Journal of Affective Disorders 246 (2019) 486–497

Contents lists available at ScienceDirect

Journal of Affective Disorders

journal homepage: www.elsevier.com/locate/jad

Research paper

Possible role of GLP-1 in antidepressant effects of metformin and exercise in CUMS mice

Liu Weina[a,b,1,*], Liu Jiatong[a,b,1], Huang Zhuochun[a,b], Cui Zhiming[a,b], Li Lingxia[a,b], Liu Wenbin[a,b], Qi Zhengtang[a,b,*]

[a] Key Laboratory of Adolescent Health Assessment and Exercise Intervention of Ministry of Education, East China Normal University, Shanghai 200241, China
[b] School of Physical Education & Health Care, East China Normal University, Shanghai 200241, China

ABSTRACT

Background: Both depression itself and antidepressant medication have been reported to be significantly related to the risk of type 2 diabetes mellitus (T2DM). Glucagon-like peptide-1 (GLP-1), a treatment target for T2DM, has a neuroprotective effect. As an enhancer and sensitiser of GLP-1, metformin has been reported to be safe for the neurodevelopment. The present study aimed to determine whether and how GLP-1 mediates antidepressant effects of metformin and exercise in mice.

Methods: Male C57BL/6 mice were exposed to chronic unpredictable mild stress (CUMS) for 8 weeks. From the 4th week, CUMS mice were subjected to oral metformin treatment and/or treadmill running. A videocomputerized tracking system was used to record behaviors of mice for a 5-min session. ELISA, western blotting and immunohistochemistry were used to examine serum protein concentrations, protein levels in whole hippocampus, protein distribution and expression in dorsal and ventral hippocampus, respectively.

Results: Our results supported the validity of metformin as a useful antidepressant; moreover, treadmill running favored metformin effects on exploratory behaviors and serum corticosterone levels. CUMS reduced GLP-1 protein levels and phosphorylation levels of extracellular signal-regulated kinase 1/2 (ERK1/2), but increased protein levels of B-cell lymphoma 2-associated X-protein (BAX) in mice hippocampus. All these changes were restored by both single and combined treatment with metformin and exercise.

Limitations: We did not establish a causal relationship between GLP-1 expression and related signaling, using GLP-1 agonist and antagonist or knockout techniques.

Conclusions: Our findings have demonstrated that protein levels of pERK and BAX may be relevant to the role of GLP-1 in antidepressant effects of metformin and exercise, which may provide a novel topic for future clinical research.

1. Introduction

The chronic and festering nature of depression substantially contributes to the global burden of disease and disability (Krishnan and Nestler, 2008). Significantly, the 2017 World Health Organization (WHO) report said that more than 300 million people suffer from depression globally. It is urgent to seek the treatments they need to live healthy and productive lives. However, there is no one dominant medication for depression (Hripcsak et al., 2016). Moreover, both depression itself (Deschenes et al., 2018) and antidepressant medication (de Groot et al., 2018) have been reported to be significantly related to the risk of type 2 diabetes mellitus (T2DM). Thus, further trials of diabetes treatment as potential antidepressant are needed (Moulton et al., 2018).

For T2DM, metformin is the most commonly prescribed medication, being favored a single first-line medication (Hripcsak et al., 2016). More important, metformin has been recently found to improve depressive symptoms and potentiate antidepressant effects of fluoxetine in chronic restraint stress rats (Khedr et al., 2018). Conversely, a recent meta-analysis has shown little promise of metformin in improving depression (Moulton et al., 2018). In view of inconsistent effects of metformin on depression, further study is necessary to clarify antidepressant action of metformin treatment. Clinically, it has been reported that adding exercise to antidepressant drug sertraline may offer significant advantages over affective symptoms of depressed patients (Murri et al., 2018). Therefore, the first aim of the present study is

Abbreviations: BAX, BCL-2-associated X protein; BCL-2, B-cell lymphoma-2; BDNF, brain derived neurotrophic factor; CORT, corticosterone; CUMS, chronic unpredictable mild stress; DH, dorsal hippocampus; ERK, extracellular signal-regulated kinase; FST, forced swim test; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor; OFT, open field test; SPT, sucrose preference test; T2DM, type 2 diabetes mellitus; VH, ventral hippocampus

* Corresponding authors at: Key Laboratory of Adolescent Health Assessment and Exercise Intervention of Ministry of Education, School of Physical Education & Health Care, East China Normal University, 500 Dongchuan Road, Shanghai 200241, China.

E-mail addresses: weina1978@126.com (W. Liu), qzht79@163.com (Z. Qi).

[1] Theses authors contributed equally to this work.

https://doi.org/10.1016/j.jad.2018.12.112
Received 29 June 2018; Received in revised form 18 December 2018; Accepted 24 December 2018
Available online 26 December 2018
0165-0327/

to identify whether metformin ameliorates depression-like behaviors in mice exposed to chronic unpredictable mild stress (CUMS), thus being employed as a useful antidepressant; and whether treadmill running favors metformin effects on depression.

Metformin has been reported to enhance adult neural precursor proliferation/self-renewal dependent upon the p53 family transcription factor TAp73 and promote neuronal differentiation of these cells by activating the AMP kinase (AMPK)-atypical protein kinase C (aPKC)-CREB binding protein (CBP) pathway in mice brain (Fatt et al., 2015). Metformin can permeate pituitary gland, frontal cortex, and hippocampus after chronic oral administration, and exerts anti-inflammatory and neuroprotective effects in brain (Labuzek et al., 2010). Besides the mediating roles in emotion and memory, the hippocampus has recently emerged as an important control center for feeding behavior through glucagon-like peptide-1 (GLP-1) signaling (Hsu et al., 2017). GLP-1, produced both in peripheral L-cells of gastrointestinal tract and in brain, is an anorexigenic peptide and treatment target for T2DM. GLP-1 analog liraglutide has been recently reported to attenuate depressive behaviors via improving hippocampal plasticity in mice (Weina et al., 2018). GLP-1 receptor (GLP-1R) agonists have also been shown neuroprotective effects both in diabetic (Gault and Holscher, 2018) and depressed (Zhao et al., 2018) mice. Metformin is regarded as an enhancer and sensitiser of GLP-1 (Cho and Kieffer, 2011). Thus we infer that metformin may exert an antidepressant effect through GLP-1 signaling.

GLP-1 analogue has been reported to improve cognitive functions in T2DM with up-regulation of brain derived neurotrophic factor (BDNF) (Abdelwahed et al., 2018). GLP-1R agonists can prevent nerve dysfunction via extracellular signal-regulated kinase (ERK) in diabetic mice (Jolivalt et al., 2011). Importantly, antidepressant effects of quetiapine have been found to be related to BDNF/ERK signaling activation in hippocampus (Chen et al., 2015). Besides increased BDNF level, GLP-1 mimetic can also enhance B-cell lymphoma-2 (BCL-2, a growth factor signaling molecule) and reduce BCL-2-associated X protein (BAX, an apoptosis signaling molecule) in Parkinson's disease mice (Ji et al., 2016). In addition, treadmill exercise has been reported to exert neuroprotective effects by activating BDNF/ERK signaling in depressed rats (Wu et al., 2017) and BCL-2/BAX signaling in Alzheimer's disease mice (Koo et al., 2013). Therefore, the second aim of the current study is to investigate whether GLP-1 is involved in antidepressant effects of metformin and exercise, and protein expression changes in related pathways in CUMS mice.

2. Materials and methods

2.1. Animals and groups

Male C57BL/6 mice (4-5-week old, 18–20 g) were obtained from Shanghai SLAC Experimental Animal Center (Shanghai, China). For chronic unpredictable mild stress (CUMS) model, BALB/c mice are typically used since they are more stress sensitive and responsive to antidepressants (Yalcin et al., 2008). Similarly, C57BL/6 mice have also been shown their sensitivity to the effects of CUMS (Lopes et al., 2016; Xu et al., 2018) and other chronic stress (Dias et al., 2014; Pena et al., 2017). Moreover, C57BL/6 mice are more sensitive to noise and odours than BALB/c mice (Connor et al., 2016). Only male mice were used in this study to avoid potential effects on behavioral or molecular testing when female mice are at different estrous cycle stages (Naik et al., 2018). Mice were housed with a 12-h light: dark cycle under controlled temperature (22 ± 2 °C) and humidity (50 ± 10%), and were given standard diet and water ad libitum. All mice were divided into five groups: control (Con), CUMS, CUMS + Met, CUMS + Run, CUMS + Met + Run; $n = 8$ per group. In our previous study, we did not find swimming effects on depression-like behaviors in control rats (Liu et al., 2013). Similarly, there were no significant differences of depressive behaviors between control mice subjected to treadmill running or not (Leem and Chang, 2017). Moreover, we aimed to examine the effects of metformin/running in CUMS animals. Therefore, we did not assess intervention effects in control animals. Of course, it remains possible exercise may induce improvement and biochemical changes on its own or with metformin in the control mice. It is thus necessary to design positive control groups (e.g., Con + Met/Run) in future study.

All procedures were in accordance with the guidelines for the use of laboratory animals published by the People's Republic of China Ministry of Health (No. 55 order, January 25, 1998) and were approved by the Experimental Animal Care and Use Committee at East China Normal University (ECNU 2006-05).

2.2. Treatment protocol

2.2.1. Chronic unpredictable mild stress procedure

The CUMS procedure was performed as described in our previous study (Liu et al., 2018). Mice in CUMS group were subjected to different stressors: cage tilting for 24 h (45°), wet bedding for 24 h (200 ml of water per cage), cold swimming for 5 min (at 10 °C), swimming in hot water for 5 min (at 40 °C), fasting for 48 h, water deprivation for 24 h, level shaking for 10 min, tail nip for 1 min (1 cm from the end of the tail), and inversion of the light/dark cycle for 24 h. These stressors were applied for 56 days, during which each stressor was applied 5–6 times. Mice received one of these stressors at different time every day and the same stressor was not applied consecutively over two days so that mice could not predict the occurrence of stimulation. Control group was undisturbed except for necessary procedures such as routine cage cleaning.

2.2.2. Drug treatments

From the 4th week during CUMS exposure, mice in Met group were subjected to metformin treatment. Metformin was purchased from Shandong Corelle Pharmaceutical Industry (Liaocheng City, Shandong Province, China). Mice received metformin 5 mg/ml in drinking water for 5 weeks, and water bottles were replaced with refresh metformin solution every day (Lu et al., 2016). Metformin is more effective with oral treatment than intravenous administration, because oral administration prolongs the time residing in the gut to make metformin formulations the most potent (Bahne et al., 2018).

2.2.3. Treadmill exercise protocol

The exercise program also began from the 4th week during CUMS exposure, including two phases: adaptation and training. During the first week for adaptation, mice in Run group were accustomed to running on a treadmill at 13 m/min on a 0% incline for 10 min × 2 d, 20 min × 2 d, and 30 min × 2 d Then, the training period was consisted of 30 min of treadmill running at 13 m/min, 5 d/week, for a total of 4 weeks with no inclination (Bocco et al., 2016). Exercise was performed at the same time every day (between 4:00 and 6:00 p.m.).

2.3. Behavioral testing

Except sucrose preference, a videocomputerized tracking system (DigBehav, Jiliang Co. Ltd., Shanghai, China) was used to record depression-like behaviors of the animals. All testing equipment was thoroughly cleaned between each session. These behavioral tests were performed as described in our previous study (Liu et al., 2018).

2.3.1. Sucrose preference test (SPT)

Briefly, 72 h before the test mice were trained to adapt 1% sucrose solution (w/v): two bottles of 1% sucrose solution were placed in each cage, and 24 h later 1% sucrose in one bottle was replaced with tap water for 24 h. After adaptation, mice were deprived of water and food for 24 h, followed by the sucrose preference test, in which mice housed in individual cages had free access to two bottles containing 200 ml of

sucrose solution (1% w/v) and 200 ml of water, respectively. At the end of 24 h, the sucrose preference was calculated as a percentage of the consumed 1% sucrose solution relative to the total number of liquid intake.

2.3.2. Forced swim test (FST)

Among all animal models, the FST remains one of the most used tools for screening antidepressants (Petit-Demouliere et al., 2005), since it has good predictive validity and allows rapid and economical detection of substances with potential antidepressant-like activity (Castagne et al., 2011). The swimming sessions were conducted by placing the mice in cylinders (30 cm height × 10 cm diameters) containing 25 °C water 20 cm deep so that the mice could not support themselves by touching the bottom with their feet. The FST was conducted for 5 min and immobility time was recorded. Floating in the water without struggling and only making movements necessary to keep its head above the water were regarded as immobility.

2.3.3. Open field test (OFT)

The OFT is routinely used to study anxiety-like behaviors in mouse (Carola et al., 2002). In addition, the OFT is also employed to evaluate the effects of antidepressant treatment (Shyong et al., 2017). This study aimed to examine antidepressant effects of metformin and exercise, so the OFT was used to measure exploratory behavior and general activity in mice. Each mouse was placed in the center of the open field (30 × 30 × 30 cm chamber, with 16 holes in its floor) for 5 min in a quiet room after weighed. Parameters assessed were the number of poking into holes and the traveled distance and time in the center. Poking number can be considered as an exploratory parameter (Gupta et al., 2014), and central distance and time can be used to measure locomotor activity (Moghadam et al., 2018).

2.4. Blood and tissue sample collection

Mice were decapitated and blood was kept in room temperature, and then was centrifuged at 3000 rpm for 10 min to separate the serum and blood cells. Blood sampling were collected between 09:00 and 12:00 a.m. It has been reported that corticosterone (CORT) concentration remains constant from 03:00 a.m. to 15:00 p.m. (Gong et al., 2015). Simultaneously, the hippocampus was rapidly and carefully separated on ice-plate. The whole hippocampus was collected, because it has been reported to be associated with chronic stress by western blot analysis (Kinoshita et al., 2014). Serum (ELISA) and hippocampus (WB) were stored at −80 °C until assays, respectively. For IHC, mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and transcardially perfused with phosphate-buffered saline followed by 4% paraformaldehyde (PFA) in phosphate-buffered saline. Brains were immediately removed and post-fixed in 4% PFA for 48 h. Brains were embedded in paraffin and their coronal sections (6 μm thick) were cut on a Leica microtome for IHC detection.

2.5. Molecular biological experiments

2.5.1. Enzyme-linked immunosorbent assay (ELISA)

Serum concentrations of CORT and GLP-1 were determined using commercially ELISA kits (CORT: Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China; GLP-1: Invitrogen, Carlsbad, California, USA) following the manufacturer's instructions.

2.5.2. Western blotting (WB)

The total protein concentration was assessed using the Bradford assay and run on an SDS-PAGE gel. Western blot analysis was performed using the primary antibodies (anti-GLP-1, #AF0166, 1:1000, Affinity; anti-BDNF, #ab203573, 1:1000, Abcam; anti-ERK1/2, #ab196883, 1:1000, Abcam; anti-phosphorylated ERK1/2, #ab214362, 1:500, Abcam; anti-BCL-2, #ab182858, 1:2000, Abcam; anti-BAX, #ab32503, 1:1000, Abcam) in TBST overnight at 4 °C. Monoclonal anti-β-Tubulin (#sc-9104, 1:500, Santa Cruz) was used as an internal control. WB quantification was normalized to β-Tubulin. 30 μl samples were electrophoresed and transferred to PVDF membranes. After blocking, membranes were incubated with different antibodies. The blots were incubated HRP-conjugated secondary antibodies and signals detected by enhanced chemiluminescence (ECL) WB detection reagents.

2.5.3. Immunohistochemistry (IHC)

The coronal sections of brains were pretreated with 3% hydrogenperoxide (H_2O_2) for 10 min at room temperature to remove endogenous peroxidase activity. Then, sections were incubated with primary antibody (rabbit anti-mice monoclonal Aβ, 1:100, Beyotime Biotechnology); diluted in PBS in 4 °C for overnight. After this, sections were incubated with their corresponding secondary antibody (goat anti-rabbit IgG conjugated to horse radish peroxidase, 1:100, Beyotime Biotechnology) diluted in PBS for 30 min at 37 °C. In each treatment, the slides were washed at least 3 times with PBS each for 5 min. The immunoreactivity was visualized with 3,3′-diaminobenzidine (DAB) (Beyotime Biotechnology, Shanghai, China) color reaction. Sections were chosen in accordance with stereological rules: the first section was taken at random and every fifth section afterward (Zhang et al., 2015). Dorsal hippocampus (DH) and ventral hippocampus (VH) are functionally distinct structures. Spatial memory mainly depends on DH, whereas VH relates to stress and emotion (Cope et al., 2018; Fanselow and Dong, 2010). Therefore, IHC measures in these regions were analyzed separately. The cumulative gray values and the number of GLP-1R positive cells (being dyed yellow or brown) were obtained by using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

2.6. Statistical analysis

Data are presented as mean ± SEM. Analyses were performed using GraphPad Prism, version 6.01 (GraphPad Software). Body weight was analyzed by the means of a repeated measurement ANOVA. Differences among experimental groups were determined by unpaired *t* test, or ANOVA followed by Tukey test for *post hoc* comparisons. Statistical significance was set at $P < 0.05$ or 0.01.

3. Results

3.1. CUMS induces depression-like behaviors in mice

With respect to effects of CUMS on body weight in mice (Fig. 1A), a repeated measurement ANOVA yield a main effect of time ($F_{(8,56)} = 45.127$, $p < 0.01$), a main effect of group ($F_{(1,14)} = 145.662$, $p < 0.01$) and significant group by time interaction ($F_{(8,112)} = 4.587$, $p < 0.01$). No significant differences were found in the baseline body weight between control group and CUMS group, but the body weight of CUMS group (from the 1st week till the 8th week) was significantly lower than that of control group ($p < 0.01$).

Then, we examined the effects of CUMS on depression-like behaviors in mice. As shown in Fig. 1B-E, unpaired *t* test indicated that CUMS induced depression-like behaviors, including reduced percentage of sucrose preference ($t = 5.187$, $p < 0.01$; Fig. 1B) in SPT and increased immobility time ($t = 5.152$, $p < 0.01$; Fig. 1C) in FST, as well as reduced poking number ($t = 2.417$, $p < 0.05$; Fig. 1D) and distance ($t = 3.604$, $p < 0.01$; Fig. 1E) and time ($t = 4.942$, $p < 0.01$; Fig. 1F) traveled in center in OFT, compared to the control. In addition, no significant difference in corticosterone levels was observed between control group and CUMS group (Fig. 1G). These results confirmed CUMS effects on depression-like behaviors in mice.

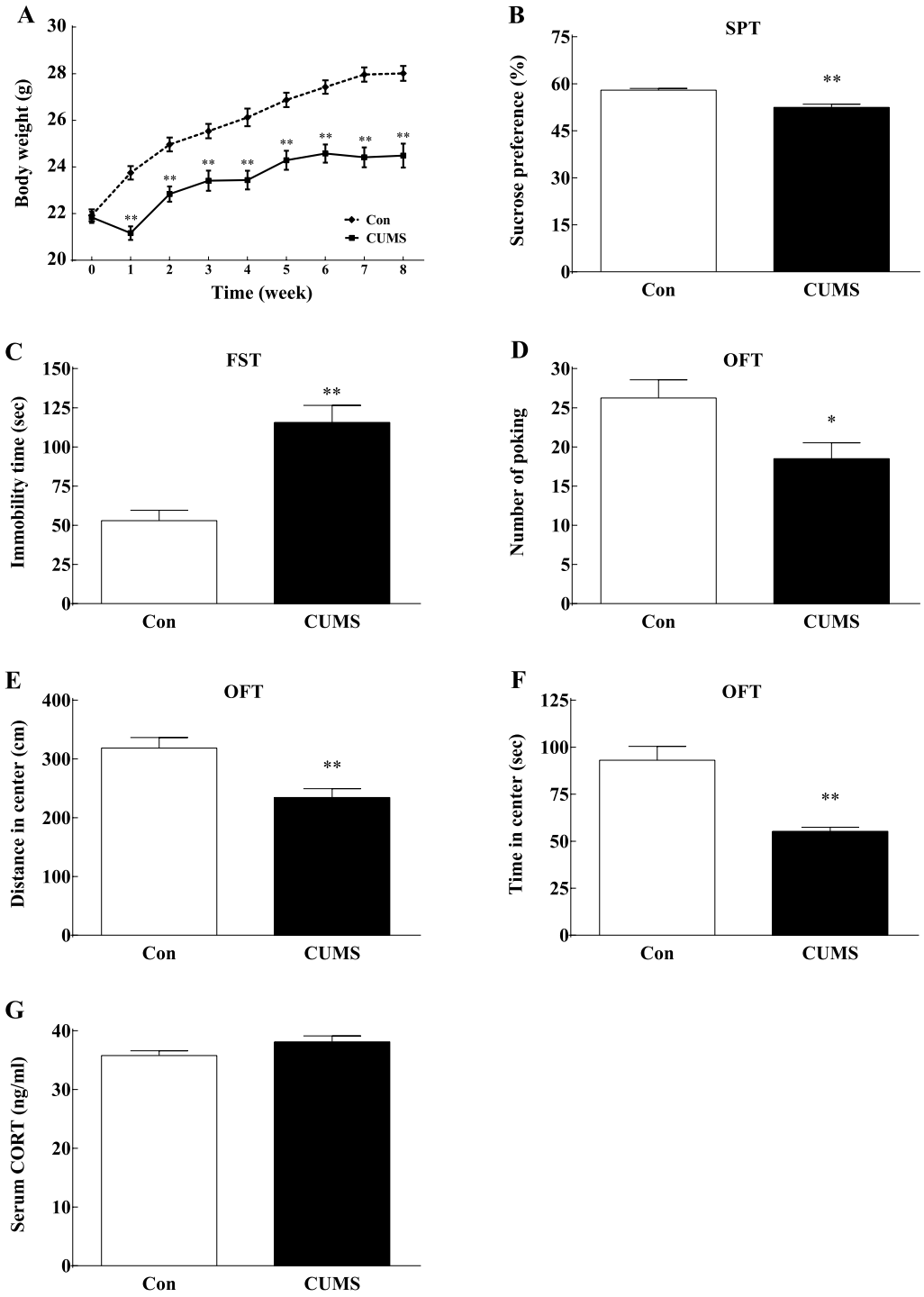

Fig. 1. CUMS induces depression-like behaviors in mice. (A) Changes in body weight during 8-week treatment; (B-E) Behavioral changes: B-Sucrose preference in sucrose preference test (SPT), C-Immobility time in forced swim test (FST), D-Poking number and E-Central distance and F-Central time in open field test (OFT); (G) Corticosterone (CORT) levels in serum. Data are presented as means ± SEM (n = 6–8 per group). *$p < 0.05$, **$p < 0.01$ versus control (Con).

3.2. Metformin and exercise ameliorate depression-like behaviors in CUMS mice

As shown in Fig. 2A, there were no significant differences of body weight among CUMS group, metformin treated group (Met), or treadmill running group (Run) over the 8-week period. The body weight of co-treatment group of Met and Run was significantly lower than that of CUMS group ($p < 0.05$) at the end of the 5th and 6th week. In addition, metformin or exercise did not block CUMS-induced body weight loss. Similarly, it has been reported that swimming training decrease body weight significantly compared to sedentary mice (Kim et al., 2014). In our previous study, CUMS rats with treadmill preconditioning have also been found to gain less weight and growth rate than sedentary CUMS rats (Liu and Zhou, 2012). The reason for these results may be that physical activities consume more energy with no difference in levels of food intake (Kim et al., 2014).

Then, we examined the effects of metformin and exercise on depression-like behaviors in CUMS mice. As shown in Fig. 2B-E, one-way ANOVA revealed a significant effect of treatment on sucrose preference test ($F_{(3,20)} = 4.932$, $p < 0.01$); forced swim test ($F_{(3,20)} = 6.256$,

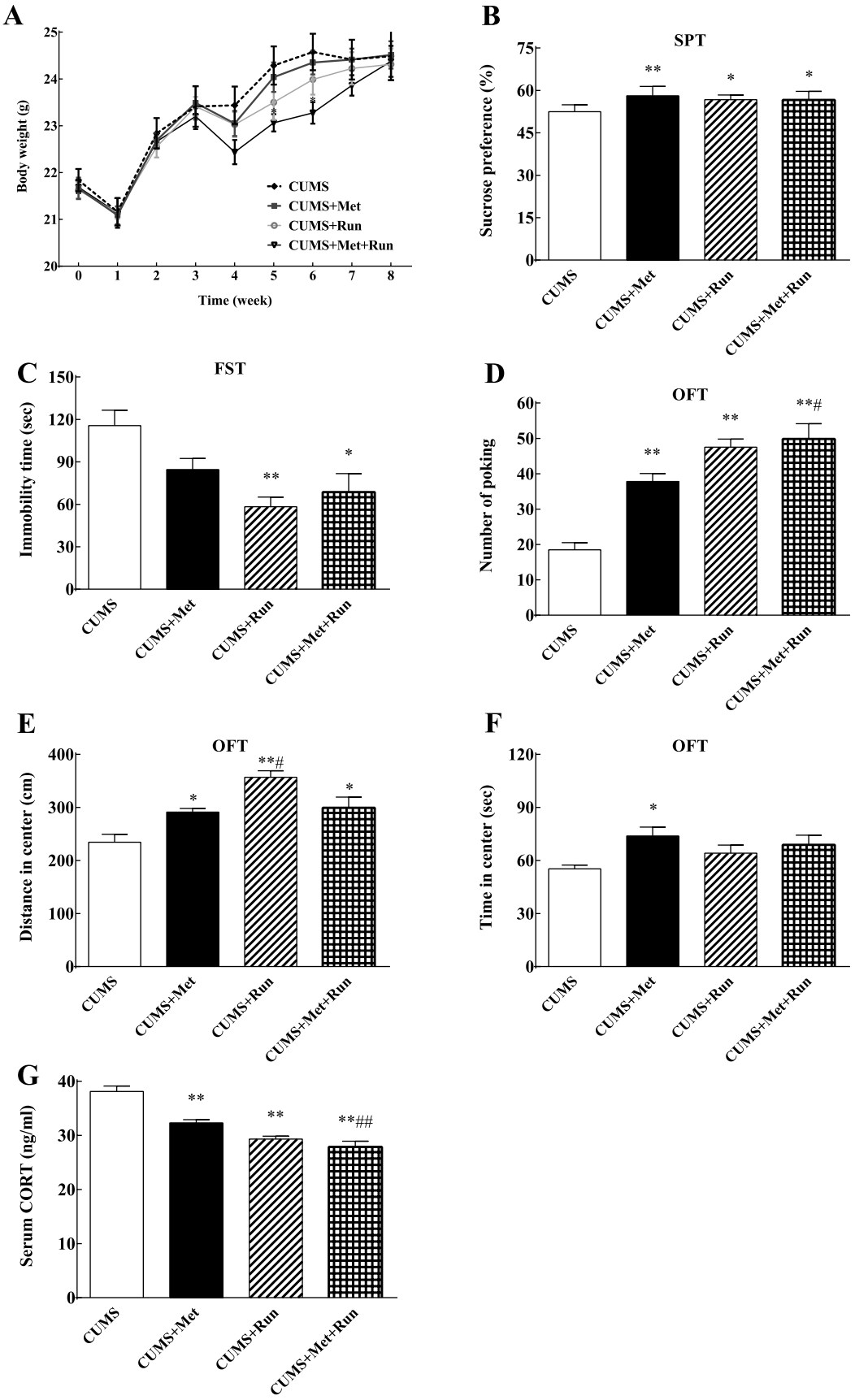

Fig. 2. Metformin and exercise ameliorate depression-like behaviors in CUMS mice. (A) Changes in body weight during 8-week treatment; (B-E) Behavioral changes: B-Sucrose preference in sucrose preference test (SPT), C-Immobility time in forced swim test (FST), D-Poking number and E-Central distance and F-Central time in open field test (OFT); (G) Corticosterone (CORT) levels in serum. Data are presented as means ± SEM (n = 6–8 per group). *$p < 0.05$, **$p < 0.01$ versus Con; #$p < 0.05$, ##$p < 0.01$ versus CUMS + Met.

$p < 0.01$); open field test ($F_{(3,20)} = 24.480$, $p < 0.01$ for poking; $F_{(3,28)} = 12.150$, $p < 0.01$ for distance; $F_{(3,28)} = 3.090$, $p < 0.05$ for time), as well as serum corticosterone levels ($F_{(3,26)} = 27.310$, $p < 0.01$). Post hoc analysis indicated that metformin ameliorated depression-like behaviors in CUMS mice, including increased percentage of sucrose preference ($p < 0.01$, Fig. 2B) in SPT, and poking number ($p < 0.01$, Fig. 2D) and central distance ($p < 0.05$, Fig. 2E) and time ($p < 0.05$, Fig. 2F) in OFT, as well as decreased corticosterone levels ($p < 0.01$, Fig. 2G) in serum. Besides the above-mentioned behavioral changes, exercise alone ($p < 0.01$) or co-treatment of Met and Run ($p < 0.05$) also decreased immobility time in FST (Fig. 2C). In addition, effect of exercise on central distance ($p < 0.05$, Fig. 2E) in OFT was better than that of metformin. Moreover, effects of co-treatment of Met and Run on poking number ($p < 0.05$, Fig. 2D) in OFT and corticosterone levels ($p < 0.01$, Fig. 2G) in serum were better than that of metformin alone. These results confirmed the improving effects of metformin and exercise on depression-like behaviors in CUMS mice.

3.3. CUMS reduces protein levels of hippocampal GLP-1 in mice

To determine whether GLP-1 and its receptor (GLP-1R) are involved in CUMS effect on depression-like behaviors, ELISA and WB were used to examine serum concentration and hippocampal protein levels of GLP-1, respectively; GLP-1R expression in hippocampus was detected using IHC. Unpaired t test indicated that CUMS reduced hippocampal protein levels of GLP-1 ($t = 2.786$, $p < 0.05$; Fig. 3B), compared to the control. However, no significant differences in serum GLP-1 concentration (Fig. 3A), GLP-1R expression in VH or DH (Fig. 3C-E) were observed between control group and CUMS group. These results suggested that GLP-1 but not GLP-1R might be involved in CUMS-induced depression.

3.4. Metformin and exercise increase protein levels of serum and hippocampal GLP-1 in CUMS mice

Then, we examined the effects of metformin and exercise on levels of GLP-1 and GLP-1R in CUMS mice. As shown in Fig. 4A-B, one-way ANOVA revealed a significant effect of treatment on serum GLP-1 concentration ($F_{(3,28)} = 25.680$, $p < 0.01$) and hippocampal protein levels of GLP-1 ($F_{(3,10)} = 3.057$, $p < 0.05$) in CUMS mice. Post hoc analysis indicated that both single and combined treatment with metformin and exercise increased serum GLP-1 concentration ($p < 0.01$, Fig. 4A). Moreover, effect of exercise on serum GLP-1 concentration was better than that of co-treatment of Met and Run ($p < 0.01$, Fig. 4A). In addition, single treatment with metformin or exercise also increased hippocampal protein levels of GLP-1 ($p < 0.05$, Fig. 4B). However, neither single nor combined treatment with metformin or exercise changed hippocampal GLP-1R expression in VH or DH (Fig. 4C-E). These results showed that GLP-1R may be involved to mediate GLP actions in antidepressant effects of metformin and exercise in CUMS mice, but change in hippocampal GLP-1R protein levels is not involved.

3.5. CUMS affects phosphorylation of ERK1/2 and protein levels of BAX in mice

To determine whether BDNF/ERK and BCL-2/BAX pathway is involved in CUMS effects on depression-like behaviors, WB was used to examine the related protein expression in hippocampus. As shown in Fig. 5, unpaired t test indicated that CUMS reduced phosphorylation levels of ERK1/2 ($t = 3.370$, $p < 0.05$; Fig. 5C and F) and increased BAX protein levels ($t = 1.813$, $p < 0.05$; Fig. 5E and F), compared to the control. No significant differences in protein levels of BDNF (Fig. 5A and F), ERK1/2 (Fig. 5B and F), or BCL-2 (Fig. 5D and F) were observed between control group and CUMS group. These results suggested that protein changes of p-ERK and BAX might be involved in CUMS-induced depression.

3.6. Metformin and exercise restore protein expression of pERK1/2 and BAX in CUMS mice

Then, we examined the effects of metformin and exercise on BDNF/ERK and BCL-2/BAX pathway in CUMS mice. As shown in Fig. 6, one-way ANOVA revealed a significant effect of treatment on phosphorylation levels of ERK1/2 ($F_{(3,12)} = 5.132$, $p < 0.05$) and BAX protein levels ($F_{(3,11)} = 3.415$, $p < 0.05$). Post hoc analysis indicated that both single and combined treatment with metformin and exercise increased phosphorylation levels of ERK1/2 ($p < 0.05$, Fig. 6C and F) and reduced BAX protein levels ($p < 0.05$, Fig. 6E and F). CUMS-associated increase in BAX is not very obvious, as seen from protein bands (one per group). Based on data analysis, CUMS did increase BAX expression in total ($n = 3$–4 per group). WB of both ERK1/2 and pERK1/2 were performed following the antibody guide. ERK protein band was divided into two subzones-ERK1 (44 KD) and ERK2 (42 KD). Protein molecular weight of ERK1/2 was too close to be separated after phosphorylation (being activated simultaneously), thus only one band was seen for pERK1/2. In view of more sensitive of total ERK1/2 than p-ERK/total ERK ratio (Natalini et al., 2016), protein levels of total ERK1/2 and pERK1/2 can be examined separately (Huelter-Hassler et al., 2017). Neither single nor combined treatment with metformin or exercise changed protein levels of BDNF (Fig. 6A and F), ERK1/2 (Fig. 6B and F), or BCL-2 (Fig. 6D and F). These results showed that protein changes of p-ERK and BAX might be involved in antidepressant effects of metformin and exercise in CUMS mice.

4. Discussion

CUMS has been widely used as an animal model of depression. Besides lower body weight gain, the behavioral changes in the present study are also consistent with previous studies, including anhedonia in SPT, despaired behaviors in FST, as well as reduced exploratory activities in OFT. Both single and combined treatment with metformin and exercise can ameliorate CUMS-induced depression-like behaviors and decrease serum levels of corticosterone, a major stress hormone and depression marker (Franklin et al., 2012). Similarly, chronic treatment with metformin, fluoxetine and their combination has been reported to reverse the changes of depression-like behaviors and serum corticosterone level in chronic restraint stress rats, with a significant preferable effect toward the combination (Khedr et al., 2018). We also found that treadmill running favored metformin effects on poking number in OFT and serum corticosterone levels.

To address the potential molecular pathology of antidepressant effects of metformin and exercise, we investigated the levels of GLP-1 and GLP-1R in CUMS mice. Our results demonstrated that CUMS reduced protein levels of hippocampal GLP-1; moreover, metformin and exercise increased protein levels of serum and hippocampal GLP-1 in CUMS mice. Specific binding sites for GLP-1 are widely distributed in central nervous system including hippocampus (Merchenthaler et al., 1999). Both GLP-1 analogs (Weina et al., 2018) and GLP-1R agonists (Zhao et al., 2018) have been shown to have antidepressant activities. In addition, it has been reported that moderate intensity exercise can increase plasma GLP-1 level in healthy (Ueda et al., 2009a) and obese (Ueda et al., 2009b) subjects. Exercise has been known to have an antidepressant effect (Yau et al., 2014). Thus, our findings suggested that regulating GLP-1 expression might be a promising approach to treat depression and novel mechanism of metformin or exercise effects on depression.

Chronic treatment with GLP-1 (7-36) and GLP-1R (liraglutide) has been shown to elevate the level of BDNF (Ando et al., 2017), which has been widely associated with depression (Castren and Antila, 2017). Metformin, fluoxetine, and their combination can reverse decreased hippocampal BDNF mRNA level induced by chronic restraint stress (Khedr et al., 2018). It has also been reported that metformin can alter BDNF transcription but not protein level in older mice (Allard et al.,

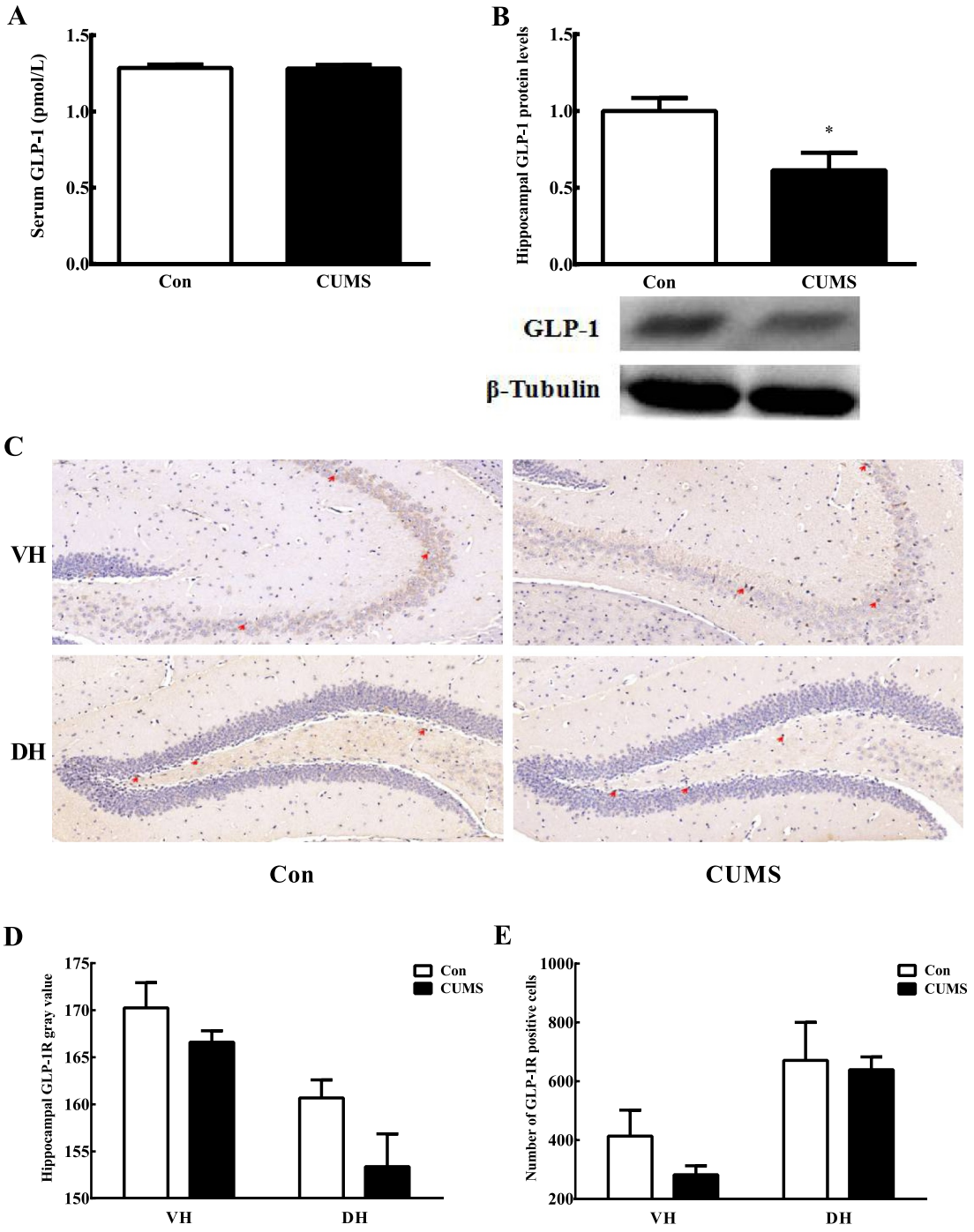

Fig. 3. CUMS reduces protein levels of hippocampal GLP-1 in mice. GLP-1 protein levels in serum (A, $n = 8$ per group) and hippocampus (B, $n = 3$–4 per group). Hippocampal GLP-1R distribution (C: top, ventral hippocampus; bottom, dorsal hippocampus), gray value (D) and positive cell number (E) using IHC. Magnification 200 × , Scale bars = 50 μm. $N = 3$ sections per brain were analyzed, $n = 3$ per group. Data are presented as means ± SEM. $^{*}p < 0.05$ versus Con.

2016). Moreover, our previous study has also demonstrated mRNA changes of hippocampal BDNF in CUMS rats with exercise preconditioning (Liu and Zhou, 2012). Similarly, we did not reveal the changes of BDNF protein expression in this study. Actually, BDNF protein but not its RNA is generally thought to mediate the neurotrophic effects of BDNF. Therefore, it is necessary to further examine BDNF changes in neurotrophic effects. Hippocampal BDNF/ERK signaling may be involved in chronic stress-induced depression and antidepressant treatment (Chen et al., 2015). Our results also showed that metformin and exercise, alone or in combination, increased phosphorylation levels but not protein levels of ERK1/2 in CUMS mice hippocampus. Similarly, proangiogenic effect of metformin has been reported to be mediated by increasing phospho-ERK1/2, with no change in ERK1/2 activity (Bakhashab et al., 2018). GLP-1 analog liraglutide has been found to promote neurite outgrowth through increasing p-ERK/ERK expression (Li et al., 2015). It has also been reported that GLP-1R agonists cannot signal via ERK1/2 in normal rats, but can significantly increase ERK1/2 phosphorylation levels in diabetic rats (Jolivalt et al., 2011). In addition, treadmill exercise can increase hippocampal p-ERK/ERK ratio and BCL-2 expression but decrease BAX expression in traumatic brain injury-induced rats (Shin et al., 2016).

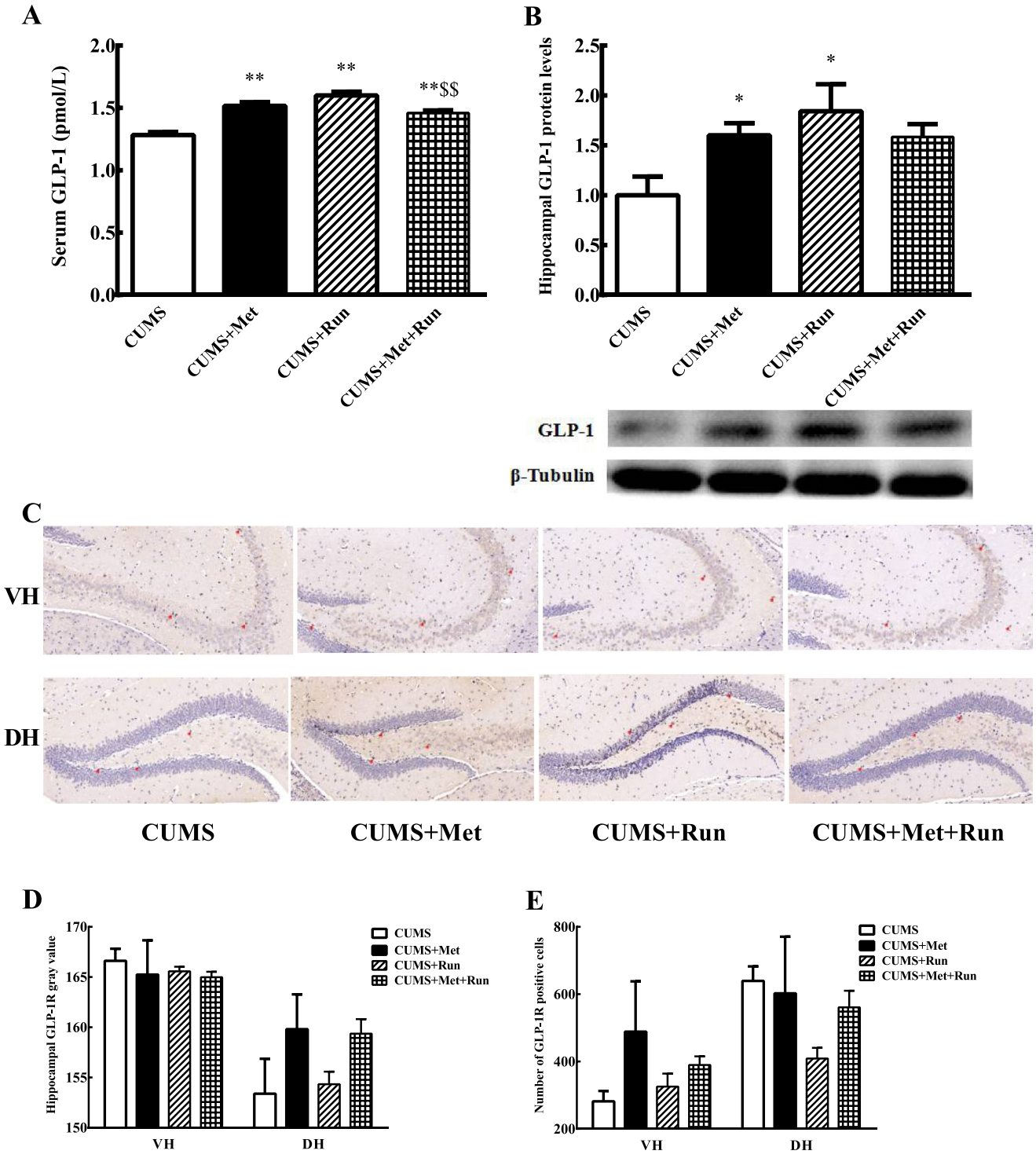

Fig. 4. Metformin and exercise increase protein levels of serum and hippocampal GLP-1 in CUMS mice. GLP-1 protein levels in serum (A, $n = 8$ per group) and hippocampus (B, $n = 3$–4 per group). Hippocampal GLP-1R distribution (C: top, ventral hippocampus; bottom, dorsal hippocampus), gray value (D) and positive cell number (E) using IHC. Magnification 200 × , Scale bars = 50 μm. $N = 3$ sections per brain were analyzed, $n = 3$ per group. Data are presented as means ± SEM. *$p < 0.05$, **$p < 0.01$ versus Con; $$$p < 0.01$ versus CUMS + Run.

We also found that CUMS-affected hippocampal BCL-2/BAX signaling was reversed by single and combined treatment with metformin and exercise. It has been recently reported that CUMS exposure can downregulate BCL-2/BAX ratio, whereas antidepressants can upregulate protein expression of BCL-2 and downregulate protein expression of BAX, thus increase the ratio of BCL-2/BAX (Li et al., 2018). Similarly, advanced glycation end products have been shown to increase BAX protein expression and decrease BCL-2 protein expression with a lowered BCL-2/BAX ratio, which can be reversed by metformin treatment (Pang et al., 2015). Treadmill exercise can improve neuronal cell death through upregulating BCL-2/BAX ratio (Koo et al., 2013). GLP-1 analog (Liu et al., 2016) and GLP-1R agonist (Fan et al., 2014) can also exert neuroprotective effects through increasing BCL-2/BAX expression. These findings suggest that protein levels of p-ERK and BAX may be associated with antidepressant effects of metformin and exercise in CUMS mice. The AMPK-aPKC-CBP pathway has been shown to be

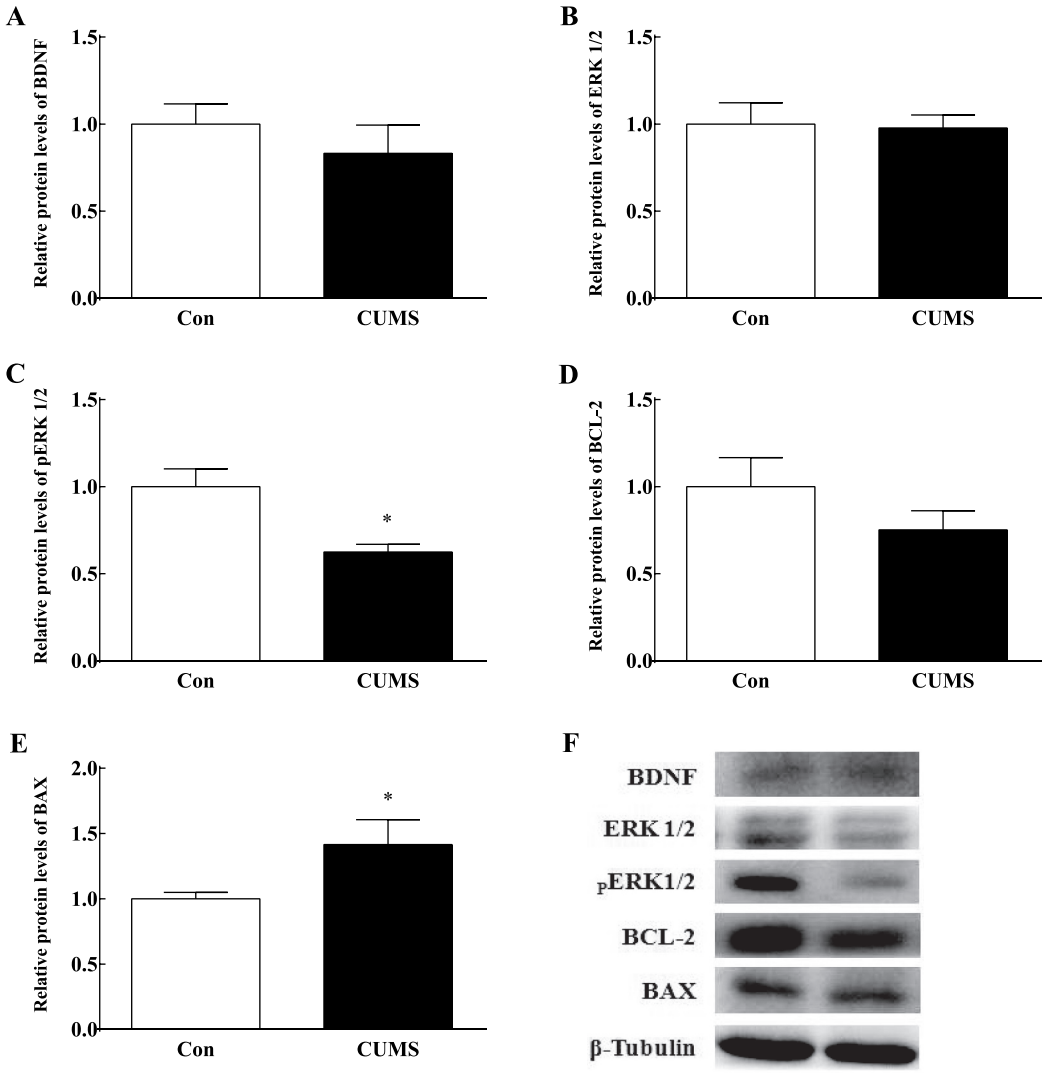

Fig. 5. CUMS affects protein levels of p-ERK and BAX in mice. The relative protein levels of BDNF (A), ERK1/2 (B), ERK1/2 phosphorylation (C), BCL-2 (D), BAX (E), and protein bands (F) in hippocampus. Data are presented as means ± SEM (n = 3–4 per group). *$p < 0.05$ versus Con.

important for metformin treatment to promote rodent and human neurogenesis in culture (Wang et al., 2012). It is therefore worthy of the most evaluation and discussion in vivo. Take our current study for example, we should further explore the role of AMPK-aPKCCBP pathway and hippocampal neurogenesis in metformin effects on depression.

5. Conclusion

In conclusion, our findings showed that protein changes of p-ERK and BAX may be involved in CUMS-induced GLP-1 reduction, which was restored by single and combined treatment with metformin and exercise. Further study is needed to establish a causal relationship between GLP-1 expression and related signaling, using GLP-1 agonist and antagonist or knockout techniques. On the other hand, the world is moving forward to more consistent therapy across diseases (Hripcsak et al., 2016). Metformin, the first-line therapy for T2DM (Viner et al., 2017), has been reported to be safe for the neurodevelopment (Van Dam et al., 2018). Exercise is also well known to promote recovery from depression and diabetes. Therefore, antidepressant therapy with metformin and/or exercise can become a topic for future clinical research.

Conflict of interest

The authors have declared that no competing interests exist.

Contributors

W.N.L. conceived and designed the experiments, analyzed the data and wrote the manuscript. J.T.L. and Z.T.Q. conceived, designed and performed these studies. Z.C.H., Z.M.C., L.X.L. and W.B.L. assisted animal model establishment and the performance of ELISA, western blotting and immunohistochemistry. Z.M.C. assisted data analysis.

Role of funding source

Funding for this study was provided by the National Natural Science Foundation of China, the Natural Science Foundation of Shanghai, the Fundamental Research Funds for the Central Universities, and the Key Laboratory Construction Project of Adolescent Health Assessment and Exercise Intervention of Ministry of Education. All funding sources had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

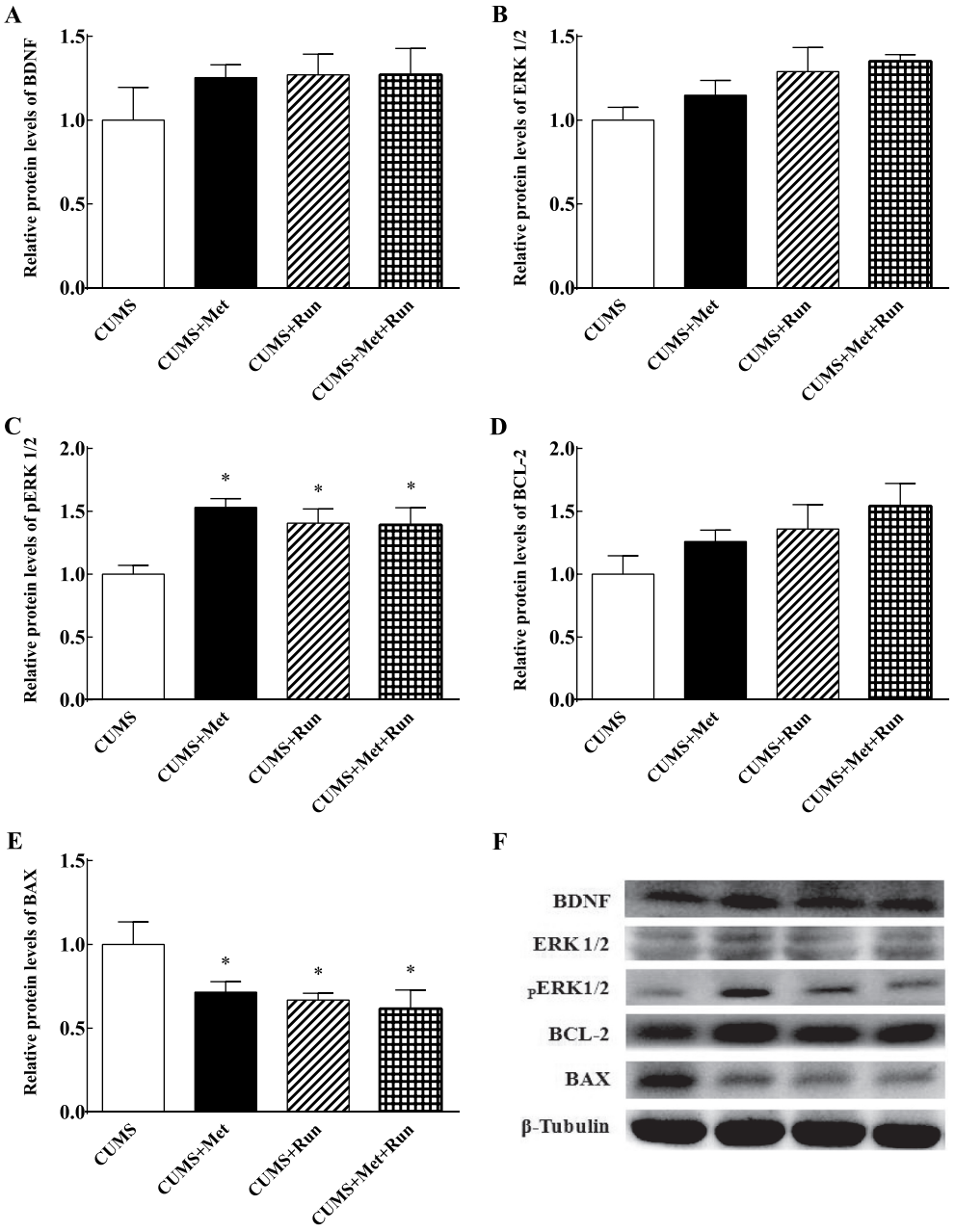

Fig. 6. Metformin and exercise restore protein levels of p-ERK and BAX in CUMS mice. The relative protein levels of BDNF (A), ERK1/2 (B), ERK1/2 phosphorylation (C), BCL-2 (D), BAX (E), and protein bands (F) in hippocampus. Data are presented as means ± SEM (n = 3–4 per group). *$p < 0.05$ versus Con.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (no. 31871208), the Natural Science Foundation of Shanghai (no. 18ZR1412000), the Fundamental Research Funds for the Central Universities (no. 2017ECNU-HWFW026), and the Key Laboratory Construction Project of Adolescent Health Assessment and Exercise Intervention of Ministry of Education (no. 40500-541235-14203/004).

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