Dear Editors,

I am submitting our manuscript entitled *"**FGF21 restores hippocampual mitochondrial dysfunction via enhancing Nrf2/HO-1 and AMPK/SirT1/PGC-1α signaling pathways to alleviate chronic unpredictable mild stress induced depressive like behaviors in mice**"* for possible publication in the "***Metabolic Brain Disease*** ". This paper is neither the entire paper nor any part of its content has been published or has been accepted elsewhere．It is not being submitted to any other journal．

We believe our paper may be of particular interest to the readers of your journal because our study suggests that Fibroblast growth factor 21 (FGF21), a member of the FGF superfamily, exert anti-depressive roles on a chronic unpredictable mild stress (CUMS)- induced model of depression. The effects were consistent with improved hippocampual mitochondrial function, reflected by FGF21-induced increases in mitochondrial membrane potential (MMP), ATP concentration and decrease of reactive oxygen species (ROS) levels. At the same time, FGF21 ameliorated oxidative stress in CUMS-exposed mice by enhancing superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase activities, and reducing malondialdehyde (MDA) level in the hippocampus. Mechanistically, we found that CUMS treatment decreased expression levels of mitochondrial fusion protein 1 (MFN1), and increased expression levels of mitochondrial dynamin-related protein 1 (DRP1). FGF21 administration increased expression of MFN1, and reduced expression of DRP1. Meanwhile, FGF21 treatment promoted the expressions of Nrf2, HO-1, phosphorylated AMPK, SirT1, PGC-1a in the hippocampus. This study revealed that FGF21 alleviates CUMS induced depressive like behaviors by restoring mitochondria function, improving oxidative stress and enhancing Nrf2/HO-1 and AMPK/SirT1/PGC-1α signaling pathways. It suggested that FGF21 would be a potential therapeutic agent in the management of depression.

All authors agreed to submit this manuscript to your journal.

Thank you very much for your kind consideration.

Sincerely yours,

You Liu, Ph.D.

Professor of Biochemistry

College of Food Science and Technology, Guangdong Ocean University, 1 Hai-Da Road, Zhanjiang, 524088, P.R. China

Tel (Fax): 86-759-2396076

Email: liuy6254282@gdou.edu.cn

FGF21 restores hippocampual mitochondrial dysfunction via enhancing Nrf2/HO-1 and AMPK/SirT1/PGC-1α signaling pathways to alleviate chronic unpredictable mild stress induced depressive like behaviors in mice

Yun-Tao Zhao [a], Ling Shen [a], Yong-Ping Zhang [a], Lulu zhang [a], Leigang Jin [c], Shaohong Chen [a], Cai Song [a], Chuanyin Hu [b*], You Liu [a*]

[a] Research Institute for Marine Drugs and Nutrition, Modern Biochemistry Experimental Center, College of Food Science and Technology, Guangdong Ocean University, Zhanjiang, 524088, P.R. China;

[b] Department of Biology, Guangdong Medical University, Zhanjiang, 524023, P.R. China;

[c] State Key Laboratory of Pharmaceutical Biotechnology, Department of Medicine, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong, China

* Corresponding authors:

You Liu, Ph.D.

Professor of Biochemistry

College of Food Science and Technology, Modern Biochemistry Experimental Center, Guangdong Ocean University,

1 Hai-Da Road, Zhanjiang, Guangdong 524088, P.R. China

Tel: 86-759-2396076

Email: liuy6254282@gdou.edu.cn

Chuanyin Hu, Ph.D.

Associate Professor of Neurobiology

Department of Biology, Guangdong Medical University,

2 East Wenming Road, Zhanjiang, Guangdong 524023, P.R. China

Tel: 86-759-2388574

Email: huchyin@126.com

Abstract:

Mitochondrial dysfunction plays a key role in the pathogenesis of depression. Ample research proves mitochondria are a promising target for depression. Fibroblast growth factor 21 (FGF21) exerts roles in neuroprotection and could enhance mitochondria function. Here, the anti-depressive effect of FGF21 was evaluated on a chronic unpredictable mild stress (CUMS)- induced model of depression. The depressive-like behaviors were assessed using sucrose preference test (SPT), forced swim test (FST) and tail suspension test (TST). The results showed that treatment of FGF21 significantly attenuated the decrease in SPT, and dramatically reduced the immobility time in the TST and FST. These effects were associated with enhanced hippocampal mitochondrial function, reflected by FGF21-induced increases in mitochondrial ATP concentration, mitochondrial membrane potential (MMP), and decrease of reactive oxygen species (ROS) levels. At the same time, FGF21 ameliorated oxidative stress in CUMS-exposed mice by enhancing superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase activities, and reducing malondialdehyde (MDA) level in the hippocampus. Mechanistically, we found that CUMS treatment decreased expression level of mitochondrial fusion protein 1 (MFN1), and increased expression level of mitochondrial dynamin-related protein 1 (DRP1). FGF21 administration increased expression of MFN1, and reduced expression of DRP1. Meanwhile, FGF21 treatment promoted the expression levels of Nrf2, HO-1, phosphorylated AMPK, SirT1, PGC-1a in the hippocampus. This study revealed that FGF21 alleviates CUMS induced depressive like behaviors by restoring mitochondria function via enhancing Nrf2/HO-1 and AMPK/SirT1/PGC-1α signaling pathways. It suggested that FGF21 would be a potential therapeutic agent in the management of depression.

Keywords: depression; FGF21; mitochondria; Nrf2/HO-1; AMPK/SirT1/PGC-1α

Introduction

Depression is a common clinical mental disorder caused by the interaction of endocrine system, monoamine neurotransmitters, nervous system and other pathogenic mechanisms (World Health 2017). According to statistics from the World Health Organization, the number of patients suffering from depression has increased by about 20% in the past 10 years (Allen et al. 2021). There are currently about 300 million patients worldwide. It is estimated that by 2030, the burden of disease

caused by it will become the world's first burden of disease (World Health 2017; Chen et al. 2018). Its high incidence, high recurrence rate, and high suicide rate have aroused people's attention (Malhi and Mann 2018; Ménard et al. 2016). At present, antidepressants commonly used in clinical practice are developed based on the "monoamine hypothesis". Only one-third of depression patients get relief immediately after taking a single antidepressant, and two-thirds of patients get relief after taking the drug for a few weeks (Ménard et al. 2016). Moreover, long-term use of antidepressants will bring serious side effects, such as increased body weight, increased suicidal tendency, sexual dysfunction, etc (Antunes et al. 2015).

The etiological mechanisms underlying depression are complicated and have yet to be elucidated. Accumulating data suggest that mitochondrial dysfunction is related to the development of depression (Khalid et al. 2016; Opie et al. 2017; Molendijk et al. 2018). Mitochondria are key organelles for cellular energy metabolism and multiple biosynthetic pathways, and are also widely involved in cellular signaling. Mitochondrial dysfunction is described as reduced mitochondrial biogenesis, diminished membrane potential, and the decrease in mitochondrial number and changed activities of oxidative proteins (Allen et al. 2018) . Mitochondrial dysfunction is considered a multifactorial phenomenon since it may have multiple causes and affects numerous neurobiological processes, such as reducing neurogenesis, altering synapsis and enhancing apoptosis, which orchestrate the process of depression (Gong et al. 2011). Epidemiological data indicate that the incidence of depression in patients with mitochondrial disease is higher than that of ordinary people. A cohort study conducted by Fattal et al. show that 54% of patients with mitochondrial disease are diagnosed with lifelong major depression, and most patients had psychiatric manifestations before the diagnosis of mitochondrial disease (Fattal et al. 2006; Fattal et al. 2007; Gardner and Boles 2011; Li et al. 2012). In many rodent depression models, impaired mitochondrial function of cortex, hippocampus and hypothalamus can be observed (Inczedy-Farkas et al. 2012). Agents capable of enhancing mitochondrial function have been studied for the treatment of mood disorders as adjuvant therapy to current pharmacological treatments (Gong et al. 2011; Inczedy-Farkas et al. 2012). Coenzyme Q10 and L-carnitine, which are drugs for the treatment of mitochondrial dysfunction, have been proven to improve the symptoms of depression (Fisher and Maratos-Flier 2016; Lewis, et al. 2019). These findings show enhancing mitochondrial function might serve as a target for depression therapy.

Fibroblast growth factor 21 (FGF21), a member of the FGF superfamily, is an endogenous regulation factor expressed mainly in the liver, pancreas, skeletal muscle and adipose tissue (Lewis et al. 2019). As a hormone, FGF21 exerts powerful modulating abilities in regulating glucose and lipid metabolism (Fisher and Maratos-Flier 2016; Lewis et al. 2019; Tezze et al. 2019). Accumulating data have shown that pharmacological administration of FGF21 exhibits good pharmacological activities on diabetes mellitus, obesity, nonalcoholic fatty liver disease and cardiovascular disease (Kwok and Lam 2017; Tezze et al. 2019; Tucker et al. 2019; Olapoju et al. 2020). Plasma FGF21 can reportedly cross the blood-brain barrier (BBB) by simple diffusion (Hsuchou et al. 2007). Growing evidence emerged indicating that FGF21 exerted neuroprotective effect in Alzheimer's disease (Taliyan et al. 2019), traumatic brain injury (Chen et al. 2018), ischemic stoke (Wang et al. 2020), obesity induced cognitive decline (Sa-Nguanmoo et al. 2016), age induced neurodegeneration (Kang et al. 2020) and Parkinson's disease (Fang et al. 2020). Recent studies show that FGF21 exerts antidepressant effects in mice with depression induced by LPS and chronic social defeat stress (Wang et al. 2020; Usui et al. 2021). The neuroprotective effects of FGF21 were proposed to be associated with the anti-oxidative, anti-inflammatory, anti-apoptosis, anti- endoplasmic reticulum stress activities, and enhancing of mitochondrial dysfunction.

To date, the effect and underlying mechanisms of FGF21 in a model of chronic unpredictable mild stress (CUMS)-induced depression are unclear. In this study, a mice depression model was established by exposing mice to CUMS and the effects of FGF21 on the depressive-like behaviors via restoring mitochondria dysfunction were tested. Our results unraveled that FGF21 possessed anti-depressive property owing to its efficacy in reversing mitochondria dysfunction.

Materials and Methods

Reagents

Recombinant Murine FGF-21 was purchased from PrimeGene (Shanghai, China). 2′,7′-dichlorofluorescindiacetate (DCF) were obtained from Molecular Probes (Carlsbad, CA, USA). 5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) mitochondrial membrane potential assay kit, mitochondria isolation kit and ATP assay kit were purchased from Beyotime Biotechnology Co., Ltd. Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) and malondialdehyde (MDA) assay kits were purchased from Nanjing Jiancheng

Biotechnology Co., Ltd. (Nanjing, China). Antibodies against mitochondrial fusion protein 1 (MFN1), mitochondrial dynamin-related protein 1 (DRP1) and nuclear factor E2-related factor 2 (Nrf2) were obtained from Abcam (Cambridge, MA, USA). Anti-peroxisome proliferator-activated receptor γ co-activator-1α (PGC-1α) antibody was got from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Antibodies against AMP-activated protein kinase (AMPK), phosphorylated AMPK (p-AMPK), Sirtuin1 (SirT1), heme oxygenase 1(HO-1) and Actin were purchased from Cell Signaling Technology (Danvers, MA, USA).

Animals and treatments

Male C57BL/6 mice (20±2 g) were obtained from Guangdong Medical Laboratory Animal Center (Guangzhou, China). The mice were housed under standard animal room conditions (temperature 22 ± 1 °C, humidity 55–60%, 12/12 h light/dark cycle). Mice were randomly divided into three groups with fifteen mice in each group: Control, CUMS and CUMS + FGF21 groups. The CUMS + FGF21 group was administrated with FGF21 (0.1 mg/kg) by intraperitoneal injection once every day for 35 days. The experimental procedures were illustrated in Fig. 1A.

Chronic unpredictable mild stress (CUMS) induced depression model in Mice

CUMS protocol was performed as described previously (Zhao et al. 2021). In brief, , mice of CUMS group and CUMS + FGF21 group were treated with the following mild stressors for 63 days randomly: 4℃ for 1.5 h; swimming in 18℃ cold water for 10 min; bondage for 1.5 h; 45° cage tilting for 12 h; strobe light for 12 h; inversion of the light/dark cycle for 12 h; water shortage for 12 h; food shortage for 12 h; wet bedding for 12 h; different bedding for 12 h; cage shaking for 2.5 h (150 rpm). These eleven stressors were used continuously and individually, the same stressor does not reuse within three days. Control group mice were not received any kind of stressors and were raised in normal conditions.

Behavioral tests

Sucrose preference test (SPT)

The SPT was done to operationally define the anhedonia behavior as previously described (Zhao et al. 2021). In the adaption phase, mice were placed individually in cages supplied with one bottle of 1% sucrose water (w/v) and one bottle of pure water for 48 h. Then, mice were deprived of food and water for 24 h. Then, each mouse was allowed to drink water from the two bottles freely for 4 h, one with pure water and the other with 1 % sucrose water. After 4 h, the consumption of sucrose

121 water and pure water was calculated, and the sucrose preference was measured as follows: (sucrose

122 water consumption/total water consumption] × 100%).

Forced swimming test (FST)

124 The FST was performed as previously described (Zhao et al. 2021). The experiment was carried

125 out within 2 days. On day 1, mice were individually placed in a swimming tank (50-cm height, 20-

126 cm diameter) filled with water (temperature 25 ℃ ± 1 ℃) to a depth of 35 cm for 10 min. On day

127 2, the mice were individually placed in a swimming tank for 6 min, and the immobility time was

128 recorded during the last 5 mins by experimenters blinded to the design.

Tail suspending test (TST)

130 In the TST, each mouse was suspended individually by its tail's tip at a height of 50 cm above the

131 floor. The test was performed for a period of 5 min, and the total duration of immobility was scored

132 by an observer blinded to the experimental conditions.

Isolation of hippocampal mitochondria

134 Mitochondria in the hippocampus blocks were isolated using the tissue mitochondria isolation

135 kit according to the instructions. All centrifugation procedures were performed at 4 ℃. Briefly,

136 fresh tissues were homogenized using a handheld Tissue-Tearor homogenizers using an isolation

137 buffer (1:10, w/v). The tissue homogenizations were centrifuged at $1000 \times g$ for 5 min.

138 Supernatants were transferred to separate tubes and centrifuged at $11000 \times g$ for 10 min. The

139 sediment consisted of mitochondria. The sediments were resuspended with appropriate amounts of

140 mitochondrial stock solution and the protein concentrations were measured by BCA assay kit.

Determination of the mitochondrial membrane potential (MMP)

142 MMP in the mitochondrial suspensions was determined by JC-1 MMP assay kit. Briefly, 20 μL of

143 mitochondrial suspension and 180 μL of JC-1 working solution were added to the wells of a 96-well

144 black microplate. The microplate was incubated at room temperature for 15 min and fluorescence was

145 measured using VARIOSKAN Flash Microplate Reader (Thermo Scientific, USA) with an

146 excitation/emission wave length of 525/590 nm.

ATP assay in isolated mitochondria

148 ATP concentration was measured by a firefly luciferase- based ATP assay kit following the

149 manufacturer's protocol. Briefly, 100 μL of ATP testing working solution was added to the wells of a

150 96-well black microplate and incubated at room temperature for 5 min, and then 20 μL mitochondrial

151 suspension or ATP standard were added into detection wells quickly. Emitted light was measured using

152 VARIOSKAN Flash Microplate Reader (Thermo Scientific, USA). ATP concentration was determined

153 according to a standard curve.

Mitochondrial reactive oxygen species (ROS) assay

155 Mitochondrial ROS production was measured using dichloro-hydrofluoresceindiacetate (DCF)

156 reagent. Briefly, mitochondria (0.4 mg protein / ml) were incubated with 5 μM DCF at room

157 temperature for 20 min. The fluorescence intensity was read using VARIOSKAN Flash Microplate

158 Reader (Thermo Scientific, USA) with an excitation/emission wave length of 485/530 nm.

159

Biochemical analysis

161 Hippocampal tissue blocks were homogenized with phosphate buffer (PBS, pH = 7.2) (1:10, w/v)

162 and centrifuged at $12000 \times g$ for 15 min at 4°C. The supernatant was collected and stored at -70°C

163 until used for biochemical assays. The protein concentrations were detected with the BCA assay kit.

164 SOD, GSH-PX and CAT activities, and MDA levels in the supernatant were assessed according to

165 the instructions of the corresponding detection kits.

Western blotting

167 Hippocampal tissue homogenates were prepared in ice-cold RIPA lysis buffer con-taining a

168 mixture of phosphatase inhibitor and protease inhibitors and were centrifuged at 12 000 rpm for 15

169 min at 4 °C. Supernatants were collected and total protein concen-trations were measured with the

170 BCA assay kit.

171 Samples containing 30 μg proteins were separated by 10% sodium dodecyl sulfate-

172 polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes. Following

173 blocking with 5% non-fat milk or BSA at room temperature for 1 h, the membranes were incubated

174 overnight at 4 °C with primary antibodies p-AMPK (dilution 1:1000), AMPK (dilution 1:1000),

175 PGC-1α (dilution 1:700), SirT1 (dilution 1:1000), Actin (dilution 1:1000), DRP1 (dilution 1:1000),

176 MFN1 (dilution 1:2000), Nrf2 (dilution 1:2000), HO-1 (dilution 1:1000). After several washes with

177 Tris-buffered saline with 0.1% Tween 20 (TBST) buffer the membranes were incubated with

178 horseradish peroxidase-conjugated secondary antibodies (dilution: 1:2000) for 2 h. The signal was

179 measured by chemiluminescent ECL Substrate kit (Millipore) with ChemiDoc XRS+ System

180 (BioRad, USA). The intensity of the protein bands was quantified using Image J software after

181   normalization to Actin.

182   **Statistical analysis**

183   Results are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was

184   determined by one-way analysis of variance (ANOVA) followed by Tukey's test when analyzing

185   more than two groups by the GraphPad Prism software version 8.0 (GraphPad Software, Inc., La

186   Jolla, CA, USA). $p < 0.05$ was considered statistically significant.

187   **Results**

188   **FGF21 alleviated CUMS-induced depressive-like behaviors**

189   To explore antidepressant effects of FGF21, mice were subjected to the CUMS stimuli for 63

190   days and treated with FGF21 for 35 days (Fig. 1A). At the end of treatments, behavioral experiments

191   were performed. Results in the SPT (Fig. 1B) showed an overall significantly reduced preference

192   for sucrose in CUMS-induced mice when compared to that of control mice, which was partially

193   reversed by FGF21 treatment ($p < 0.01$).

194   In the FST (Fig. 1C), a significant increase in immobility time was found in CUMS-induced mice

195   compared to that of control mice ($p < 0.01$), which was dramatically reversed by administration of

196   FGF21 ($p < 0.01$). The administration of FGF21 markedly reversed CUMS-induced depressive-like

197   behavior in mice.

198   In the TST (Fig. 1D), CUMS-induced mice exhibited significantly longer immobility time

199   compared with those of control group ($p < 0.01$), which was significantly reversed by FGF21

200   administration ($p < 0.01$, Fig.1D).
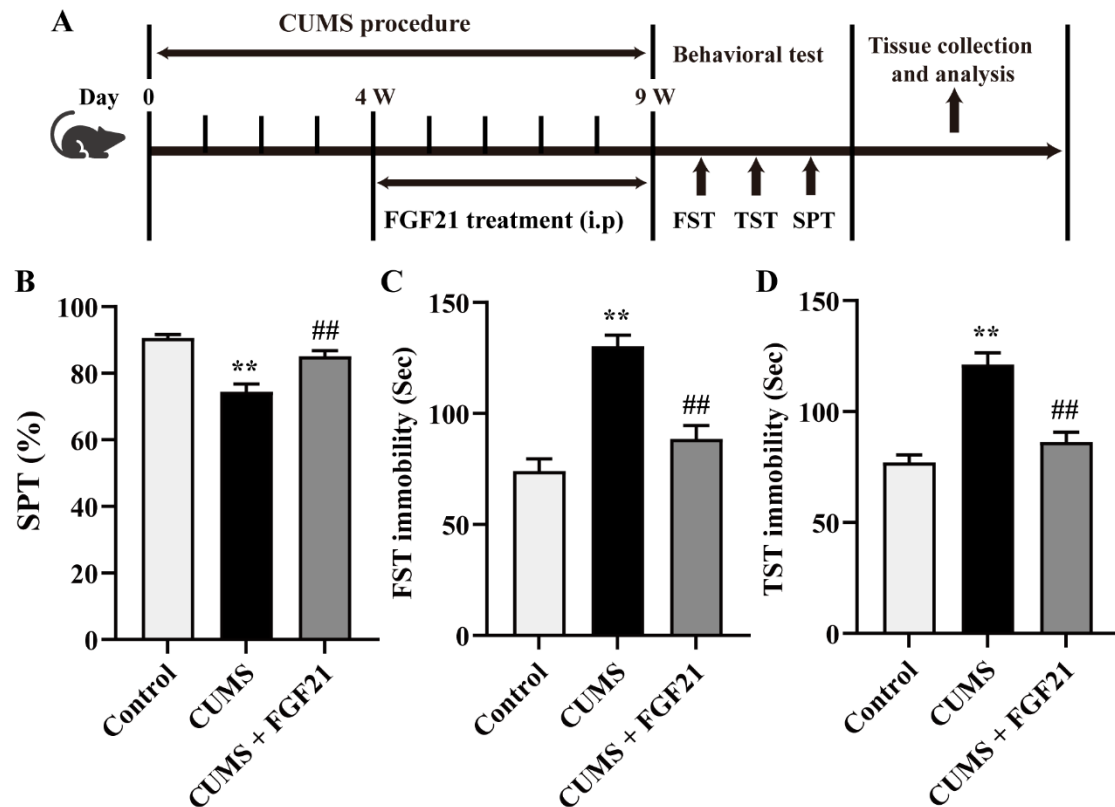
Fig. 1 Experimental protocol and the results of behavioral assessments. (A) Schematic illustration of the experimental procedures. (B) The treatment with FGF21 significantly improved the decreased sucrose preference observed in mice exposed to CUMS. (C–D) FGF21 exposure obviously reduced the immobility time in the FST and TST compared with that of the CUMS group. All data are expressed as mean ± SEM (n = 15). **$p < 0.01$ vs. control group; ## $p < 0.01$ vs. CUMS group.

FGF21 improved mitochondrial function in the hippocampus

The pathogenesis of depression is strongly related to mitochondrial dysfunction in the hippocampus. To assess the effects of FGF21 on mitochondrial function in the hippocampus, brain mitochondrial ATP production, mitochondrial membrane potential changes and mitochondrial ROS production were measured. Mitochondrial ATP production and mitochondrial membrane potential were decreased markedly (Fig. 2A, B), whereas ROS production was significantly increased in the hippocampus obtained from the CUMS group mice compared with those from the control group mice (Fig. 2C). FGF21 treatment remarkably reversed the declining mitochondrial ATP production and membrane potential (Fig. 2A, B, $p < 0.05$ vs CUMS group), and decreased mitochondrial ROS

218   production (Fig. 2C, $p < 0.01$ vs CUMS group) in the hippocampus of the CUMS-induced

219   depressive mice. These findings suggested that FGF21 treatment led to restored brain mitochondrial

220   function in CUMS-induced depressive mice.

221
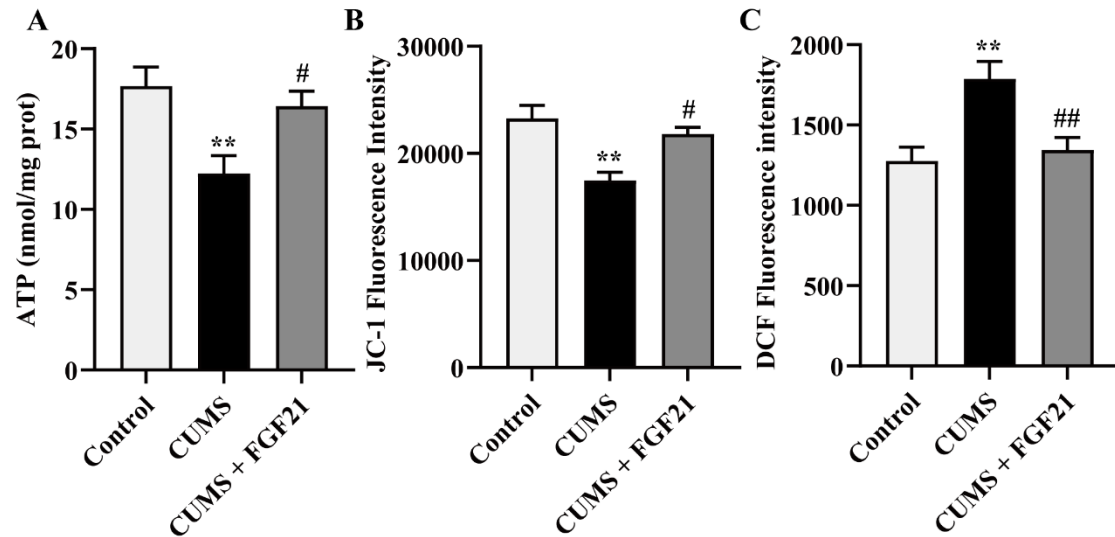
222   **Fig. 2 Effects of FGF21 on mitochondrial dysfunction in the hippocampus of CUMS-induced**

223   **mice**. Mitochondria were isolated from the hippocampus and mitochondrial ATP production (A),

224   membrane potential levels (B), and ROS production (C) were measured. Data were expressed as

225   mean ± SEM (n = 5). $**p < 0.01$ vs. control group. [#]p < 0.05, [##]p < 0.01 vs. CUMS group.

226

227   **FGF21 inhibited oxidative stress in the hippocampus**

228   Mitochondrial dysfunction disrupts respiratory chain function and accelerates ROS production.

229   The changes of oxidative stress related parameters in the hippocampus of mice were detected.

230   Results showed that SOD, CAT and GSH-Px activities were markedly declined in the hippocampus

231   of CUMS mice, whereas FGF21 treatment restored these changes ($p < 0.05$, Fig. 3A-C). The content

232   of MDA in the hippocampus was markedly elevated in the hippocampus of CUMS mice. After

233   treatment with FGF21, MDA levels decreased significantly compared to that of CUMS group ($p <$

234   0.01, Fig. 3D).
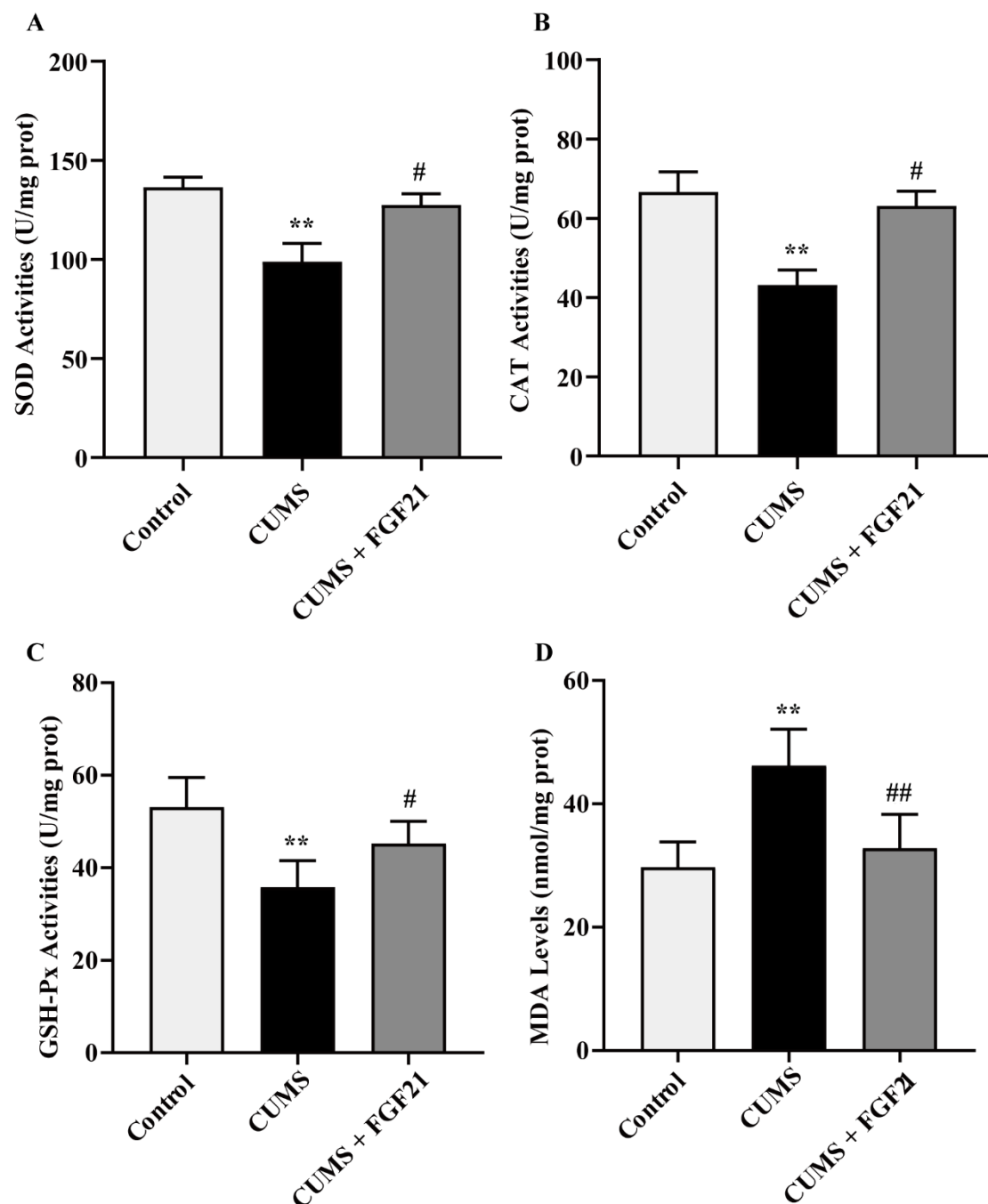
Fig. 3 Effects of FGF21 on oxidative stress in the hippocampus. Treatment of FGF21 significantly increased (A) SOD (B) CAT (C) GSH-PX activities, and decreased the content of MDA (D) in the hippocampus. All data are expressed as mean ± SEM (n = 5). **$p < 0.01$ vs. control group; [#]$p < 0.05$, [##]$p < 0.01$ vs. CUMS group.

FGF21 modified expression of MFN1 and DRP-1

Imbalance in mitochondrial fission and fusion involves in mitochondrial morphology change and dysfunction. The expression levels of mitochondrial fusion-related protein MFN1, and the fission

244   protein DRP-1 were measured. The results revealed that CUMS observably downregulated MFN1(p

245   < 0.01, Fig. 4A and B), and upregulated DRP-1 expression ($p < 0.01$, Fig. 4A and C) in the

246   hippocampus compared with that in the control group. Administration with FGF21 reversed these

247   changes dramatically ($p < 0.05$, Fig. 4A-C). The results suggested that FGF21 treatment restored

248   mitochondrial dynamics imbalance induced by CUMS in mice.
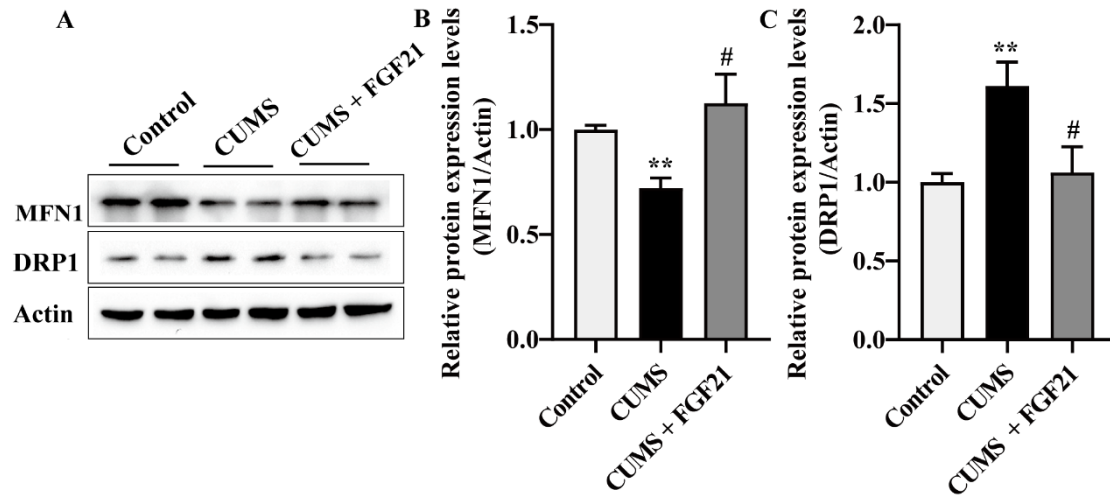
250 **Fig. 4 Effects of FGF21 on the expression of MFN-1 and DRP-1 in the hippocampus**. (A)

251 Representative images of western blot of MFN-1 and DRP-1. (B-C) Quantitative analysis of MFN1

252 and DRP-1 expression in the hippocampus. All data are expressed as mean $\pm$ SEM (n = 4). **$p <$

253 0.01 vs. control group; #$p < 0.05$ vs. CUMS group.

FGF21 enhanced Nrf2/HO-1 signaling pathway in the hippocampus

256   A growing body of studies have showed that Nrf2/HO-1 antioxidant pathway exerts pivotal roles

257 in protection of cell from oxidative stress damage. To investigate the effect of FGF21 on Nrf2/HO-

258 1 signaling pathway, the protein expression of Nrf2 and HO-1 was detected by Western blot analysis.

259 The results showed that CUMS treatment significantly decreased the protein expression of Nrf2 and

260 HO-1 in hippocampal tissues of mice compared with the control group ($p < 0.01$, Fig. 5).

261 Administration with FGF21 ameliorated these changes dramatically ($p < 0.05$, Fig. 5).
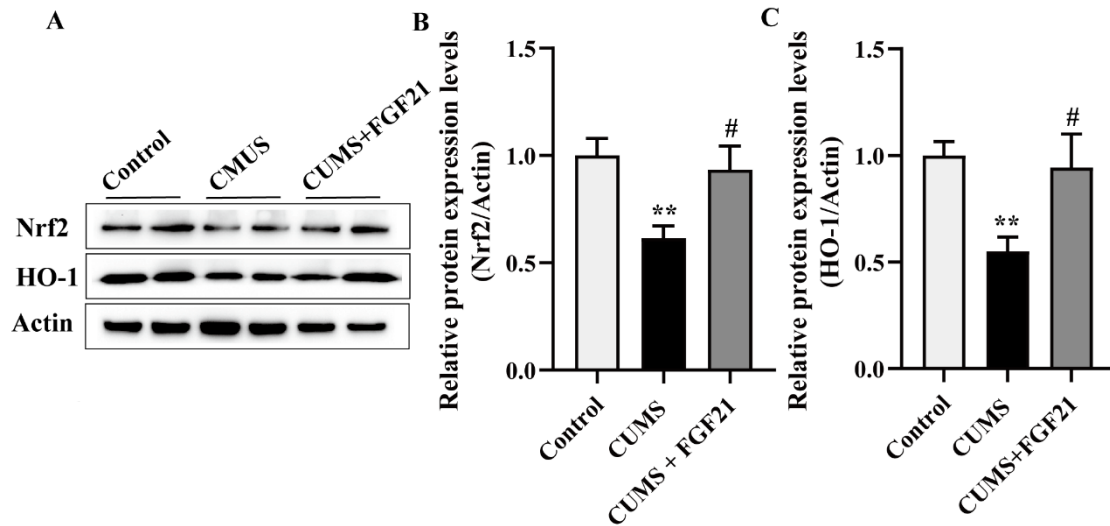
Fig. 5 Effects of FGF21 on the expression of Nrf2 and HO-1 in the hippocampus. (A) Representative images of western blot of Nrf2 and HO-1. (B-C) Quantitative analysis of Nrf2 and HO-1 expression in the hippocampus. All data are expressed as mean ± SEM (n = 4). $**p < 0.01$ vs. control group; $^{\#}p < 0.05$ vs. CUMS group.

FGF21 activates the AMPK/SirT1/PGC-1α signaling pathway in the hippocampus

To better understand the underlying mechanism of FGF21 on CUMS-induced depressive-like behaviors, the protein expressions of SirT1, AMPK and PGC-1α in hippocampal tissues were evaluated. The results revealed that CUMS dramatically suppressed p-AMPK ($p < 0.01$, Fig. 6A and B), SirT1 ($p < 0.05$, Fig. 6A and C), and PGC-1α ($p < 0.05$, Fig. 6A and D) expression in the hippocampus compared with that in the control group. Administration with FGF21 reversed these changes significantly ($p < 0.05$, Fig. 6A-D). These data suggest that FGF21 enhanced AMPK/SirT1/PGC-1α signaling pathway, which trigger signaling cascade for regulation of mitochondria function.
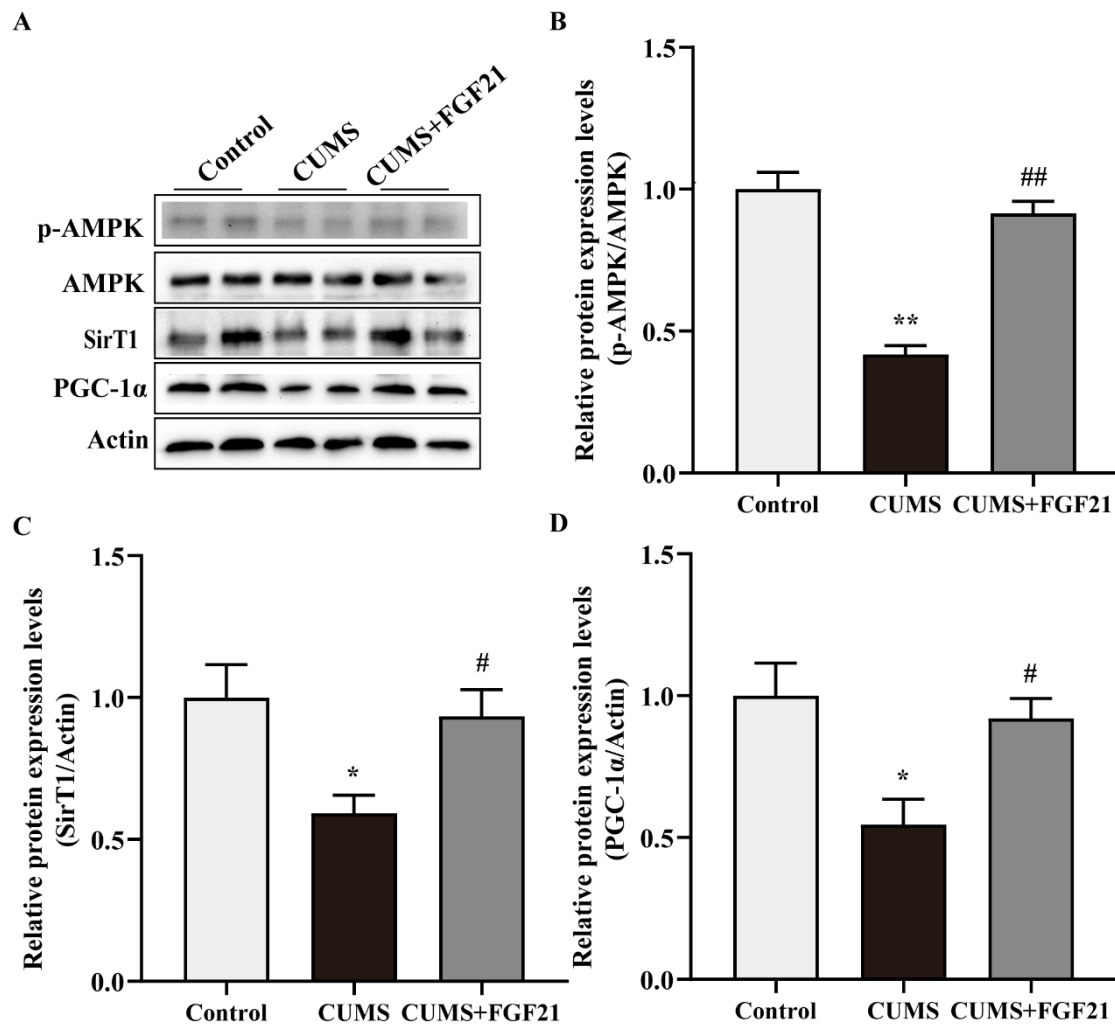
Fig. 6 Effects of FGF21 on the expression of p-AMPK, SirT1 and PGC-1α in the hippocampus. (A) Representative images of western blot of SirT1, p-AMPK and PGC-1α. (B–D) Quantitative analysis of p-AMPK, SirT1 and PGC-1α expression in the hippocampus. All data are expressed as mean ± SEM (n = 4). $*p < 0.05$, $**P < 0.01$ vs. control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. CUMS group.

Discussion

FGF 21 exerts diverse pharmacological effects on metabolic disorders, brain diseases, cardiovascular disease, reproductive dysfunction etc. It is noteworthy that FGF21 inhibit LPS and chronic social defeat stress induced depressive behaviors (Wang et al. 2020; Usui, Yoshida et al. 2021). However, whether FGF21 plays anti-depressive effect on mice with depression produced by CUMS remains unclear, and the underlying molecular mechanisms are not fully understood. The present experiments extended investigations into the antidepressant action of FGF21 on CUMS-induced depression. Our findings demonstrated that FGF21 was able to prevent behavioral

291 alterations induced by CUMS in the SPT, FST and TST. Anti-depressive effect of FGF21 was

292 associated with the restoration of mitochondrial dysfunction induced by CUMS.

293   Mitochondria, "the energy factories in the cells", are the pivotal organelle in maintaining energy

294 metabolism. Mitochondrial dysfunctions are always associated with neurological damages (Kausar et al.

295 2018). Hypothesis of "mitochondrial dysfunction in depression" opens a new avenue for

296 understanding the pathogenesis of depression (Kato 2017). Currently, the role of mitochondrial

297 dysfunction in chronic mild stress-induced experimental models of depression has gained increasing

298 attention. Rezin et al. indicate that complexes I, III and IV is inhibited in stress groups in their

299 cerebral cortex and cerebellum (Rezin et al. 2008). Chronic mild stress induces the collapse of the

300 mitochondrial membrane potential, inhibits mitochondrial respiration rates and destroys

301 mitochondrial ultrastructure (Chen et al. 2017). There is also evidence demonstrating that

302 mitochondrial dysfunction might have an important role in impaired hippocampal neurogenesis in

303 depression (Quiroz et al. 2008; Kirby et al. 2009). Mitochondrial dysfunction in the hippocampus

304 have been reported in patients with depression. Some drugs for the treatment of mitochondrial

305 dysfunction, such as coenzyme Q and L-carnitine, alleviate the symptoms of depression

306 significantly (Calingasan, et al. 2008; Fisher and Maratos-Flier 2016; Tezze et al. 2019). In this

307 study, we confirmed that CUMS treatment in mice induced hippocampal mitochondrial dysfunction

308 that was reflected by decreased ATP production and mitochondrial membrane potential as well as

309 increased mitochondrial ROS production. These changes were reversed by FGF21 treatment. The

310 results suggest that modifying of mitochondrial dysfunction plays a pivotal role in effects of FGF21

311 on CUMS-induced depressive behavior.

312   Oxidative stress refers to the imbalance between production of ROS and antioxidants.

313 Mitochondria are the most important source of ROS in most of the mammalian cells (Kausar et al.

314 2018). ROS produced in mitochondria during oxidative phosphorylation (OXPHOS) process

315 primarily triggered mitochondrial dysfunction by interacting with mitochondrial and cellular

316 components such as DNA, proteins, lipids, and other molecules. Alterations in mitochondrial

317 functions such as OXPHOS and membrane polarity, which increase oxidative stress and apoptosis,

318 may precede the development of depressive symptoms. Here, we found that SOD, CAT and GSH-Px

319 activities were decreased, and MDA content was increased, in the hippocampus of mice exposed to

320 CUMS. These changes were reversed by FGF21 treatment.

Nrf2, a potent transcriptional activator, is responsible for the regulation of diverse antioxidant and antiapoptotic related genes in response to oxidative stress, and thus enhances the levels of endogenous antioxidants and improves cell apoptosis (Zgorzynska et al. 2021). HO-1, an antioxidant and detoxifying protein regulated by Nrf2 activation, acts and functions as a rate-limiting enzyme of heme catabolism for reducing ROS production (Zgorzynska et al. 2021). Nrf2/HO-1 signaling pathway exerts anti-inflammatory, antioxidant, anti-apoptosis, and cytoprotective action. Enhancing Nrf2/HO-1 signaling pathway has been suggested as a promising therapeutic target for neuropsychiatric disorders (Hashimoto 2018; Morris et al. 2021). Here, we found that CUMS treatment obviously reduced the protein expression levels of Nrf2 and HO-1 in the hippocampus, suggesting CUMS inhibited the Nrf2/HO-1 signal pathway. FGF21 significantly activated the Nrf2/HO-1 pathway, as evidenced by increased expression of Nrf2 and HO-1 in CUMS-induced hippocampus. Therefore, enhancing the activation of Nrf2/HO-1 signal pathway by FGF21 may be a potential mechanism to protect CUMS-induced mitochondrial dysfunction.

Mitochondria are dynamic organelles that undergo fusion, fission, and transport. Mitochondrial dynamics are crucial for regulating morphology, function, number, and subcellular distribution (Burté et al. 2015). Mitochondrial fusion allows mitochondria to increase ATP and OXPHOS production, to exchange mitochondrial DNA, and to dilute possible organelle's damage (Messina et al. 2020). On the other hand, mitochondrial fission results into increased ROS production and signaling toward cell death by apoptosis (Messina et al. 2020). Mitochondrial fusion and fission require the regulation of key proteins, including the mitochondrial fusion proteins such as MFN1, MFN2 and Opa1，and the mitochondrial fission proteins such as DRP1 and Fis1, which are adjusted by PGC-1 α (Bertholet et al. 2016). Abnormalities in these proteins can lead to the accumulation of disabled mitochondria that increase ROS production (Bertholet et al. 2016). In the present study, we found that CUMS treatment decreased MFN1 expression, and increased expression of DRP1. FGF21 treatment increased expression of MFN1, and reduced expression of DRP1, suggesting that it produced benefits on CUMS induced depression by promoting Mitochondrial dynamics.

Growing evidence shows that AMPK and SirT-1 enhance mitochondrial biogenesis and oxidation by regulating PGC-1α, and prevent mitochondrial dysfunction. AMPK, a serine/threonine protein kinase, is a pivotal protein in regulating mitochondrial biogenesis and oxidation (Herzig and Shaw 2018). AMPK can indirectly activate SirT1 by regulating the level of NAD^+. SirT1, an NAD^+-

dependent protein histone deacetylase, has been shown to be involved in many physiological processes in brain, including control of gene expression, metabolism, senescence, neurogenesis and synaptic plasticity. SirT1 activation reportedly improves mitochondrial function and attenuates brain injury by increasing the level of PGC-1α (Lu et al. 2018). Pharmacological intervene of the AMPK/SirT1/PGC-1α signaling pathway may be of benefit in neuroprotection by restoration of mitochondria dysfunction and reducing mitochondria-mediated oxidative stress (Yang et al. 2020). In the present study, we found that CUMS treatment decreased expression of p-AMPK, SirT1 and PGC-1α. After treatment with FGF21, expression levels of p-AMPK, SirT1 and PGC-1α increased, which demonstrated that FGF21 exerts anti-depressive effect through enhancing AMPK/SirT-1/PGC-1α signal pathway to restore mitochondrial dysfunction.

In conclusion, this study shows that FGF21 reverses CUMS-induced depressive-like behaviors. The anti-depressive effects of FGF21 may be realized through enhancing of Nrf2/HO-1 signaling and AMPK/SirT-1/PGC-1α signaling to restore hippocampal mitochondrial dysfunction partly. The study advances a novel understanding and the underlying mechanisms of FGF21 in depression and provides a theoretical foundation for the use of FGF21 in depression. Further work will be needed to understand its specific molecular mechanism of action and clinical applications.

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380 **Ethics approval:** All animal experiments protocols were approved by the Animal Ethics Committee

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