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Moxibustion upregulates hippocampal progranulin expression

Tao Yi[1, 2], Li Qi[3], Ji Li[1, 2], Jing-jing Le[1, 2], Lei Shao[1, 2], Xin Du[1, 2], Jing-cheng Dong[1, 2, *]

1 Department of Integrative Medicine, Huashan Hospital, Fudan University, Shanghai, China
2 College of Integrated Traditional Chinese and Western Medicine, Fudan University, Shanghai , China
3 E-institute of Shanghai Municipal Education Committee, Shanghai University of Traditional Chinese Medicine, Shanghai, China

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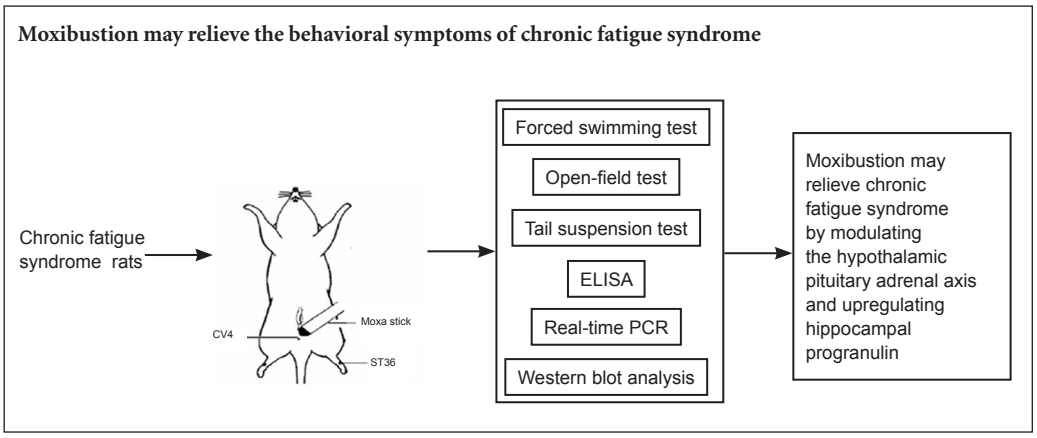
Graphical Abstract



Correspondence to:
Jing-cheng Dong, Ph.D., jcdong2004@126.com.

orcid:
0000-0002-3645-0324 (Jing-cheng Dong)

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Abstract

In China, moxibustion is reported to be useful and has few side effects for chronic fatigue syndrome, but its mechanisms are largely unknown. More recently, the focus has been on the wealth of information supporting stress as a factor in chronic fatigue syndrome, and largely concerns dysregulation in the stress-related hypothalamic-pituitary-adrenal axis. In the present study, we aimed to determine the effect of moxibustion on behavioral symptoms in chronic fatigue syndrome rats and examine possible mechanisms. Rats were subjected to a combination of chronic restraint stress and forced swimming to induce chronic fatigue syndrome. The acupoints *Guanyuan* (CV4) and *Zusanli* (ST36, bilateral) were simultaneously administered moxibustion. Untreated chronic fatigue syndrome rats and normal rats were used as controls. Results from the forced swimming test, open field test, tail suspension test, real-time PCR, enzyme-linked immunosorbent assay, and western blot assay showed that moxibustion treatment decreased mRNA expression of corticotropin-releasing hormone in the hypothalamus, and adrenocorticotropic hormone and corticosterone levels in plasma, and markedly increased progranulin mRNA and protein expression in the hippocampus. These findings suggest that moxibustion may relieve the behavioral symptoms of chronic fatigue syndrome, at least in part, by modulating the hypothalamic-pituitary-adrenal axis and upregulating hippocampal progranulin.

Key Words: *nerve regeneration; traditional Chinese medicine; moxibustion; chronic fatigue syndrome; hypothalamic-pituitary-adrenal axis; corticotrophin-releasing hormone; adrenocorticotropic hormone; behavioral symptoms; corticosterone; hippocampus; progranulin; neural regeneration*

Introduction

Chronic fatigue syndrome (CFS) is a complex, debilitating disorder characterized by unexplained physical and/or mental fatigue that lasts at least 6 months. CFS is often associated with neuropsychiatric problems (*e.g.*, depression, anxiety), neuroendocrine abnormalities, and various other complaints (*e.g.*, headache, joint pain, gastrointestinal disturbance, cognitive dysfunction, visual disturbance, and paresthesia) (Fukuda et al., 1994; Afari and Buchwald, 2003; Stouten, 2005). The pathophysiological mechanism underlying CFS is still unclear. Current therapies (*e.g.*, antidepressants and cognitive behavioral therapy), which are directed toward relieving symptoms, often have limited success and deleterious side effects (Schonfeldt-Lecuona et al., 2006; Thomas and Smith, 2006). Thus, seeking an alternative therapy for CFS needs to be addressed.

Moxibustion has been adopted as an anti-fatigue method for thousands of years in China (*Yellow Emperor's Canon of*

Medicine), and is still frequently used in present-day clinical practice. Systematic reviews have found that many of the studies show that acupuncture-moxibustion treatment is effective in treating CFS (Wang et al., 2008). However, little is known about the mechanism of moxibustion in treating CFS. Therefore, using a well-characterized rat model of CFS to clarify the possible effects of moxibustion in CFS and identify the underlying mechanisms may be of clinical benefit.

There is increasing evidence that CFS belongs to the spectrum of "stress intolerance and pain hypersensitivity" syndromes (Houdenhove and Luyten, 2007). Physical and mental stress play an important role in the pathophysiology of CFS (Luyten et al., 2008). More recent focus has been on the wealth of information supporting stress as a factor in CFS, and largely concerns dysregulation in the stress-related hypothalamic-pituitary-adrenal (HPA) axis (Tomas et al., 2013). Our previous studies have shown that moxibustion alleviates chronic hypersensitivity in adult rats by reducing HPA axis hyperactivity, providing evidence that moxibustion can exert its effect *via* HPA axis regulation (Zhou et al., 2011). From this, we can ask: what role does the HPA axis play in the anti-CFS effect of moxibustion?

The hippocampus regulates HPA axis function (Mahar et al., 2014). Neuroimaging evidence has shown structural and/or functional abnormalities in the hippocampus of CFS patients and animal models (Brooks et al., 2000; Cleare et al., 2005; de Lange et al., 2005; Cook et al., 2007; Moriya et al., 2011). Recently, progranulin was reported to function as a neurotrophic factor in the hippocampus (Van et al., 2008). Moreover, others have reported increased depressive behavior in progranulin-deficient mice (Chiba et al., 2009). Additionally, progranulin involvement in enhancement of hippocampal neurogenesis by voluntary exercise has been shown (Asakura et al., 2011). Therefore, we believe that hippocampal progranulin may be crucial for the development of CFS and the anti-CFS effect of moxibustion.

Here, we assessed the efficacy of moxibustion in a rat CFS model induced by a combination of restraint stress and forced swimming. Furthermore, we discuss the impact of moxibustion on HPA axis regulation and expression of hippocampal progranulin.

Materials and Methods

Animals

Twenty-four male Sprague-Dawley rats (180 ± 20 g body weight), specific-pathogen-free class, were supplied by the Experiment Animal Center, School of Pharmacy, Fudan University, China (license No. 2008001628826). All rats were housed at a constant temperature in a humid environment with free access to food and water. The present study conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996; http://www.nap.edu/readingroom/books/labrats/index.html). All protocols were approved by the Committee on the Use of Human and Animal Subjects in Teaching and Research, Fudan University, China. All efforts were made to minimize the number of animals used and their suffering.

Chronic fatigue syndrome induced by a combination of restraint stress and forced swimming

To induce CFS, rats were subjected to a combination of restraint stress and forced swimming. Animals were subjected to restraint stress in a glass restrainer for 21 days (3 hours/day). The restrainer size could be adjusted according to the size of the rats to ensure that each rat was immobile. After the restraint stress procedure, the animals were forced to swim until exhaustion (defined as the point at which the rat's nose remained below the water surface for 10 seconds), daily for 21 days, in a cylindrical glass jar (self-made; diameter, 40 cm and height, 80 cm) containing water up to 30 cm height at room temperature (24–28°C). The depth of the water was adequate to prevent the animals from touching the bottom of the floor with their tails. When signs of marked exhaustion became apparent, the animals were removed from the water, dried, and returned to their home cages. This chronic exposure of restraint stress and forced swimming produced physical and mental fatigue that represented CFS (Fukuda et al., 1994).

Moxibustion treatment

After 1 week of adaptation, rats (n = 24) were randomly divided according to their weight into normal, CFS, and moxibustion groups, with eight rats in each group. Moxibustion treatment was performed after 21 days of stress exposure. The detailed moxibustion procedure has been previously described (Qi et al., 2013). As shown in **Figure 1**, moxibustion was administered at bilateral *Zusanli* (ST36, located 5 mm below and lateral to the anterior tubercle of the tibia) and *Guanyuan* (CV4, located on the midline 3 cun below the umbilicus). ST36 and CV4 were located according to Lin's report (Lin, 1994). Refined moxa sticks (Hanyi Co., Ltd., Nanyang., China; 0.5 cm in diameter, made of refined mugwort floss; 2 cm high from acupoints) were ignited for 10 minutes, once daily for 14 consecutive days. Rats were not anesthetized before moxibustion treatment, and were held in a supine position on one gloved hand. As controls, rats in both the normal and CFS groups were held similarly but were not given moxibustion treatment.

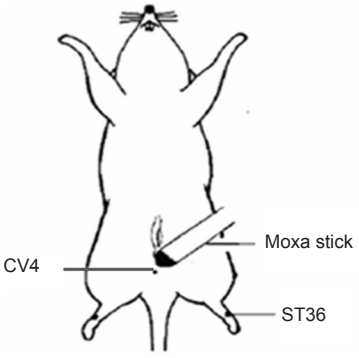

Figure 1 Moxibustion at the *Zusanli* (CV4) and *Guanyuan* (ST36) acupoints.

Behavioral assessment

The following behavioral tests were performed within 3 hours after the last moxibustion treatment.

Forced swimming test

The forced swimming test is widely used to assess fatigue behavior in animal experiments (Liu et al., 2011). To evaluate the effect of moxibustion on endurance capacity in CFS rats, maximal swimming time was measured in the forced swimming test. The test was performed according to a modification of the traditional method, suggested by Porsolt et al. (1977). Rats were placed individually into a container (80 cm high and 40 cm in diameter) containing 30 cm^3 of water at room temperature. The water depth was adequate to prevent the animals from touching the bottom of the floor with their tails. Swimming endurance capacity was assessed by forcing the rats to swim until exhaustion, which was defined as the point at which the rat's nose remained below the water surface for 10 seconds.

Open field test

The open field test is effectively used to assess the neurobehavioral profile of animals under the influence of anxiogenic/anxiolytic agents (Garcia, 2002). The open field apparatus consisted of a black square arena (100 cm × 100 cm) with a black wall 40 cm high. The floor was marked with a grid dividing the floor into 25 equal squares. During a 6-minute observation period, the rats were placed at the center of the apparatus. Horizontal locomotion (number of total squares crossed) and frequency of rearing (defined as standing upright on their hind legs) were recorded (Cai et al., 2010).

Tail suspension test

The tail suspension test was performed according to the method of Yamawaki et al. (2012), with minor modifications. Briefly, rats were suspended using bands and hung from a mounted hook 50 cm above the floor for 5 minutes. The immobile time during the last 4-minute testing period was measured. Immobility time was defined as a lack of all movement.

Preparation of samples

Rats in each group were deeply anaesthetized by intraperitoneal injection of chloral hydrate (3 mL/kg). Blood samples were taken from all rats in each group, and then the rats were decapitated. Blood samples were collected into heparin-coated tubes, centrifuged at 3,000 r/minute at 4°C for 10 minutes to separate the plasma, and stored at –80°C until analysis. The hypothalamus and hippocampus were rapidly isolated from the brain on an ice-cold platform, immediately frozen in liquid nitrogen, and then stored at –80°C for RNA and protein extraction.

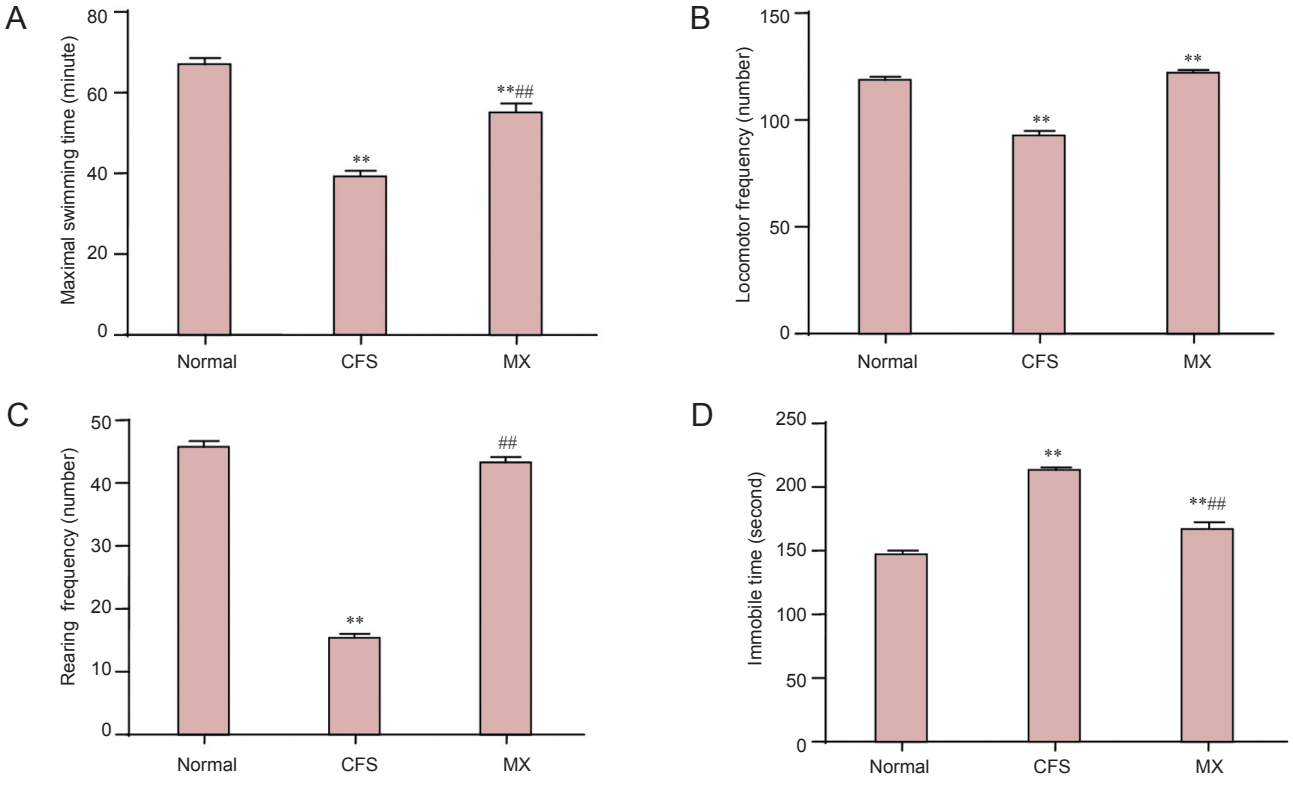

Figure 2 Behavioral performance of CFS rats after moxibustion treatment in the forced swimming test, open field test, and tail suspension test.
Data are presented as the mean ± SEM (n = 8 per group; one-way analysis of variance followed by the least significant difference *post hoc* test). (A) Maximal swimming time in the forced swimming test. (B) Locomotion frequency (number of total squares crossed) in the open field test. (C) Rearing frequency (rat stood upright on its hind legs) in the open field test. (D) Immobile time (lack of all movement) in the tail suspension test. **P < 0.01, *vs.* normal; ##P < 0.01, *vs.* CFS. MX: Moxibustion; CFS: chronic fatigue syndrome.

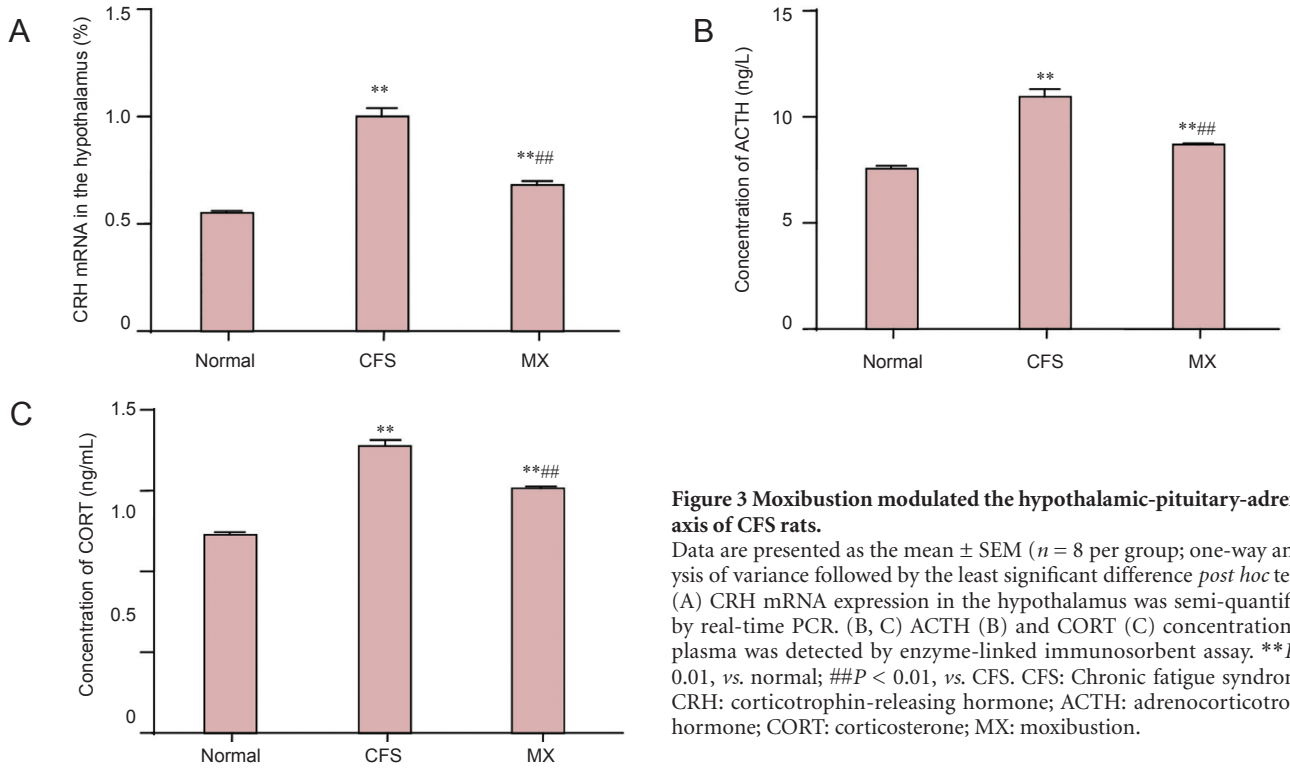

Figure 3 Moxibustion modulated the hypothalamic-pituitary-adrenal axis of CFS rats.
Data are presented as the mean ± SEM (n = 8 per group; one-way analysis of variance followed by the least significant difference *post hoc* test). (A) CRH mRNA expression in the hypothalamus was semi-quantified by real-time PCR. (B, C) ACTH (B) and CORT (C) concentration in plasma was detected by enzyme-linked immunosorbent assay. **P < 0.01, *vs.* normal; ##P < 0.01, *vs.* CFS. CFS: Chronic fatigue syndrome; CRH: corticotrophin-releasing hormone; ACTH: adrenocorticotropic hormone; CORT: corticosterone; MX: moxibustion.

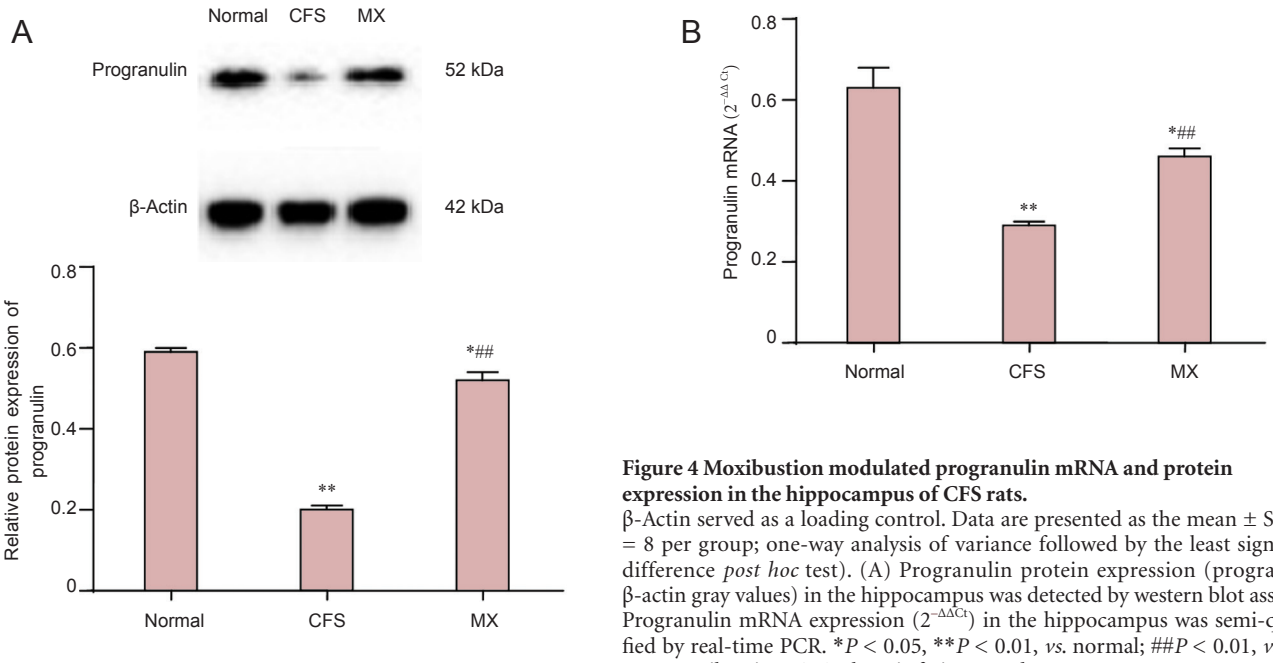

Figure 4 Moxibustion modulated progranulin mRNA and protein expression in the hippocampus of CFS rats.
β-Actin served as a loading control. Data are presented as the mean ± SEM (n = 8 per group; one-way analysis of variance followed by the least significant difference *post hoc* test). (A) Progranulin protein expression (progranulin/β-actin gray values) in the hippocampus was detected by western blot assay. (B) Progranulin mRNA expression ($2^{-\Delta\Delta Ct}$) in the hippocampus was semi-quantified by real-time PCR. *P < 0.05, **P < 0.01, *vs.* normal; ##P < 0.01, *vs.* CFS. MX: Moxibustion; CFS: chronic fatigue syndrome.

Enzyme-linked immunosorbent assay (ELISA)
Plasma levels of adrenocorticotropic hormone (ACTH) and corticosterone (CORT) were measured 2 days after blood was taken using a commercial ELISA kit (USCN-LIFE™, Wuhan, China), following the manufacturer's instructions. Both ACTH and CORT levels were calculated using the following equation: ACTH/CORT levels in plasma (ng/L) = concentration × sample dilution.

Real-time PCR
mRNA expression of corticotrophin-releasing hormone (CRH) in the hypothalamus and progranulin in the hippocampus were semi-quantified by real-time PCR. Total RNA was extracted from frozen tissue using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The primers for CRH, progranulin, and β-actin are shown in **Table 1**.

Table 1 Sequences of primer pairs used for real-time PCR

Gene	Forward primer (5'–3')	Product size (bp)
CRH	Forward: TGG ATC TCA CCT TCC ACC TT Reverse: TTC ATT TCC CGA TAA TCT CCA	111
Progranulin	Forward: TTC AGC CAA GGG AAC CAA GTG Reverse: CCC AGG ACT GTG GAG TTC TTT AG	125
β-Actin	Forward: CAC TAT CGG CAA TGA GCG GTT CC Reverse: CAG CAC TGT GTT GGC ATA GAG GTC	178

CRH: Corticotrophin-releasing hormone.

RNA concentration was determined by absorbance at 260 nm, and RNA quality by agarose gel electrophoresis and 260 nm: 280 nm absorbance ratios. For the PCR process, reactions were first incubated at 95°C for 3 minutes, followed by 40 cycles of thermal cycling at 95°C for 15 seconds and 60°C for 30 seconds. Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 7300 Sequence Detection System. Relative amounts of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method with β-actin as an internal control (Li et al., 2015).

Western blot analysis

Progranulin protein was analyzed by western blot assay. Protein was obtained by centrifugation at 15,000 r/minute for 15 minutes at 4°C. Supernatants were harvested and the protein concentration of each sample determined by bicinchoninic acid assay (Beyotime Biotechnology, Haimen, China). For gel electrophoresis, samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gels. Separated proteins were electrotransferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Membranes were blocked for 1 hour at room temperature with 5% non-fat milk (BD, Franklin Lakes, NJ, USA), and then incubated overnight at 4°C with primary rabbit anti-progranulin polyclonal antibody (1:800; R&D, Minneapolis, MN, USA). After three washes, membranes were incubated for 2 hours at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG (1:5,000; Jackson, West Grove, PA, USA). Detection was performed using an ECL kit (Millipore). Blots were subsequently probed using β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as an internal control for normalization of protein loading. Gray values for specific bands were measured using image analysis software (Labworks 4.6, Shanghai, China).

Statistical analysis

All data were analyzed using SPSS 16.0 statistical software (SPSS, Chicago, IL, USA) and are expressed as the mean ± SEM. One-way analysis of variance was used when data were in accordance with a normal distribution. Least significant difference was used to compare inter-class variation when data were in accordance with homogeneity of variance.

Games-Howell was used to compare inter-class variation when data were in accordance with heterogeneity of variance. Values of $P < 0.05$ were considered statistically significant.

Results

Moxibustion improved behavioral symptoms of CFS rats

In the forced swimming test, the maximal swimming time was notably shorter in the CFS group than in the normal group ($P < 0.01$), and longer in the moxibustion group than in the CFS group ($P < 0.01$; **Figure 2A**). In the open field test, the locomotion and rearing frequency of CFS rats were remarkably decreased compared with the normal group, while moxibustion increased both parameters ($P < 0.01$; **Figure 2B**, **C**). In the tail suspension test, immobility time was markedly increased in the CFS group compared with the normal group ($P < 0.01$), and decreased in the moxibustion group compared with the CFS group ($P < 0.01$; **Figure 2D**). These findings show that moxibustion treatment ameliorates behavioral symptoms of CFS rats.

Moxibustion regulated the HPA axis in CFS rats

Hypothalamic CRH mRNA and plasma ACTH and CORT were estimated as indices of HPA axis activity. Relative CRH mRNA expression levels were significantly higher in the CFS group than in the normal group ($P < 0.01$), yet markedly lower in the moxibustion group compared with the CFS group ($P < 0.01$; **Figure 3A**). ACTH and CORT concentrations were significantly higher in the CFS group compared with the normal group ($P < 0.01$), and significantly lower after moxibustion treatment ($P < 0.01$) (**Figure 3B**, **C**). These findings indicate that moxibustion treatment inhibits HPA axis hyperactivity in CFS rats.

Moxibustion increased progranulin mRNA and protein expression in the hippocampus of CFS rats

Next, we examined progranulin mRNA and protein levels in the hippocampus by real-time PCR and western blot assay, respectively. In the CFS group there was a significant decrease in progranulin mRNA expression compared with the normal group ($P < 0.01$), while the moxibustion group showed a significant increase compared with the CFS group ($P < 0.01$; **Figure 4B**). Western blot showed notably decreased progranulin protein expression in the CFS group compared with the normal group ($P < 0.01$), which was increased significantly in the moxibustion group compared with the CFS group ($P < 0.01$; **Figure 4A**).

Discussion

Our results are similar to the behavioral changes observed in CFS rats in previous studies (Liu et al., 2011). Moreover, all our observed effects mimic the clinical symptoms of CFS. Thus, our CFS rat model induced by a combination of restraint stress and forced swimming was successful.

Furthermore, our behavioral results show that CFS rats treated with moxibustion exhibit improved recovery from physical and mental fatigue. According to traditional Chinese

medicine (TCM) theory, the organs of the body are connected to each other by channels called meridians, in which "*qi*" and "blood" circulate. Disease is a state that indicates imbalance in "*qi*" and "blood". Retrospective studies and similarities in the basic pathogenesis indicate that CFS is similar to "*qi*-deficiency" caused by overstrain, excessive emotions, improper diet, or congenital inadequacy. The recommended therapy for "*qi*-deficiency" is to "nourish-*qi*" (Yiu and Qiu, 2005). In TCM theory, ST36 and CV4 are the two key acupoints used to "nourish-*qi*" (*Yellow Emperor's Canon of Medicine*). ST36 is the *he* point of the stomach and CV4 the *mu* point of the small intestine. Both ST36 and CV4 are the most commonly used tonic points to "nourish-*qi*" (Lu et al., 2014). The behavioral results obtained here provide evidence that 14 treatments of moxibustion at ST36 and CV4 can protect against the behavioral symptoms of CFS rats, which supports the anti-CFS effects of moxibustion. It is worth noting that in the moxibustion group, locomotion in the open field test appeared to increase somewhat, even more than in the normal group, which provides further evidence that moxibustion effectively improves physical and mental ability in CFS rats.

CFS represents a disorder of multifactorial etiology, with the exact pathophysiological mechanisms not yet fully understood. Evidence from animal and human studies shows that stressful life events and consequent HPA axis dysfunction are among the most potent factors triggering CFS (Luyten et al., 2008; Tomas et al., 2013). In the past, stress has generally been associated with HPA axis in hypercortisolism. Here, increased hypothalamic CRH mRNA expression and plasma ACTH and CORT levels in CFS rats demonstrate that a combination of restraint stress and forced swimming for 21 days significantly activates the HPA axis, which is in favor of HPA axis hyperactivity associated with stress (Wang et al., 2014). However, several lines of evidence have shown significantly reduced HPA axis function in stress-related CFS (Gold and Chrousos, 2002, Fries et al., 2005). We suspect that restraint stress and forced swimming act as stressors that activate the HPA axis response, while the contradictory results of other studies may reflect different animal models involving alternative stressors inducing CFS (Surapaneni et al., 2012). Our RT-PCR and ELISA results suggest that moxibustion modulates the stress response via the HPA axis, which is manifested by reduced hypothalamic CRH mRNA expression and plasma ACTH and CORT levels.

Early studies found that chronic stress potently decreases adult hippocampal neurogenesis (Dranovsky and Hen, 2006). Given that hippocampal neurogenesis can regulate the HPA axis (Schloesser et al., 2009; Snyder et al., 2011), this consequence of chronic stress may exacerbate the affective and behavioral responses to stress (Raison and Miller, 2003). Progranulin, also known as proepithelin, acrogranin, or prostate cancer cell-derived growth factor, is a secreted pleiotropic protein expressed in a wide variety of tissues including the hippocampus, cerebral cortex, and cerebellum (Daniel et al., 2000; Daniel et al., 2003; Liu and Bosch, 2012). Progranulin, like other growth factors, has multiple bioactivities in the central nervous system such as neurotrophic, neurogenerative, and neuroprotective effects, and is involved in neuronal function and survival (Nedachi et al., 2011). Recently, much attention has been given to the functional role of progranulin in the hippocampus. It has been demonstrated that hippocampal progranulin functions as a neurotrophic factor and plays an indispensable role in enhancing hippocampal neurogenesis (Moriya et al., 2011). We found that a combination of restraint stress and forced swimming decreases hippocampal progranulin mRNA and protein levels, while moxibustion reverses it. This indicates involvement of hippocampal progranulin in CFS and the anti-CFS effect of moxibustion.

Under the combined stress of restraint stress and forced swimming, we observed HPA axis excitation and decreased hippocampal progranulin in CFS rats. Although our findings are as yet equivocal, based on our study and previous reports, we assume that hippocampal progranulin interacts with the HPA axis in CFS development, which is worthy of further study. With moxibustion stimulation, HPA axis hyperactivity was inhibited and hippocampal progranulin expression upregulated. Taken together, and based on the acu-moxibustion effect, we propose that moxibustion at ST36 and CV4 stimulates receptors in acupoints and produces heat signals that project to the central nervous system. These thermal signals may exert an effect on structures within the HPA axis, including the paraventricular nucleus of the hypothalamus, which release CRH and arginine vasopressin, and in turn stimulate the pituitary to secrete ACTH into the systemic circulation. Consequently, ACTH acts at the adrenal gland to stimulate CORT synthesis and secretion. Additionally, the heat signals may directly initiate release of hippocampal progranulin, which promotes growth and development of hippocampal neurons and inhibits HPA axis hyperfunction.

In conclusion, our results show that moxibustion ameliorates CFS-induced behavioral symptoms by stabilizing the HPA axis stress response system and increasing hippocampal progranulin. These findings may shed light on the mechanism underlying the efficacy of moxibustion in the treatment of CFS.

Author contributions: *JCD and TY designed the study. TY, LQ, JL, and LS implemented the experiments. XD and JJL analyzed the data. TY and LQ wrote and revised the manuscript. JCD supervised all the research and edited the manuscript. All authors approved the final version of this paper.*
Conflicts of interest: *None declared.*
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Peer review: *This paper was double-blinded and stringently reviewed by international expert reviewers.*

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