognition memory with reduced alternations and number of arm entries, but the spatial memory deficits were eliminated in rFGF21-treated HFD mice (Fig. 1a, b). In another recognition test, the novel objective recognition test, there was no significant difference in the recognition index between the three groups, indicated by an RI value close to 50.0% in the acquisition phase (Fig. 1c). During the trial phase at 24 h later, HFD mice did not increase preference for the novel object (RI value was about 49.6%), while the increased preference was observed in rFGF21-treated HFD mice (RI value was about 60.5%) compared to the SD mice (Fig. 1d). In an anxiety-like behavior test, the elevated plus maze test, HFD mice showed increased anxiety with significantly less time spent within the open arms and number of open arm entries compared with SD mice, but the increased anxiety-like behavior was eliminated in rFGF21-treated mice (Fig. 1e, f). These results demonstrate that rFGF21 treatment (0.5 mg/kg, i.p. daily for 3 weeks) is able to reverse the HFD-induced cognitive impairment and anxiety-like behavior of obese mice.

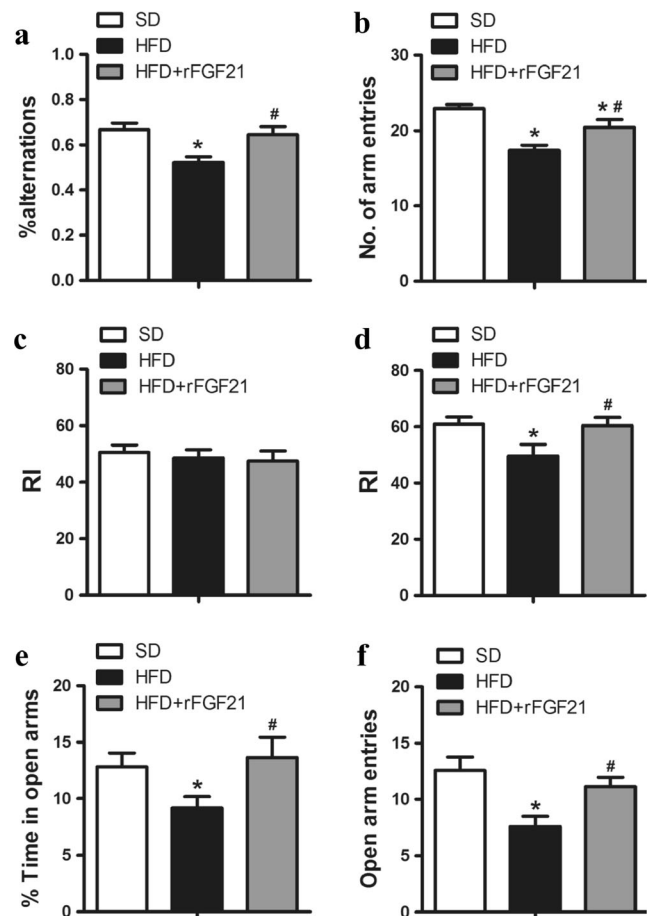


Fig. 1 rFGF21 corrects HFD-induced cognitive impairment and anxiety-like behavior in mice. **a** Y-maze: alternations of mice; **b** number of arm entries. **c** Novel object recognition test: recognition index (RI) in acquisition phase; **d** recognition index (RI) in trial phase. **e** Elevated plus maze: time spent in open arms (%); **f** numbers of open arm entries. Data are expressed as mean \pm SE, *n* = 14 per group. **P* < 0.05 versus SD; #*P* < 0.05 versus HFD

rFGF21 Corrects Glucose Metabolic Disorder and Insulin Resistance in HFD Obese Mice

After 33 weeks of HFD feeding, HFD mice (38 weeks old) were approximately 58.9% heavier than SD mice (Fig. 2a), and blood glucose were approximately 30.2% higher than SD mice (Fig. 2b), respectively. Blood HbA1c was also significantly increased in HFD mice, but lower than the cutoff point for diagnosing diabetes (Fig. 2c). During the 3 weeks of rFGF21 treatment period (0.5 mg/kg, i.p. daily for 3 weeks), the body weight and blood glucose of HFD mice were gradually decreased. At the end of the 3-week treatment, rFGF21 decreased the body weight and blood glucose level of HFD mice in about 24.1% (Fig. 2a) and 24.7% compared with nontreatment HFD control mice (Fig. 2b), respectively. rFGF21 also significantly reduced the elevated blood HbA1c level of HFD mice from 5.2 to 4.9% (Fig. 2c). These data demonstrate that rFGF21 treatment in the

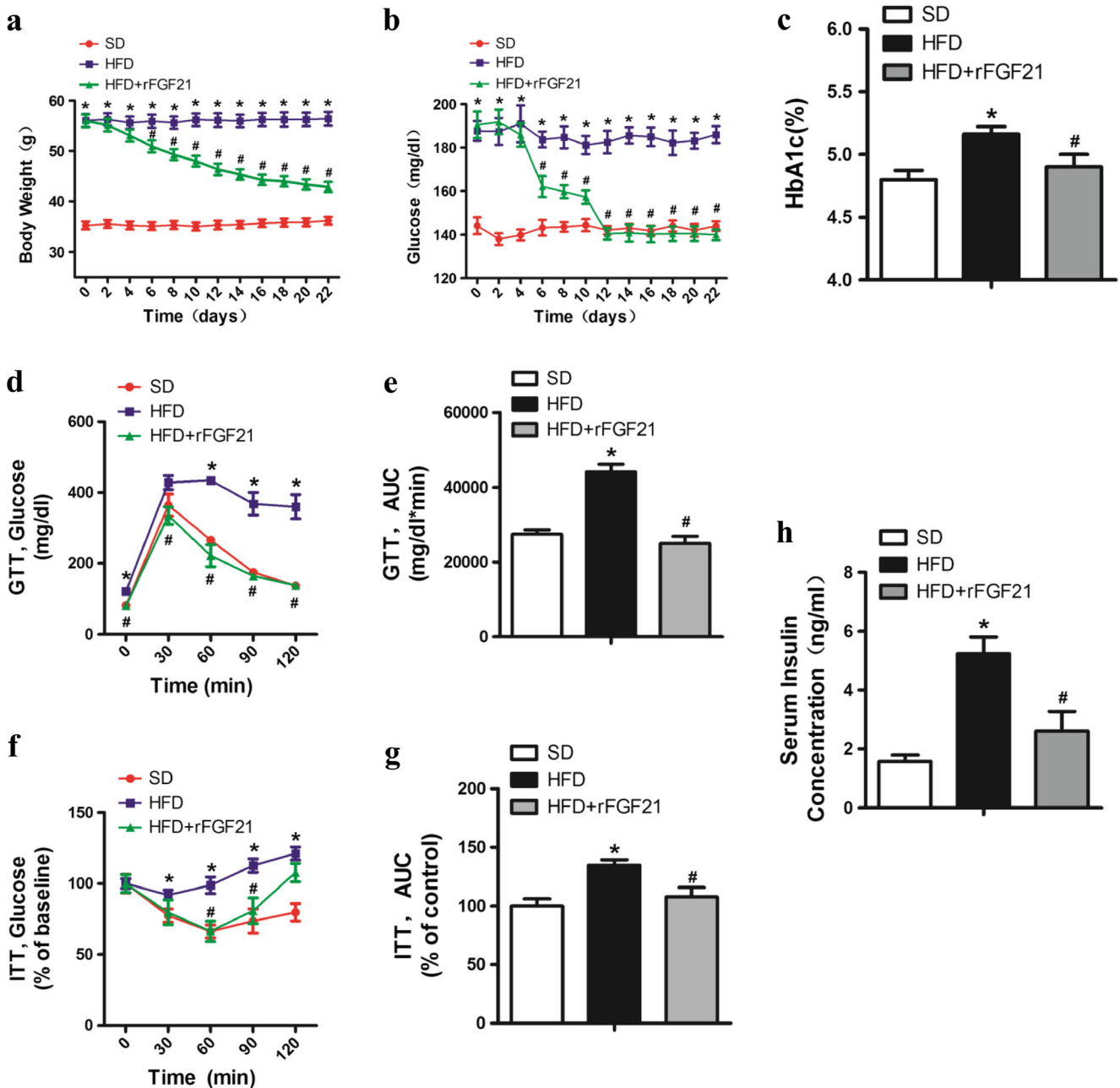


Fig. 2 rFGF21 corrects glucose metabolic disorder and insulin resistance in HFD mice. **a** Body weight changes of mice fed with standard diet (SD), high-fat diet (HFD), HFD mice treated with rFGF21 (HFD + rFGF21). *n* = 14 per group. **b** Blood glucose concentration changes of SD-, HFD-, and rFGF21-treated mice under feeding condition. *n* = 14 per group. **c** Blood HbA1c changes of SD-, HFD-, and rFGF21-treated mice. *n* = 8 per group. **d** Blood glucose concentration changes at indicated times after intraperitoneal injection of glucose (2 mg/g), *n* = 4 per group. **e** Values of glucose tolerance test (GTT) were calculated and compared by area

under the curve (AUC) of each group of mice. **f** Blood glucose concentration changes over the baseline level at indicated times after intraperitoneal injection of insulin (0.75 U/kg), *n* = 4 per group. **g** Values of insulin tolerance test (ITT) were calculated and compared by the percentage changes of area under the curve (AUC) over the SD mouse controls. **h** Serum insulin concentrations of each group of mice, *n* = 8 per group. Data are expressed as mean \pm SE. **P* < 0.05 versus SD; #*P* < 0.05 versus HFD

HFD-induced obese mouse model shows beneficial glucose metabolic modulation of elevated body weight, blood glucose, and blood HbA1c level.

To investigate HFD-induced glucose tolerance and insulin tolerance impairment and the rFGF21 effects, we measured

and compared blood insulin concentration and glucose tolerance and insulin tolerance tests between the SD-, HFD-, and FGF21-treated HFD mice. Since treatment with FGF21 for about 1 week already significantly reduced body weight and blood glucose levels in HFD mice as shown in Fig. 2a, b, we

thus selected the 1-week time point of rFGF21 treatment to assess glucose tolerance and insulin tolerance tests. Our experimental data showed that HFD mice had glucose tolerance impairment compared to SD mice, where rFGF21 treatment for 1 week completely rescued the glucose tolerance impairment (Fig. 2d, e). Consistently, HFD mice showed insulin tolerance impairment compared to SD mice, and rFGF21 treatment for 1 week significantly corrected the insulin tolerance impairment or insulin resistance (Fig. 2f, g). Lastly, we found that HFD-induced obese mice in this experiment had hyperinsulinemia (231.1% increase compared to SD mice), and rFGF21 treatment for 21 days significantly reduced hyperinsulinemia (65.0% increase compared to SD mice) (Fig. 2h). Together, these data demonstrate that HFD-induced obese mice in this study have significant glucose metabolic dysregulation indicated by glucose tolerance impairment and insulin resistance, and rFGF21 shows potent glucose metabolic modulation in these HFD mice.

rFGF21 Corrects Hyperlipidemia in HFD Obese Mice

Long-term HFD feeding in mice may also induce abnormal lipid metabolism in mice. We thus investigated the alteration of lipid metabolism and the effects of rFGF21 in these HFD mice. Compared to SD mice, HFD mice showed significantly elevated serum concentrations of TC (Fig. 3a), HDL (Fig. 3b), LDL (Fig. 3c), and LDL/HDL ratio (Fig. 3d), but no significant change for TG (Fig. 3e) was detected. These data indicate hyperlipidemia to be present in these obese mouse models. However, rFGF21 treatment significantly reduced the elevation of serum TC (38.7% reduction), HDL (35.6% reduction), LDL (43.5% reduction), and LDL/HDL ratio (12.5% reduction), respectively, in HFD mice (Fig. 3a–d). These results demonstrate a potent and beneficial effect of rFGF21 in the modulation of hyperlipidemia in HFD obese mice.

rFGF21 Reduces Systemic Pro-inflammation of HFD Obese Mice

Chronic systemic inflammation initiated in white adipose tissue plays a crucial role in the development of obesity-related insulin resistance and neuroinflammation [2, 48, 49]. We thus examined and compared messenger RNA (mRNA) levels in WAT and protein levels in circulation of the pro-inflammatory cytokines IL-1 β , IL-6, and TNF α . Our results showed in WAT of HFD mice that IL-1 β , IL-6, and TNF α mRNA levels are about 0.98-, 2.16-, and 3.48-fold higher than those in WAT of HFD mice compared to SD control mice, respectively. rFGF21 treatment for 3 weeks significantly reduced these three elevated cytokine mRNA levels by about 33.5, 48.9, and 27.6%, respectively (Fig. 4a). Using ELISA assays, we detected significant blood concentration increases of pro-

inflammatory cytokines TNF α (95.5% increase) and IL-1 β (73.0% increase) in HFD mice compared to the SD control mice, but the increases were completely eliminated by the rFGF21 treatment (Fig. 4b, c), demonstrating a potent antisystemic inflammation effect of rFGF21 in the HFD obese mouse model.

rFGF21 Can Cross the Blood-Brain Barrier and Activates FGF Receptor 1 in the Hippocampus of HFD Obese Mice

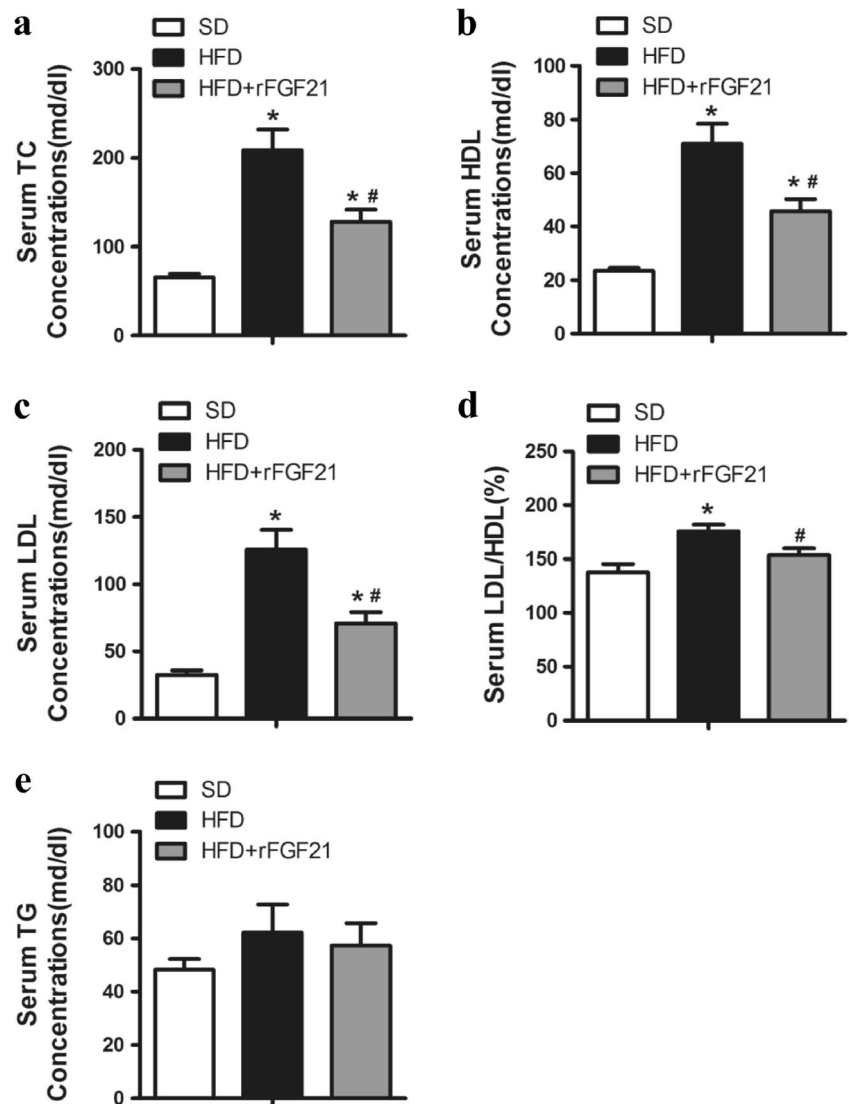
The baseline blood FGF21 level in HFD mice was significantly elevated to about 5.6-fold compared to the SD controls, demonstrating FGF21 resistance in the HFD obese mouse model (Fig. 5a). We also detected a low but slightly higher baseline level of FGF21 in the CSF of HFD mice (7.84 pg/ml) than SD mice (6.04 pg/ml), but there was no significant difference between the two groups of mice (Fig. 5b). We then examined BBB penetration capability of rFGF21 in both HFD and SD mice. At 4 h after the first injection of rFGF21 (0.5 kg/ml, i.p.), FGF21 concentration in CSF was significantly increased by about 9.27 times in SD mice and 9.69 times in HFD mice, respectively, compared to the baseline levels, but there was no significant difference between the SD and HFD mice (Fig. 5b).

To further evaluate FGF21 signaling alterations in the brain after rFGF21 injection, hippocampus tissues were harvested for protein extraction and western blot analysis. There was no significant difference in FGF21 levels of hippocampus tissues between SD and HFD mice; however, there was a significant increase of FGF21 (30.0% increase) at 4 h after rFGF21 injection in the HFD mice. We did not detect significant difference in FGF21 co-receptors FGF receptor 1 (FGFR1) and β -Klotho protein levels between SD and HFD mice, before and after rFGF21 injection. However, there was a significant increase of p-FGFR1 protein expression (47.8% increase) at 4 h after initial rFGF21 injection, suggesting increased FGFR1 activation (Fig. 5c, d). The results indicated that HFD mice were FGF21 resistant, characterized by higher FGF21 concentration in the blood but not in the CSF and brain hippocampus tissues. rFGF21 was able to cross the BBB and activate its receptor FGFR1 in the hippocampus.

rFGF21 Suppresses Microglia Activation and Pro-inflammatory Cytokine Expression in the Hippocampus of HFD Obese Mice

To investigate neuroinflammation in the brain hippocampus of HFD mice and the effects of rFGF21, we examined microglia activation using co-labeling for microglia cell marker Iba1 and activated microglia cell (M1-type microglia/macrophage) marker MHCII in the hippocampal dentate gyrus of SD-, HFD-, and rFGF21-treated HFD mice [50]. We found that the Iba1-positive cell numbers were significantly increased

Fig. 3 rFGF21 corrects hyperlipidemia in HFD mice. **a** Serum total cholesterol (TC) concentrations. **b** Serum high-density lipoprotein (HDL) concentrations. **c** Serum low-density lipoprotein (LDL) concentrations. **d** Serum LDL/HDL ratios. **e** Serum triglyceride (TG) concentrations. Data are expressed as mean \pm SE, $n = 8$ per group. * $P < 0.05$ versus SD; # $P < 0.05$ versus HFD



in HFD mice (48.5% increase) compared to SD mice, indicating an increase of microglia/macrophage activation (Fig. 6a, b). Additionally, the ratio of positive staining for MHCII to Iba1-positive cells was also significantly increased in HFD mice compared with SD mice, further suggesting the increase of M1-like microglia/macrophage (Fig. 6a, c). However, M2-like microglia/macrophage-positive cells were rarely detectable, and no clear difference was detected between the three groups of mice (data not shown here). Importantly, rFGF21 treatment significantly reduced the Iba1-positive cell numbers (20.1% reduction) and MHCII/Iba1 double-positive cell numbers (20.3% reduction) (Fig. 6a–c). These results suggest that the majority of the increased activation of microglia/macrophage in the hippocampus of HFD mice was M1-polarized microglia/macrophage, which can be significantly suppressed by rFGF21 treatment.

To further evaluate the inflammatory profiles in the hippocampal tissues, we measured mRNA levels of pro-inflammatory cytokines and anti-inflammatory trophic factors using RT-PCR. In HFD mice, hippocampal IL-1 β , IL-6, and TNF α mRNA levels were significantly higher by about 59.2, 19.9, and 60.9%, respectively, compared to SD mice. rFGF21 treatment significantly reduced the elevation by about 60.6% for IL-1 β , 26.8% for IL-6, and 25.8% for TNF α mRNA levels, respectively (Fig. 6d). While mRNA levels of BDNF, IGF-1, and CD206 were not significantly altered in HFD mice compared to SD mice, rFGF21 treatment had no effects on these mRNA levels of HFD mice (Fig. 6e). These results suggest that there was an elevated pro-inflammatory response in the hippocampus of HFD mouse brains; rFGF21 can eliminate the pro-inflammatory cytokine gene expression elevation. Additionally, we also observed similar elevation of pro-inflammatory cytokine mRNA levels in the cortex and

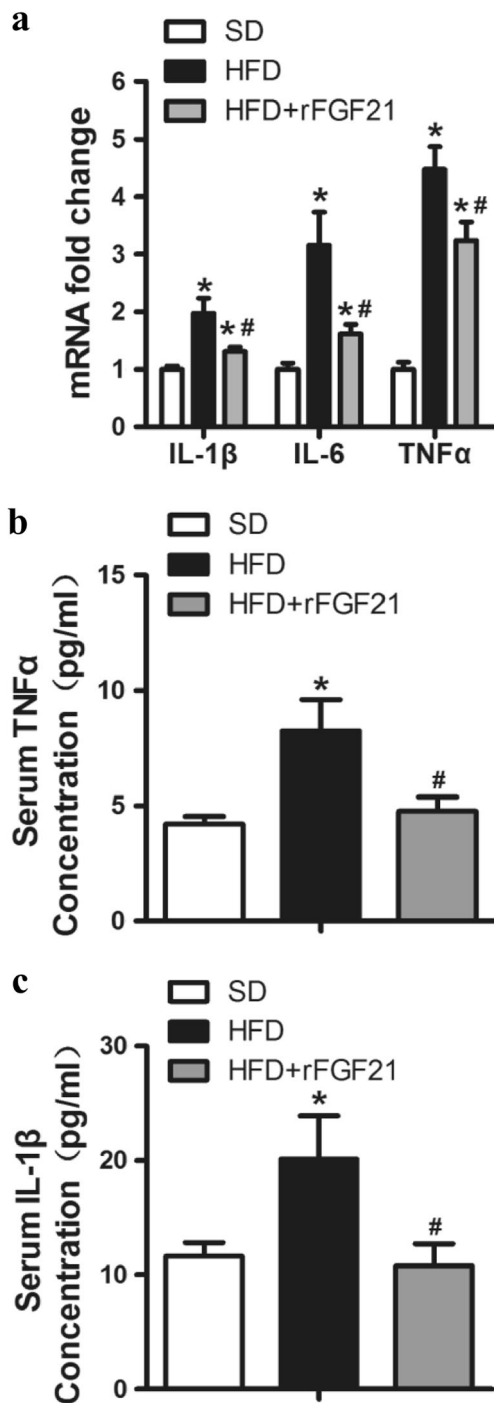


Fig. 4 rFGF21 reduces systemic pro-inflammation of HFD mice. **a** Relative fold change of IL-1 β , IL-6, and TNF α mRNA levels in white adipose tissue (WAT), $n = 5$ per group. **b** Serum TNF α concentrations, $n = 8$ per group. **c** Serum IL-1 β concentrations, $n = 8$ per group. Data are expressed as mean \pm SE. * $P < 0.05$ versus SD; # $P < 0.05$ versus HFD

hypothalamus of HFD mice, and they were also significantly reduced by rFGF21, indicating a broad pro-inflammatory status of the central nervous system, and protective effects of rFGF21 in the HFD obese mice (Supplement Fig. 1). Furthermore, by western blot analysis, we confirmed that

protein levels of hippocampal IL-1 β , IL-6, and TNF α were significantly increased in HFD mice compared to SD mice by about 24.5, 88.1, and 52.8%, respectively. Importantly, the IL-1 β , IL-6, and TNF α protein expression increases were almost eliminated by rFGF21 treatment (Fig. 6f, g).

rFGF21 Modulates Akt/GSK-3 β Signaling in the Hippocampus of the HFD Obese Mice

It has been reported that the Akt/GSK-3 β pathway plays a vital role in mediating neuroinflammation in the hippocampus, which likely contributes to obesity and diabetes in aging mice [51, 52]. We thus measured activation of Akt/GSK-3 β signaling in the hippocampus by western blot analysis. In hippocampal tissues of HFD mice, Akt was deactivated by reducing its phosphorylation, while GSK-3 β was activated by decreasing its phosphorylation compared to SD mice. rFGF21 treatment rescued Akt activation level but reversed GSK-3 β activation status (Fig. 7a, b), demonstrating a potent role of rFGF21 in modulating Akt/GSK-3 β signaling in the hippocampus of the HFD obese mouse model.

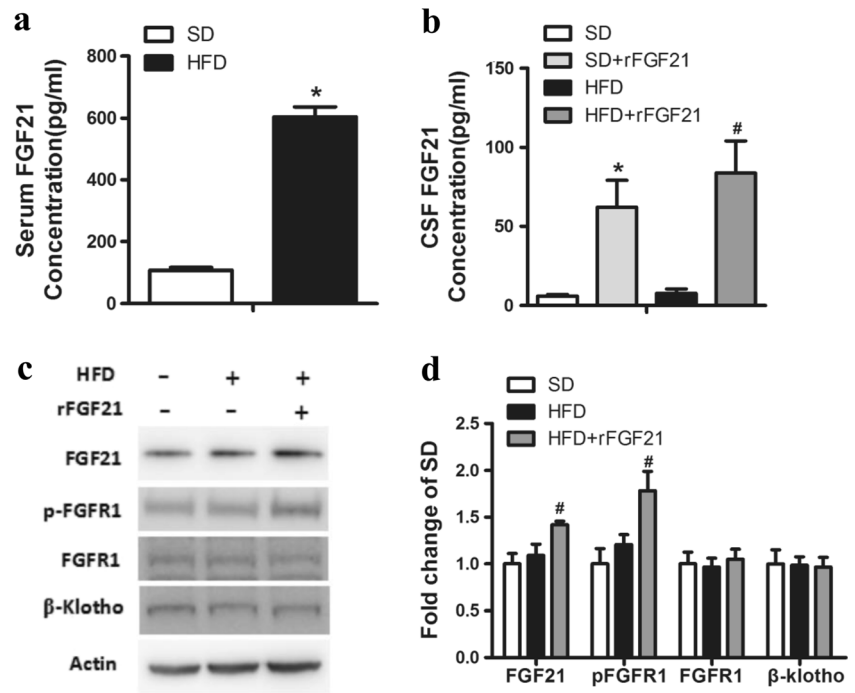
rFGF21 Promotes Hippocampal Neurogenesis but not Synaptic Plasticity in HFD Obese Mice

It has been reported that HFD may impair hippocampal synaptic plasticity and neurogenesis [2, 9, 10, 12, 53]. Thus, we examined the deficits of hippocampal synaptic plasticity and neurogenesis and the effects of rFGF21. For hippocampal synaptic plasticity in HFD mice, we measured mRNA and protein levels of the presynaptic marker synaptophysin and the postsynaptic marker PSD95 using RT-PCR and western blot analysis. Compared to SD mice, mRNA and protein levels of hippocampal synaptophysin and PSD95 were not significantly altered in HFD mice, and no effects by rFGF21 treatment in the synaptic markers in HFD mice were detected (Fig. 8a–c), indicating that there were no or limited hippocampal synaptic plasticity deficits in our HFD obese mouse model. For hippocampal neurogenesis in HFD mice, we used immunohistochemistry to quantify changes of neuronal precursor cell marker DCX-positive cell numbers in the hippocampal dentate molecular layer of SD-, HFD-, and rFGF21-treated HFD mice. DCX-positive cell numbers were significantly reduced in HFD mice compared to SD mice, whereas rFGF21 treatment significantly increased the DCX-positive cell numbers. The results suggest an impaired hippocampal neurogenesis in HFD mice, which can be reversed by rFGF21.

Discussion

Obesity and the consequent metabolic dysfunctions are strongly linked to metabolic syndrome-associated cognitive

Fig. 5 rFGF21 can cross blood-brain barrier (BBB) and activate FGF receptor 1 (FGFR1) in hippocampus of mice. **a** Baseline levels of serum FGF21 concentrations in SD and HFD mice. **b** CSF FGF21 concentrations at 4 h after initial rFGF21 injection. **c** Representative gel images of western blot analysis for FGF21, FGFR1, p-FGFR1, and β -Klotho protein expression. Actin served as an equal loading control. **d** Quantification of western blot analysis (fold change of SD mouse controls). Data are expressed as mean \pm SE, $n = 5$ per group. * $P < 0.05$ versus SD; # $P < 0.05$ versus HFD



impairment and dementia [4]. In this study, 5-week-old male mice were fed with HFD for 30 weeks. As expected, these HFD mice exhibited obesity and cognitive function decline (Figs. 1 and 2). The major findings of this study are (1) rFGF21 is a potent metabolic regulator, which is able to quickly reverse HFD-induced metabolic disorders including obesity, glucose tolerance impairment, insulin resistance, hyperlipidemia, and systemic pro-inflammation. (2) Higher FGF21 concentration in circulation, but not in CSF and brain tissues of HFD obese mice, indicates systemic FGF21 resistance in HFD mice; systemic administration of exogenous rFGF21 can cross the BBB and activate FGF21 receptor FGFR1 in the brain, suggesting its biological action in the brain. (3) There was an increased activation of M1-polarized microglia/macrophage and elevated pro-inflammatory cytokine expression in the hippocampus of HFD obese mice, and these changes can be reversed by rFGF21. (4) rFGF21 modulates Akt deactivation/GSK-3 β activation-mediated neuroinflammation signaling, promoting neurogenesis but not synaptic plasticity in the hippocampus of HFD obese mice.

One of the most unique biological features of FGF21 is its ability to exert potent and multiple tropic metabolic effects [22, 23]. Both glucose and insulin tolerance impairments are demonstrated to be essential and important pathogenic factors in the development of metabolic dysfunction-associated cognitive impairment [54]; here we show that rFGF21 treatment for only 1 week completely reversed glucose tolerance and insulin tolerance impairments in HFD obese mice (Fig. 2d–g). Importantly, insulin resistance is one major risk factor of cognitive impairment in patients with obesity/diabetes or

metabolic syndrome [55]. Here we show as expected that HFD-induced hyperinsulinemia was dramatically reduced by rFGF21 treatment (Fig. 2h), which confirmed the strong metabolic regulation of rFGF21 for reversing insulin resistance in the HFD obese mice [56]. Additionally, we found that rFGF21 treatment can also significantly reduce the elevation of serum total cholesterol and LDL/HDL ratio (Fig. 3a–d), and further confirmed a potent and beneficial effect of rFGF21 for modulating hyperlipidemia in the HFD obese mice as previously reported [57]. While hyperlipidemia is an independent risk factor for cognitive impairment which is not defined, its association with dementia has been established [58]. Hence, it has been demonstrated that dyslipidemia or lipid metabolic dysfunctions are typical features of the metabolic syndromes and may be associated with a pro-inflammatory gradient, which in part may originate in the adipose tissue itself and directly affect the endothelium and brain functions [59]. Taken together, all of these potent and beneficial metabolic regulation effects of rFGF21 may play a key role for improving cognitive impairment of HFD-induced obese mice [5, 60].

In addition to metabolic dysfunction, pro-inflammation initiated in white adipose tissue plays a crucial role in metabolic syndrome-associated cognitive function decline [2]. In this study, we found that there was an elevation of pro-inflammatory cytokines IL-1 β , IL-6, and TNF α mRNA levels in WAT of HFD obese mice; for the first time, we showed that increased mRNA levels of these pro-inflammatory cytokines were significantly inhibited by rFGF21 (Fig. 4a). Importantly, rFGF21 also significantly eliminated the increases of circulating pro-inflammatory cytokines TNF α and IL-1 β protein

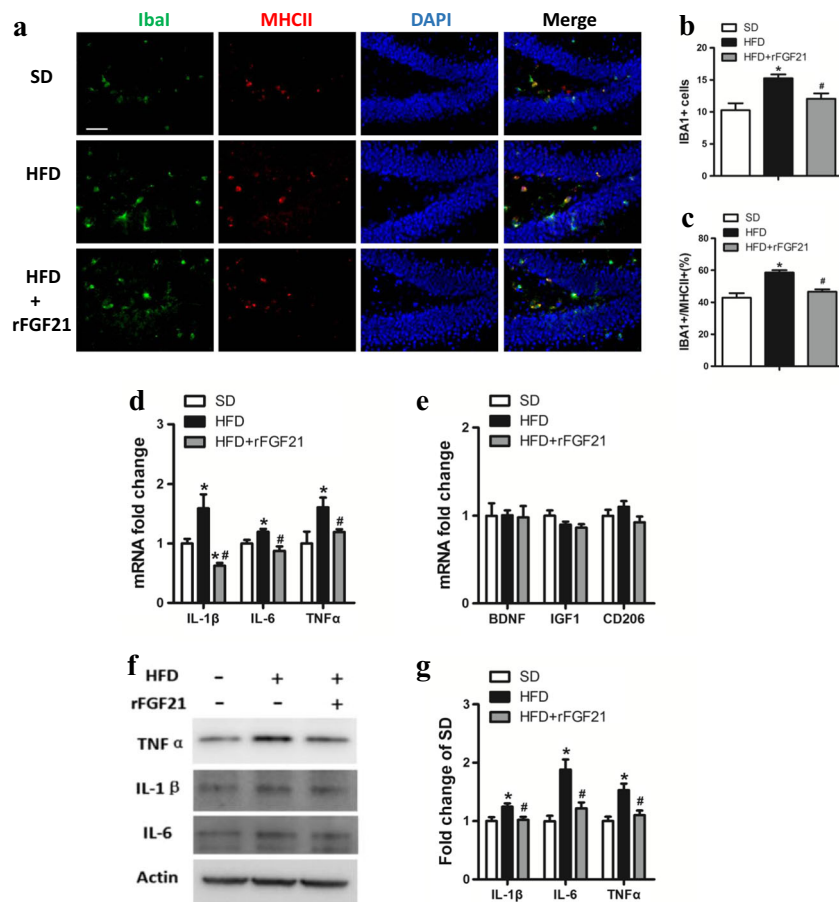


Fig. 6 rFGF21 reduces microglia activation and pro-inflammatory cytokine expression in hippocampus of HFD mice. **a** Representative immunostaining images of ionized calcium binding adapter molecule 1 (*Iba1*; green), major histocompatibility complex II (*MHCII*; red), 4',6-diamidino-2-phenylindole (*DAPI*; blue), and their merged ones in dentate gyrus (DG) of hippocampus. Scale bar = 50 μ m. **b** Quantification of Iba1-positive microglia cell numbers in the hippocampal dentate gyrus. **c** Quantification of Iba1 and MHCII double-positive microglia cell

numbers in the hippocampal dentate gyrus. **d** Relative fold change of IL-1 β , IL-6, and TNF α mRNA levels in brain hippocampus. **e** Relative fold change of BDNF, IGF-1, and CD206 mRNA levels in brain hippocampus. **f** Representative gel images of western blot analysis for IL-1 β , IL-6, and TNF α protein expression. Actin served as an equal loading control. **g** Quantification of western blot analysis (fold change of SD mouse controls). Data are expressed as mean \pm SE, $n = 5$ per group. * $P < 0.05$ versus SD; # $P < 0.05$ versus HFD

levels (Fig. 4b, c), demonstrating a potent antisystemic inflammation effect of rFGF21 in the HFD obese mouse model.

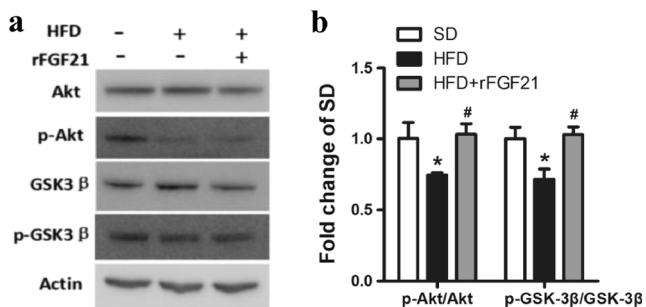
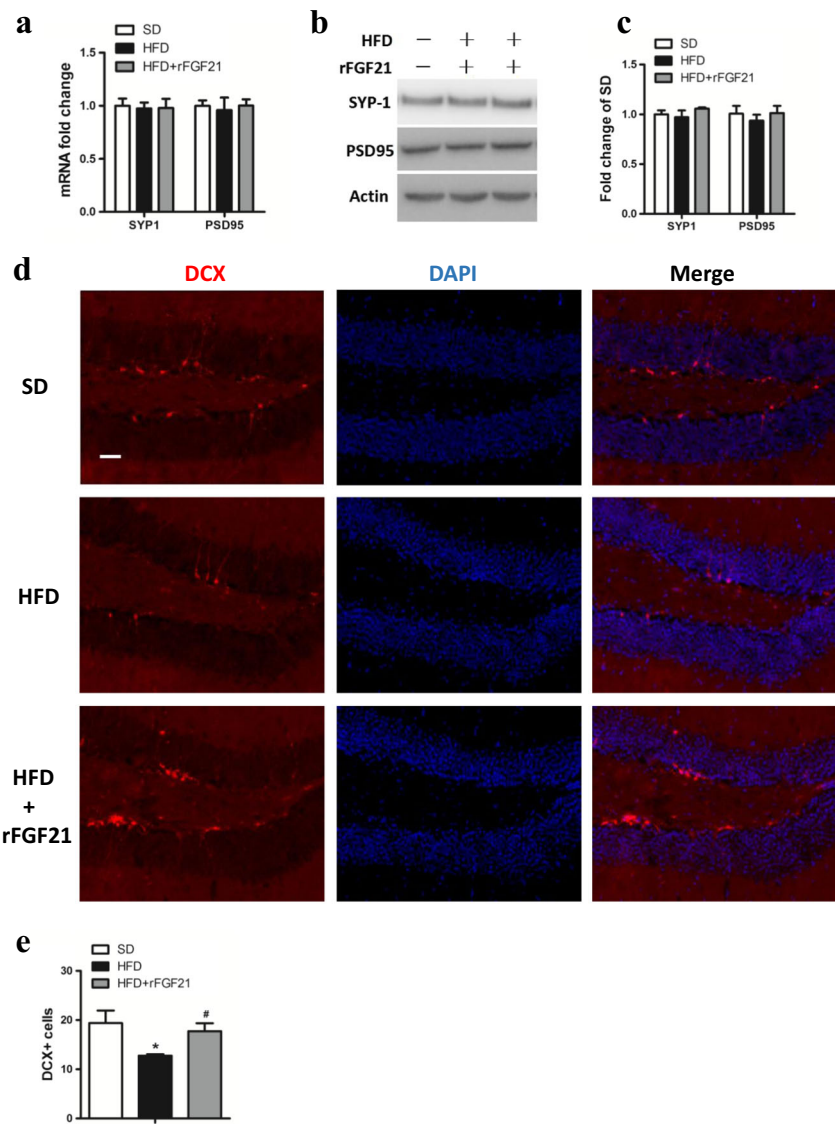


Fig. 7 rFGF21 modulates Akt/GSK-3 β signaling in hippocampus of HFD mice. **a** Representative gel images of western blot analysis for Akt, p-Akt, GSK-3 β , and p-GSK-3 β protein expression. Actin served as an equal loading control. **b** Quantification of western blot analysis (fold change of SD mouse controls). Data are expressed as mean \pm SE, $n = 6$ per group. * $P < 0.05$ versus SD; # $P < 0.05$ versus HFD

Interestingly, FGF21 levels are paradoxically increased in the serum of human subjects with T2D and obesity, as well as in obese diabetic rodents [19]. The elevation of FGF21 could be a compensatory protective response to underlying metabolic stress or might be due to impaired FGF21 signaling of β -Klotho and FGFR downregulation [61], called FGF21 resistance that implies the need for supraphysiological doses of rFGF21 to achieve therapeutic efficacy [19, 22, 62]. Thus, in this study, we for the first time tested whether there are altered FGF21 levels in the circulation versus the brain of obese mice. We found that HFD obese mice had about 6 times higher serum FGF21 concentration than the SD mice (Fig. 5a); however, we did not detect a significant difference of FGF21 concentration in the CSF and brain tissues between HFD mice and control SD mice (Fig. 5b–d). Since FGF21 signaling requires activation of FGFRs, specifically FGFR1 and its co-receptor β -Klotho complex [63], they are broadly expressed in the brain [64–66]. However, we did not detect significant

Fig. 8 rFGF21 promotes hippocampal neurogenesis but not synaptic plasticity of HFD mice. **a** Relative fold change of hippocampal synaptophysin (*SYP-1*) and PSD95 mRNA levels. **b** Representative gel images of western blot analysis for SYP-1 and PSD95 protein expression in hippocampus. Actin served as an equal loading control. **c** Quantification of western blot analysis (fold change of SD mouse controls). **d** Representative immunostaining images of newly born neuron marker doublecortin (*DCX*; red) in dentate gyrus (DG) of hippocampus. Scale bar = 50 μ m. **e** Quantification of DCX positive cells in the hippocampal dentate gyrus. Data are expressed as mean \pm SE, $n = 6$ per group. * $P < 0.05$ versus SD; # $P < 0.05$ versus HFD



differences in FGF21 co-receptors FGF receptor 1 (FEFR1) and β -Klotho protein levels between SD and HFD mice (Fig. 5c, d). All these new experimental findings suggest that HFD-induced obese mice develop a systemic FGF21 resistance. This systemic resistance is not seen in the brain, or there might be a separate developmental progress in the FGF21 resistance, at least in the early metabolic disorder stage of obesity.

Importantly, it has been speculated that FGF21 might have direct brain actions, because a very low heparin-binding affinity makes FGF21 capable of crossing the BBB by simple diffusion [21]; it is detectable in both human and rodent CSF [21, 67]. For the first time, we found FGF21 concentrations in the CSF and hippocampal tissues were significantly increased in both HFD and SD mice at 4 h after rFGF21 injection, and the rFGF21 injection also significantly increased p-FGFR1 expression, demonstrating that rFGF21

could cross the BBB and activate FGF21-FGFR1 signaling in the hippocampus (Fig. 5b–d).

It has been thought that hippocampus dysfunction is considered to be a key and central mechanism that underlies cognitive impairment, including neuroinflammation, synaptic plasticity, and neurogenesis impairment in the hippocampus, although brain pathophysiology related to obesity-associated cognitive impairment is complex and poorly known [10, 12, 13]. To investigate hippocampal neuroinflammation and rFGF21 effects in HFD obese mice, we evaluated microglia activation, pro-inflammatory cytokine expression, and Akt/GSK-3 β signaling in the hippocampus. We detected significant increases of microglia/macrophage marker Iba1-positive cells and M1-type microglia/macrophage marker MHCII-positive cells in the hippocampal dentate gyrus of HFD mice, and importantly, microglia/macrophage activation can be significantly suppressed by rFGF21 treatment (Fig. 6a–c). Since

increased M1-type microglia/macrophage activation evokes pro-inflammatory cytokine expression [68], we next measured mRNA levels of pro-inflammatory cytokines and anti-inflammatory trophic factors using RT-PCR. In HFD mice, we found that hippocampal IL-1 β , IL-6, and TNF α mRNA and protein levels were significantly increased, but the increases were significantly reduced by rFGF21 treatment (Fig. 6d–g). On the other hand, mRNA levels of anti-inflammatory trophic factors BDNF, IGF-1, and CD206 were not significantly altered in HFD mice (Fig. 6e). To explore the potential signaling regulation of rFGF21, we then examined the Akt/GSK-3 β pathway alteration. It has been known that Akt deactivation and GSK-3 β activation play dominant roles in promoting neuroinflammation [69, 70]. We found that Akt was deactivated by reducing its phosphorylation, while GSK-3 β was activated by decreasing its phosphorylation in hippocampal tissues of HFD mice; however, rFGF21 treatment rescued Akt deactivation and reversed GSK-3 β activation status (Fig. 7a, b), demonstrating a potent role of rFGF21 in Akt/GSK-3 β signaling modulation.

It has been reported that elevated pro-inflammation in the hippocampus may consequently lead to synaptic plasticity and neurogenesis impairment [2, 9, 10, 12, 53]. Surprisingly, we did not detect hippocampal synaptic plasticity deficits in HFD obese mice by measuring mRNA and protein levels of the presynaptic marker synaptophysin and the postsynaptic marker PSD95 (Fig. 8a–c). These results are not consistent with some published reports, perhaps due to the different models used or sensitivity limitation on the endpoint assessments [71]. Furthermore, we cannot exclude the possibility that other mechanistic markers of synaptic plasticity deficits might be altered in this model, such as internalization of synaptosomes, hippocampal dendritic spine density, and long-term potentiation [53, 72], and all these remain to be defined in future investigation.

The hippocampus has an important function for learning and memory, and continuous hippocampal neurogenesis implies that newborn neurons replace dying cells and form functional synapses [73]. Adult hippocampal neurogenesis may be negatively affected by aging and stresses such as oxidative damage, neuroinflammation, and cerebral metabolic dysfunction [74]. It has been reported that in high-fat diet-induced obese mice, hippocampal neurogenesis deficit plays an important role for the cognitive function impairment [75, 76]. In this study, we detected a significant neurogenesis deficit in the hippocampal dentate molecular layer, which was significantly rescued by rFGF21 treatment (Fig. 8d, e). This promoting effect of neurogenesis might be one of the important underlying mechanisms for the cognitive function improvement by rFGF21. Taken together, these new findings demonstrate that rFGF21 can significantly eliminate the HFD-induced hippocampal dysfunction, mainly by suppressing hippocampal microglia/macrophage activation and pro-inflammatory

cytokine expression, modulating Akt activation/GSK-3 β deactivation signaling, and promoting neurogenesis. These hippocampal dysfunction improvements may in part underlie the rFGF21-mediated restoration of the HFD-induced cognitive function impairment of obese mice.

Interestingly, a recent publication also showed that rFGF21 significantly inhibited cognitive function decline in HFD obese male rats (HFD consumption for 12 weeks); possible mechanisms were speculated, including restoration of synaptic plasticity, brain mitochondrial function, and cell apoptosis [77]. In the present study, we tested a relatively higher rFGF21 dose regimen (0.5 mg/kg, i.p. twice a day daily for 3 weeks) in a relatively severe obese mice model (HFD consumption for 30 weeks). We did not detect synaptic plasticity deficits, and the assessments for brain cell mitochondrial function, oxidative stress, and apoptosis were not proposed in this study. However, some data of the present study are consistent with this published work, including rFGF21 effects for improving cognitive function and metabolic modulation and reducing blood TNF α level in the HFD-induced obese insulin-resistant rats [77]. More importantly, our present study confirmed the beneficial effects of rFGF21 in attenuating cognitive decline of rodent obese models and provided new insight of the underlying mechanisms.

In addition to supporting our hypothesis, our experimental findings also provide new information related to FGF21 biology and pharmacology. For the first time, we demonstrated that rFGF21 could efficiently suppress pro-inflammatory cytokine expression in WAT of HFD obese mice, which might be its essential mechanism for systemic pro-inflammation inhibition [2]. We also found that HFD-induced obese mice develop a systemic FGF21 resistance, but the detected increase of FGF21 in circulation is not detectable in the CSF, suggesting that there might be different processes or pathological cascade for the FGF21 resistance between peripheral organs and the central nervous system, at least in the early metabolic disorder stage of obesity. Lastly, the increased CSF FGF21 concentration after rFGF21 systemic administration demonstrates that, in addition to the systemic action, rFGF21 has a direct access to the brain by crossing the BBB and biologically activating FGF21-FGFR1 signaling in the brain.

Although our experimental findings are both fundamentally and translationally significant, several limitations still exist. First, the link between systemic metabolic modulation and hippocampal dysfunction remains to be fully assessed. rFGF21 was able to correct glucose and tolerance impairment and systemic insulin resistance, eliminating hippocampus dysfunction, but how the systemic versus brain actions of rFGF21 contribute to the underlying mechanisms, and what is the crosstalk between metabolic disorders versus hippocampal dysfunctions need to be further explored. Second, similarly, it appears that exogenous rFGF21 may inhibit pro-inflammation in WAT and bloodstream, as well in brain

hippocampus, but how the systemic pro-inflammation inhibition relates to the hippocampal pro-inflammation status remains to be investigated. Third, we only examined hippocampal dysfunction that is closely related to neuroinflammation mechanisms, but other hippocampus dysfunctions and other brain regions such as the hypothalamus may also be involved [9]; all of these mechanistic questions need to be further investigated. Fourth, while rFGF21 improved systemic insulin resistance and FGF21 resistance in HFD-induced obese mice, the metabolic dysfunction in the brain and the connection to the cognitive impairment need to be thoroughly examined, at least within the context of our model system. Fifth, due to lack of available working antibody for immunohistochemistry that determines the active form of FGF21 receptor FGFR1 (phosphorylation FGFR1), we were unable to define brain regions and cellular types for FGF21-FGFR1 activation taking place, but both endogenous and exogenous FGF21-mediated receptor activation and consequent biological signaling pathways warrant investigation in the future [78]. Lastly, this study was proposed as a proof-of-concept investigation, and all translational aspects, including utilizing the FGF21 analog, should be further tested in a well-controlled preclinical translational setting [25, 79]. Moreover, using the HFD-induced obese insulin-resistant model is aimed to mimic human metabolic syndrome, but there are variable mechanisms between different obese animal models [80]; future studies testing rFGF21 in other metabolic syndrome-related animal models should be pursued.

In summary, our experimental findings support our hypothesis that rFGF21 is protective to HFD-induced cognitive impairment, at least in part by metabolic regulation in glucose tolerance impairment, insulin resistance, and hyperlipidemia; potent systemic pro-inflammation inhibition; and improvement of hippocampal dysfunction, particularly by inhibiting pro-neuroinflammation. This study suggests that FGF21 might be a novel molecular target of the disease-course-modifying strategy for early intervention of metabolic syndrome-associated cognitive decline.

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Compliance with Ethical Standards All animal experiments were performed following protocols approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee in compliance with the NIH Guide for the Care and Use of Laboratory Animals. All efforts were exerted to minimize animal suffering.

Conflict of Interest The authors declare that they have no conflict of interest.

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