Effects of Moxibustion on Amygdala -HPA Axis in Rats with Kidney-Yang Deficiency Syndrome Induced by Hydrocortisone Injection

Wen-guo Ye
Jiangxi university of traditional Chinese medicine
Hai-hua Yao
Shanghai eighth people's hospital
You-jiang Min (myj2002@126.com)
Shanghai eighth people's hospital https://orcid.org/0000-0002-4258-5196
Kai-tao Luo
Jiaxing hospital of traditional Chinese medicine
Jie Sun
Jiangxi university of traditional Chinese medicine
Zheng Yuan
Shanghai eighth people's hospital
Hui-qi Wu
Jiangxi university of traditional Chinese medicine
Li-hong Cheng
Jiangxi university of traditional Chinese medicine

Research

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Abstract

Background: The syndrome of *kidney-yang* deficiency is one of the main syndromes in traditional Chinese medicine. Modern evidences prove that the hypothalamus - pituitary - adrenal axis (HPA axis) function disorder is the main material basis of *kidney-yang* deficiency syndrome. The upper regulating center, such as hippocampus and amygdala can affected HPA axis. Although moxibustion have a therapeutic effect for *kidney-yang* deficiency syndromes, the underlying mechanism remains unclear. This study investigated the effect of suspended moxibustion on amygdala and HPA axis and elucidated the possible molecular mechanism of moxibustion in improving *kidney-yang* deficiency syndrome.

Methods: 60 male Sprague-Dawley rats were randomly divided into the normal group (n=12) and the model-building group (n=48). Rats in the model-building group were given intramuscular injection of hydrocortisone to establish the model of *kidney-yang* deficiency. The 48 rats successfully modeled were then randomly divided into model group (model, n=12) , carbenoxolone intraperitoneal injection group (CBX, n=12), moxibustion group (moxi, n=12) and moxibustion plus carbenoxolone intraperitoneal injection group (moxi+CBX, n=12). In the moxibustion group, *Shenshu* (BL23) and *Guanyuan* (CV4) points were treated with moxibustion for 14 days. After treatment, the level of corticostesone (CORT), adrenocorticotropic hormone (ACTH) and corticotropin-releasing hormone (CRH) in serum, the expressions of mineralocorticoid receptor (MR), glucocorticoid receptor (GR), 11 beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1), corticotropin releasing factor (CRF) and ACTH in rats' amygdala and (or) hypothalamus, pituitary were detected. Data were analyzed by one-way analysis of variance.

Results: Compared with the normal group, the level of CRH, ACTH and CORT in serum, and the mRNA and protein expressions of MR, GR and 11β-HSD1in amygdala, the mRNA and protein expressions of 11β-HSD1 in hypothalamus and CRF mRNA expression in amygdala and hypothalamus, and ACTH mRNA expression in pituitary of rats in the model group were all significantly decreased ($P < 0.05$ or 0.01). After treatment with moxibustion, except for 11β-HSD1 mRNA expression, the observation index mentioned above were increased to different degrees compared with those in the model group ($P < 0.05$ or 0.01).

Conclusion: Suspended moxibustion can effectively improve the serum levels of ACTH, CRH and CORT, and can up-regulate the mRNA and protein expression of MR, GR , 11β-HSD1, CRF and ACTH in amygdala and hypothalamus of *kidney-yang* deficiency rats, which may be one of the molecular mechanisms of moxibustion in improving *kidney-yang* deficiency syndrome.

Background

Hypothalamic-pituitary-adrenocortical axis (HPA axis), an important part of the neuroendocrine system, is involved in the control of stress reaction and the regulation of many physical activities, such as digestion, immune system, mood and emotion, sexual behavior, energy storage and consumption [1]. The HPA axis is not only affected by the negative feedback of hormones secreted by each organ, but also affected by the upper regulating center, such as hippocampus and amygdala. Amygdala is a part of the limbic system, which is composed of the amygdala central nucleus, medial nucleus and cortical nucleus. Stimulation of

these three nuclei in rats can induce corticosterone secretion, while damage to the amygdala can reduce the secretion of ACTH and corticosterone caused by fracture, and also reduce the secretion of ACTH after adrenalectomy [2]. Study has shown that the activation of CRH peptide in the central amygdala can positively respond to cortisol, leading to anxiety, fear and stimulate the stress system [3]. In addition, the amygdala also contains high concentrations of CRF, CRH, CRH receptors, CRH binding proteins and corticosteroid receptors [4]. This suggests that the amygdala may have the ability to synthesize and secrete CRH, which may directly activate the HPA axis and be regulated by the feedback of CRH and corticosteroid. Further studies suggest that CRH systems from hypothalamus and amygdala jointly constitute the neural circuits of stress responsiveness and anxiety generation in chronic stress [4].

Glucocorticoid (GC) is the terminal product of the HPA axis, which plays a key role in the amygdala-HPA axis and stress response [5]. Mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) participate in the regulation of the HPA axis through the combination with GC [6]. The negative feedback regulating effect of GC on target tissue (including HPA axis) depends on the content of GR and MR in the upper regulating center and the concentration of GC in target tissue cells. Besides the influence of the concentration of GC in the plasma, the concentration of GC in the target tissue is mainly adjusted by the preglucocorticosteroid metabolase 11 beta-hydroxysteroid dehydrogenase (11β-HSD) [7, 8]. 11β-HSD is a microzyme complex, a metabolic rate-limiting enzyme of glucocorticoid, which catalyzes the REDOX reaction between the ketone group (inactive) and the hydroxyl group (active) at the position 11 of glucocorticoid [9]. 11β-HSD1 is more expressed in hippocampus, amygdala, neocortical and cerebellum in adult rats, which is helpful to maintain the level of corticosterone in inner part and plays an important role in promoting the growth and maturation of tissues [10]. Carbenoxolone (CBX), a derivative of glycyrrhizae, is a non-selective inhibitor of 11β-HSD, which can inhibit both 11β-HSD1 and 11β-HSD2 [11]. The inhibition of 11β-HSD1 by CBX was similar to that of 11β-HSD1 knockout mice [12].

Kidney-yang deficiency syndrome, one of the main syndromes in traditional Chinese medicine, is a type of deficiency-cold syndrome caused by deficiency and decline of *kidney-yang*. Modern studies have confirmed that the low function of HPA axis is the main material basis of *kidney-Yang* deficiency syndrome [13]. Literature research and clinical observation [14–17] showed that moxibustion therapy in traditional Chinese medicine has a good effect on *kidney-Yang* deficiency syndrome. Our previous studies have also shown that moxibustion on *shenshu* and *guanyuan* with moxa sticks can effectively improve the dysfunction of HPA axis [18]. Can moxibustion with moxa stick affect the expression of MR, GR, 11β-HSD1, CRH and ACTH in the amygdala-HPA axis to treat *kidney-yang* deficiency syndrome? Therefore, its therapeutic mechanism is worthy of further study.

Methods

Experimental animals

Sixty healthy, clean, male, Sprague Dawley (SD) rats aged 8 weeks (weighing 200 ± 20 g) were purchased from the Experimental Animal Center of Jiangxi University of Traditional Chinese Medicine, of Jiangxi

Province, China (certificate no. JZLLSC (jiangxi) 2018-0033). The rats were fed with standard fodder and with food and water freely available in a controlled environment with a constant temperature of 20 ~ 25℃ and a 12/12-hr light/dark cycle. All procedures were conducted in accordance with guidelines reviewed and approved by the Institutional Animal Care and Use Committee of Jiangxi University of Traditional Chinese Medicine, China.

Modeling

Sixty rats were randomly divided into the normal group (norm, n = 12) and the model-building group (n = 48). Referring to literature [19] and making improvements, each rat in the model-building group was given intramuscular injection of hydrocortisone(*Huazhong* pharmaceutical co., LTD., national drug approval no. H420201507) at a dose of 0.03 mg/g·bw in left and right hind limbs of the rat alternately, and the modeling was carried out continuously for 14 days. Each rat in the normal group was given an intramuscular injection of 0.9% normal saline at a dose of 0.03 mg/g·bw for 14 days. During this period, natural light and free eating and drinking will be adopted. Criteria for model success [20[: decreases in body weight, withered clothing hair, hunched back, aversion cold, tendency to cluster, slowed reaction, weakness, decreased activity, scrotal shrinkage, and significantly increased urine output.

Grouping and treatment

The 48 rats successfully modeled were then randomly subdivided into model group (model, n = 12), carbenoxolone intraperitoneal injection group (CBX, n = 12), moxibustion group (moxi, n = 12) and moxibustion + carbenoxolone intraperitoneal injection group (moxi + CBX, n = 12).

In the moxibustion treatment group, *guanyuan (CV4)* and *shenshu(BL25)* (double laterals) were selected, and acupoints locating according to the *experimental acupuncture* [21]. Moxibustion treatment began on the 1st day after modeling finished. Before the treatment, the hair on the acupoints was cut off to expose the skin in advance. *guanyuan (CV4)* and *shenshu (BL25)* points were heated by suspended moxibustion using a moxa-stick produced from mugwort (custom-made for use with animals, length 12 cm, diameter 0.6 cm, made in the Affiliated Hospital of Jiangxi University of Traditional Chinese Medicine, Nanchang, China) at a height of approximately 3 cm over a hairless area of the skin for 20 min, once a day for 14 days.

Rats in the CBX group were given intraperitoneal injection of carbenoxolone at a dose of 10 mg /(kg·d) (11) once a day for 14 days. Fixation was performed as in the moxibustion group for a total of 14 times.

Rats in the moxi + CBX group were received moxibustion as in the moxibustion group. After moxibustion treatment, the rats were given intraperitoneal injection of carbenoxolone as in the CBX group.

Rats in the normal group and model group were fixed in the same way as the moxibustion group did, and were given intraperitoneal injection of normal saline (10 mg /(kg·d) once a day for 14 days.

Sampling

After 14 days of treatment, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital 3% (weight/volume) at a dose of 40 mg/kg. Arterial blood is extracted and centrifuged to extract the supernatant, and stored in the − 20℃ refrigerator for later use. Tissues such as amygdala, hypothalamus and pituitary were extracted and stored in the − 80℃ refrigerator for later use.

Enzyme linked immunosorbent assay (ELISA) assessment

The levels of CORT, ACTH and CRH were determined using an ELISA kit purchased from Shanghai *bogu* biotechnology co., LTD (Shanghai, China). The ELISA-based method was conducted according to protocol provided by manufacturer.

Reverse transcription real-time quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed as described previously with minor modifications [22]. Total RNA was isolated from amygdala, hypothalamus and pituitary of rats in each group using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). The mRNA expression levels of MR, GR, 11β-HSD1,CRH and ACTH were measured using an RT-qPCR system with SYBR Green (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was amplified by PCR using primers for each target gene. cDNA was synthesized from each RNA using a random primer and RevertAid™ M-MuLV reverse transcriptase (Fermentas), according to the manufacturer's instructions. PCR was performed in a final volume of 25 μL, containing 12.5 μL 2 × PCR master mix, 2 μL cDNA, 1 μL forward primer, 1 μL reverse primer, and 8.5 μL sterilized DEPC water. RT-qPCR conditions were as follows: 94 °C for 5 minutes, followed by 40 cycles of 95 °C for 15 seconds, 60 °C for 45 seconds and 72 °C for 30 seconds. The fluorescence signal was detected at 60 °C, and the samples were finally extended at 72 °C for 7 minutes. The amplification efficiency was compared between the target and reference control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the delta-delta Ct (ΔΔCt) method [23]. The primers employed are listed in Table 1.

Table 1
Primer sequences for real-time quantitative polymerase chain reaction

Gene	Full name	Sequences (5–3')	Product size (bp)
11β-HSD1	11 beta- hydroxysteroid dehydrogenase type 1	Sense primer: AAA ATA CCT CCT CCC CGT CC antisense primer: AGG CAG CGA GAC ACC ACC	219
MR	mineralocorticoid receptor	Sense primer: AGA AGC TGG GGA AGT TAA AAG G antisense primer: TCG GAG CGA TGT ATG TGG TC	102
GR	glucocorticoid receptor	Sense primer: CAT TAC CAC AGC TCA CCC CTA C antisense primer: GCA ATC ACT TGA CGC CCA C	148
CRH	corticotropin-releasing hormone	Sense primer: TGG CTC TGT CGC CCT GTC antisense primer: CAG CGG GAC TTC TGT TGA GG	186
ACTH	adrenocorticotrophic hormone	Sense primer: CTC CTG CTT CAG ACC TCC ATA G antisense primer: GGC TGT TCA TCT CCG TTG C	161
GAPDH	glyceraldehyde 3-phosphate dehydrogenase	Sense primer: GGA GTC TAC TGG CGT CTT CAC antisense primer: ATG AGC CCT TCC ACG ATG C	237

Western blot assay

Western blot analysis was performed as described previously with minor modifications [24]. All amygdala and hypothalamus tissues obtained in each group were homogenized in lysis buffer (JRDUN Biotechnology, Shanghai, China). The sample was centrifuged at 12,000 r/min for 20 minutes at 4 °C, and an aliquot of the supernatant was taken for protein concentration estimation using the BCA assay. Equal amounts of proteins were separated by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE), and then the resolved proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were incubated with primary antibodies overnight at 4 °C.

Monoclonal antibodies used for Western blotting included a rat monoclonal anti-MR antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The antibodies used in this study were rat monoclonal antibodies, 1:1000 and were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), except for those specifically indicated. The other antibodies included anti-GR, anti-11β-HSD1 and anti-GAPDH. The membranes were washed 5 minutes with Tris-buffered saline Tween 3 times and incubated with goat anti-rabbit IgG-HRP (1:1000; Beyotime Biotechnology, Shanghai, China) and goat anti-mouse IgG-HRP PS1 (C-20) (1:1000; Beyotime Biotechnology, Shanghai, China) antibodies at 37 °C for 1 hour. The immunoreactive bands were visualized using an enhanced chemiluminescence reagent (Beyotime Biotechnology, Shanghai, China). The grayscale values of bands were quantified using Image J software (Fujifilm, Tokyo, Japan). The relative expression of protein was calculated based on the ratio of target grayscale values to loading control grayscale values.

in situ hybridization

in situ hybridization was performed as described previously with minor modifications [25]. Six paraffin blocks were selected randomly and placed into a refrigerator at -20℃ for at least 30 minutes. Then, the blocks were cut into 4-7-μm-thick serial sections. After being dewaxed and dehydrated, each section was incubated in 50 μl hybridization buffer containing 10 μM oligonucleotide probe at 95℃ for 5 minutes and 37–40℃ for 12 hours, washed three times with 5×, 1 × and 0.2 × saline sodium citrate, and treated with blocking buffer at 37℃ for 15 minutes. Then, the blocking buffer was blotted with paper. Each section was then incubated in 30 μl biotinylated anti-digoxin antibody (1:50) at 37℃ for 1 hour, washed four times with 0.5 M PBS, incubated in streptavidin-biotin-peroxidase complex at 37℃ for 30 minutes, and rinsed four times in 0.5 M PBS. Subsequently, the sections were developed in 3, 3, diaminobenzidine, counterstained with hematoxylin, dehydrated in alcohol, permeabilized in xylene, mounted with proof quench mounting agent, and photographed with a fluorescence microscope [26]. The upper, middle, lower, left and right visual fields were randomly selected for each section and the image-pro Plus 6.0 software was used to detect the integrated optical density (IOD) of positive cells, the average value was used for statistical analysis. The negative control was incubated in 0.01M PBS without primary antibody. The primers employed are shown in Table 2.

Table 2
Primer sequences for Hybridization in situ

Gene	Full name	Sequences (5–3')
11β-HSD1	11 beta-hydroxysteroid dehydrogenase type 1	CAGAUGCCAGGUUUGUGCUCAAGUAAAUCCAAAAUGGAUAGCCUUACUCA
CRF	corticotropin-releasing factor	CAAUACAAAUAACGCUGUUUUGUUACUACAAAGAAACACACUUUGUGCA
MR	mineralocorticoid receptor	GGAUGGAGAGGAUAGCAAUCCCGGCAGUCGCCCUACUGACGGUGGG
GR	glucocorticoid receptor	UGCUUGUGGAGCCUUUCGAGAAAUCAAGGAGAAUCCUCUGCUGCUU

Statistical analyses

All data are presented as the mean ± SD. Data were analyzed by one-way analysis of variance followed by a post hoc Student-Newman-Keuls (SNK) test using SPSS 19.0 software (SPSS, Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

Results

The level of CORT, ACTH and CRH in serum by ELISA

Compared with the normal group, the level of CORT, ACTH and CRH in serum of rats in the model group was significantly decreased ($P < 0.01$). After treatment, except for the CBX group, the serum level of CORT and ACTH in the other two groups were significantly increased compared with those in the model group ($P < 0.05$ or 0.01). And the serum levels of CRH were significantly increased compared with that in the model group ($P < 0.05$ or 0.01).

Compared with the CBX group, the serum level of CORT and CRH in the moxibustion group was significantly higher ($P < 0.01$), and the serum level of ACTH in the moxibustion and moxi + CBX group were significantly higher ($P < 0.01$or 0.05). Compared with the moxibustion group, the serum level of CORT, ACTH and CRH in the moxi + CBX group was significantly lower ($P < 0.01$ or 0.05). As shown in Fig. 1.

The mRNA expression of MR, GR, 11β-HSD1, CRH and ACTH by qRT-PCR

Compared with the normal group, the mRNA expressions of MR and GR in the amygdala of rats in the model group were both significantly decreased ($P < 0.01$). After treatment, except for the CBX and moxi + CBX group, the mRNA expressions of MR and GR in the amygdala of rats in the moxibustion group were significantly higher than those in the model group ($P > 0.01$). Except for GR, the mRNA expressions of MR and GR in the amygdala were not significantly different among CBX, moxibustion and moxi + CBX group ($P > 0.05$). As shown in Fig. 2A.

Compared with the normal group, the mRNA expressions of 11β-HSD1 and CRH in the amygdala of rats in the model group were both significantly decreased($P < 0.01$). After treatment, except for CBX group and CRH in the CBX group, the mRNA expressions of CRH in the amygdala of rats in the moxibustion group and moxi + CBX group were significantly higher than those in the model group ($P < 0.01$ or 0.05). Compared with CBX, the mRNA expressions of 11β-HSD1 and CRH in the amygdala of rats in the moxibustion group were significantly increased ($P < 0.01$ or 0.05). As for the mRNA expressions of 11β-HSD1 and CRH, therer were no significantly different between moxibustion and moxi + CBX group ($P > 0.05$). As shown in Fig. 2B.

Compared with the normal group, the mRNA expressions of 11β-HSD1 and CRH in the hypothalamus of rats in the model group were both significantly decreased($P < 0.01$). After treatment, except for 11β-HSD1 in the CBX and moxi + CBX group, the mRNA expressions of 11β-HSD1 and CRH in the hypothalamus of rats in the CBX, moxibustion and moxi + CBX group were significantly higher than those in the model group ($P < 0.01$ or 0.05). Compared with the CBX group, except for CRH in the moxi + CBX group, the mRNA expressions of 11β-HSD1 and CRH in the hypothalamus of rats in the moxibustion and moxi + CBX group were both significantly increased ($P < 0.01$). As for the mRNA expressions of 11β-HSD1 and CRH, there were no significantly different between moxibustion group and moxi + CBX group ($P > 0.05$). As shown in Fig. 2C.

Compared with the normal group, the ACTH mRNA expression in the pituitary of rats in the model group was significantly decreased ($P < 0.01$). After treatment, except for the CBX group, the ACTH mRNA expressions in the pituitary of rats in the moxibustion and moxi + CBX group were significantly higher than that in the model group ($P < 0.01$ or 0.05). Compared with the CBX, the ACTH mRNA expressions in the moxibustion group was significantly higher($P < 0.01$). As for the ACTH mRNA expressions, there were no significantly different between moxibustion group and moxi + CBX group ($P > 0.05$). As shown in Fig. 2D.

The protein expression of MR, GR and 11β-HSD1 by Western blotting

Compared with the normal group, the protein expressions of MR, GR and 11β-HSD1 in the amygdala of rats in the model group were all decreased to different degrees ($P < 0.05$ or 0.01). After moxibustion treatment, the protein expression of MR, GR and 11β-HSD1 in the amygdala of rats were increased to different degrees compared with those in the model group ($P < 0.05$). As for the protein expressions of MR,

GR and 11β-HSD1, there were no significantly different among the CBX, moxibustion and moxi + CBX group (P > 0.05).As shown in Fig. 3A and B.

Compared with the normal group, the 11β-HSD1protein expression in the hypothalamus of rats in the model group was significantly decreased (P < 0.01). After treatment, the 11β-HSD1 protein expression in the hypothalamus of rats were significantly increased compared with that in the model group (P < 0.01). As for the 11β-HSD1 protein expression in the hypothalamus of rats, there were no significantly different among the CBX, moxibustion and moxi + CBX group (P > 0.05). As shown in Fig. 3 C and D.

mRNA expressions of MR, GR, 11β-HSD1 and CRF in amygdala, and 11β-HSD1 and CRF in hypothalamus by *in situ* hybridization

In situ hybridization-positive cells contained brown spots or particles, as indicated by the arrows. In the normal group, a large number of mRNA expressions of MR, GR, 11β-HSD1 and CRF in amygdala, and 11β-HSD1 and CRF in hypothalamus were observed (Fig. 4, 5, 6). After modeling induced by hydrocortisone injection, rare mRNA expressions of MR, GR, 11β-HSD1 and CRF in amygdala, and 11β-HSD1 and CRF in hypothalamus were observed, and the integral optical density (IOD) values of mRNA expressions of MR, GR, 11β-HSD1 and CRF in amygdala, and 11β-HSD1 and CRF in hypothalamus of rats in the model group were significantly decreased (P < 0.01) compared with those in the normal group (Fig. 4, 5, 6 and Fig. 7).

Treatment with moxibustion could increase the mRNA expressions of MR, GR, 11β-HSD1 and CRF, and the IOD values of their positive cells in the amygdala of rats in the moxibustion group were significantly higher than those in the model group and in the CBX group (P < 0.01 or 0.05) (Fig. 4, 5 and Fig. 7A, 7B).

As for MR mRNA expressions, the IOD values of its positive cells in the moxibustion group was significantly higher than that in the moxi + CBX group (P < 0.05) (Fig. 4 and Fig. 7A). As for 11β-HSD1 mRNA expression,the IOD values of its positive cells in the moxi + CBX group was higher than that in the CBX group (P < 0.05). And as for CRF mRNA expression the IOD values of its positive cells in the moxi + CBX group was higher than that in the model group (Fig. 5 and Fig. 7B).

Treatment with moxibustion could increase the mRNA expressions of 11β-HSD1, and the IOD values of its positive cells in the hypothalamus of rats in the moxibustion group and in the moxi + CBX group were significantly higher than those in the CBX group (P < 0.01 or 0.05), but not higher than those in the model group (P > 0.05). Treatment with moxibustion could increase the mRNA expressions of CRF, and the IOD values of its positive cells in the hypothalamus of rats in the moxibustion group and in the moxi + CBX group were significantly higher than those in the model group (P < 0.01 or 0.05), but not higher than those in the CBX group (P > 0.05) (Fig. 6 and Fig. 7C).

Discussion

Corticotropin releasing hormone (CRH) is one of the primary regulator of the hypothalamus - pituitary - adrenal (HPA) axis [4]. CRH, released from a central bump in the hypothalamus, is transported to the anterior pituitary via portal vein system, and stimulates the secretion of adrenocorticotropic hormone (ACTH), then ACTH stimulate adrenal cortical synthesis to secret glucocorticoid (GC) [4]. GC can also inhibit the generation of ACTH and CRH by feedback, thus forming the feedback regulating loop of HPA axis [27, 28]. In addition, the amygdala can also synthesized and secreted CRH, then directly activate the HPA axis and be regulated by the feedback of CRH and GC [29–32]. The feedback regulation of GC to the amygdala and the hypothalamus may be related to the combination of GC with GR and (or) with MR. When the level of GC was within the basis range, GC almost completely binds to MR, and only when GC's concentration higher than the basis level will bind to GR [33]. When GC combines with GR in the amygdala, it gives feedback information to the neurons in small cell group of paraventricular nucleus of hypothalamus, thus reducing the secretion of CRH. Through top-down regulation, the activity of HPA axis is further inhibited, so as to reduce the excessive GC level in the body, thus maintaining the hormone level homeostasis of the body [32]. Before GC binds to GR and MR, its activity and concentration are affected by the regulator 11β-HSD1, which converts inactive 11-dehydrocorticosterone / cortisone into active corticosterone / cortisol [34].

When a large amount of exogenous corticosteroids are used in the body for a long time, GC leads to excessive activation of GR, which eventually leads to the damage of nerve cells in the upper regulatory center, such as hippocampus and amygdala, and the HPA axis function is inhibited by the feedback. When the use of exogenous corticosteroids is stopped, the inhibition of HPA axis is exposed. The stress and adaptability of the body to the changes of the external environment significantly decrease, and a series of *yang* deficiency manifestations of deficiency and cold appear [35, 36]. Just as the treatment for some autoimmune diseases requires long-term use of a large number of hormones, when the disease improves, the patient develops hormone-dependent symptoms, showing the symptoms of *yang* deficiency of traditional Chinese medicine [37, 38]. The most commonly used rat model of corticosterone *kidney-yang* deficiency was prepared according to this pathological factor. In this study, after modeling the rats took on a series of deficiency manifestations such as withered clothing hair, decreases in body weight, slowed reaction, aversion cold, weakness, tendency to cluster, and decreased activity. In this study, detected by PCR, WB and *in situ* hybridization, we found that there was a lower expression of MR, GR, 11β-HSD1, CRH or CRF in the amygdala of rats in the model group than those in the normal group. And the expression of 11β-HSD1, CRH or CRF in the hypothalamus of rats in the model group has the same tendency as those in the amygdala. Compared with the normal group, the level of CORT, ACTH and CRH in serum, the mRNA expression of ACTH in pituitary in the model group rats were significantly decreased, which was the same as the results in our previous study [18].

11β-HSD has two isoenzymes, i.e. 11β-HSD1 and 11β-HSD2. 11β-HSD1 is a primary reductase that converts inactive 11-dehydrocorticosterone/cortisone into active corticosterone / cortisol. But the action of 11β-HSD2 is opposite to that of 11β-HSD1 [10]. When the concentration or activity of GC is high in vivo, 11β-HSD2 outweighs 11β-HSD1 in the role of conversion, in contrast, when GC low, 11β-HSD1 outweighs 11β-HSD2. Together, they assist MR and GR to maintain the relative stability of hormone levels. CBX can

inhibit both 11β-HSD1 and 11β-HSD2 [11]. After modeling in this study, GC was always at a low level in rats, 11β-HSD1 was activated and played an important role, and MR was also activated and combined with GC. Therefore, as the results of this study showed, the mRNA expression of MR in the amygdala was about three times that of GR. As a blocking agent, CBX mainly inhibits 11β-HSD1, so in this study 11β-HSD1 was selected as the observation index instead of 11β-HSD2. In this study, the expression of 11β-HSD1 in the amygdala and hypothalamus of rats in the CBX group was lower than that in the model group. Due to the effect of 11β- HSD1 was blocked by CBX, the concentration or activity of GC is lower in the CBX group, thus causing increased expression of MR in the amygdala by feedback regulation. Just as the experiment results showing that the MR expression of amygdala in rats in the CBX group is higher than that in the model group, correspondingly, CRF expession in the amygdala and hypothalamus, CRH expression in hypothalamus and ACTH expression in the pituitary of rats in the CBX group are higher than those in the model group, and the levels of CORT, ACTH and CRH in serum of rats in the CBX group is higher than that in the model group. Literature research found that there was no similar studies have been reported, but there were still some experiments that use the expressions of CRH, MR, GR, etc. in the amygdala and hypothalamus as observation indicators to study the effect of GC on the body. For example, when pregnant Wistar rats were injected with CBX (12.5 mg s.c.) daily throughout pregnancy, Welberg, et al. [39] found that CBX treatment reduced birth weight, and adult offspring of CBX-treated dams had persistently reduced body weight, showed less grooming and rearing,and reduced immobility in a forced swim test. In addition, they found that these animals had increased basal CORT levels, increased CRH and reduced GR mRNA in the hypothalamus, increased GR mRNA expression in the amygdala, with unaltered MR mRNA. The effect of CBX on the expression of CRH, MR and GR in the offspring amygdala and hypothalamus of pregnant rats was inconsistent with the results of this study. The reason mainly maybe that the observing subjects or animal models were different in the two experiments.

For the treatment of *kidney-yang* deficiency syndrome, *the introduction to medicine*,a classic work of traditional Chinese medicine, has the theory that moxibustion treating the deficiency syndrome, so as to help restoring the *yuan yang*; moxibustion treating the cold syndrome, so as to help re-warming the *qi*. Moxibustion is a traditional Chinese medical therapy using the burning of dried mugwort (moxa) and is widely used in China for the treatment of various diseases and symptoms of “deficient conditions” (weakness) [40]. Suspended moxibustion is an indirect form of moxibustion in which the moxa-stick is placed on an acupoint without skin contact to stimulate circulation by warming the acupoints and inducing smoother flow of *blood* and *qi*, to improve health and facilitate recovery from disease [40]. In our previous study, moxibustion on *shenshu* and *guanyuan* points could positively regulate the dysfunction of pituitary-adrenal and pituitary-thyroid axis in rats with *kidney-yang* deficiency syndrome [18]. In this study, compared with the model group, the mRNA and protein expressions of MR, GR and 11β-HSD1 in the amygdala of rats were significantly up-regulated after the treatment on *shenshu* and *guanyuan* point with suspended moxibustion. After moxibustion, the mRNA expressions of 11β-HSD1 and CRF(or CRH) in the hypothalamus, ACTH in the pituitary and the level of CRH, ACTH and CORT in serum of rats were all significantly increased compared with those in the model group. According to the theory of tradition Chinese medicine, *shenshu* is mainly used to repair *yang*, *guanyuan* is mainly used to repair *qi*, and the two acupoints are in harmony with each other, so as to cultivate *yuan qi*, and to improve the function of

tonifying kidney and warming *yang* [41]. One possible reason is that, according to the theory from *the Golden mirror of medicine (YiZong Jin Jian)*, another classic work of traditional Chinese medicine, *shenshu* point belongs to the *bladder meridian*, which is the *back shu point* of the kidney and *guanyuan* point belonging to *ren meridian*, is the *small intestine collection point*, and is the commonly used point for the treatment of all kinds of deficiency. Another reason may be that, according to modern neuroanatomical theory, *Shenshu* point, innervated by the 1st ~ 3rd lumbar ganglion segment, was related with genitalia by ilioinguinal nerve and genital nerve of lumbar plexus, and was closely related to the kidney, adrenal gland, internal genitalia, etc. through the traffic branch of the lumbar sympathetic trunk and the lumbar visceral nerve [18]. And *guanyuan* point is connected to the liver, spleen, kidney and other substantial organs through the twelfth thoracic nerve and related small visceral nerves. Zhou et al. [42] using HRP nerve tract tracking technology, found that the afferent projection of *guanyuan* and uterus had convergence and overlap in the spinal ganglia between lumbar 3 and sacral 5. In this study, treatment with suspended moxibustion on *shenshu* and *guanyuan* has not only a strong theoretical basis, but also a wide range of experimental basis. For example, Zhao et al. [43] found that the symptoms of *kidney-yang* deficiency were improved after a period of mild moxibustion treatment on *guanyuan* and *shenshu* point in elderly patients. Ren et al. [41] found that the treatment of moxibustion on *shenshu* and *guanyuan* could significantly improved the inhibitory state of the HPA axis in rats with *kidney-yang* deficiency, and the therapeutic effect of combination of *shenshu* and *guanyuan* was significantly better than that of combination of *shenshu* and *zusanli* or combination of *zusanli* and *guanyuan*. This was one of the reasons that *shenshu* and *guanyuan* were selected in this study.

Conclusion

In this study, we demonstrated that suspended moxibustion can effectively improve the serum levels of ACTH, CRH and CORT, and can up-regulate the mRNA and protein expression of MR, GR, 11β-HSD1, CRF and ACTH in amygdala and hypothalamus of *kidney-yang* deficiency rats. Considering the experimental model of *kidney-yang* deficiency syndrome used in this study shares several pathologic similarities to human, then MR, GR and 11β-HSD 1 could represent a novel therapeutic target in the treatment of *kidney-yang* deficiency syndrome.

List Of Abbreviations

11β-HSD1	11 beta-hydroxysteroid dehydrogenase type 1
ACTH	adrenocorticotropic hormone
CBX	carbenoxolone
CORT	corticosterone
CRF	corticotropin releasing factor
CRH	corticotropin releasing hormone
ELISA	enzyme linked immunosorbent assay
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	glucocorticoid
GR	glucocorticoid receptor
HPA axis	hypothalamus - pituitary - adrenal axis
MR	mineralocorticoid receptor
PBS	phosphate buffered solution
RT-qPCR	Reverse transcription real-time quantitative polymerase chain reaction
SD	Sprague Dawley
SNK	Student–Newman–Keuls

Declarations

Ethical approval

All procedures were conducted in accordance with guidelines reviewed and approved by the Institutional Animal Care and Use Committee of Jiangxi University of Traditional Chinese Medicine, China.

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Disclosure of interest

The authors have no conflicts of interest to declare. We confirm that this article is the authors' original work and has not been published elsewhere nor is it currently under consideration for publication elsewhere.

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Authors' contributions

YJM conceived and designed the study and KTL revised the paper. WGY and HHY wrote the paper, provided data and revised the paper. JS, ZY, HQW, LHC participated the experiment. All authors approved the final version of the paper.

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Figures

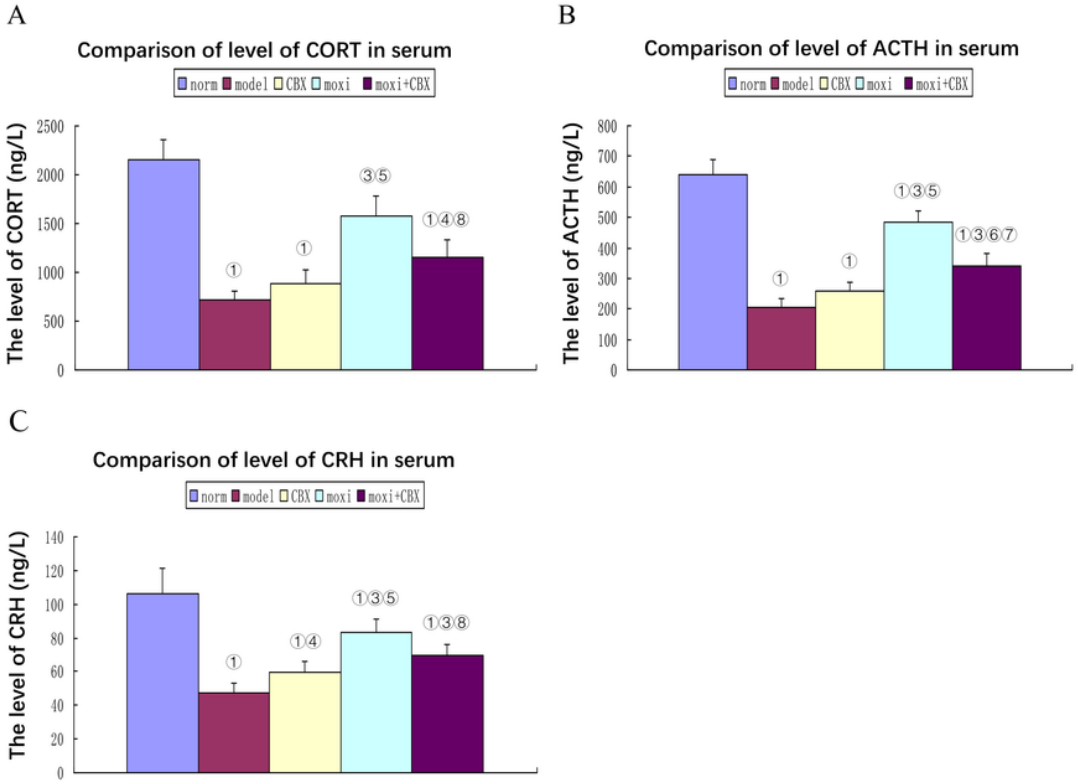

Figure 1

Comparison of the level of CORT, ACTH and CRH in serum. Compiled results in a bar graph for the level (A) of CORT, (B) of ACTH, (C) of CRH. □P < 0.01, □P < 0.05 versus norm□ □P < 0.01, □P < 0.05 versus model□□P < 0.01, □P < 0.05 versus CBX□□P < 0.01, □P < 0.05 versus moxi. Data are shown as the mean ± standard error of the mean (1-way analysis of variance and Student-Newman-Keuls post hoc test, n = 6 rats/group). norm: normal group; model: model group; CBX: carbenoxolone intraperitoneal injection group; moxi:

moxibustion group; moxi + CBX: moxibustion plus carbenoxolone intraperitoneal injection group. CORT: corticosterone; ACTH: adrenocorticotrophic hormone; CRH: corticotropin-releasing hormone.

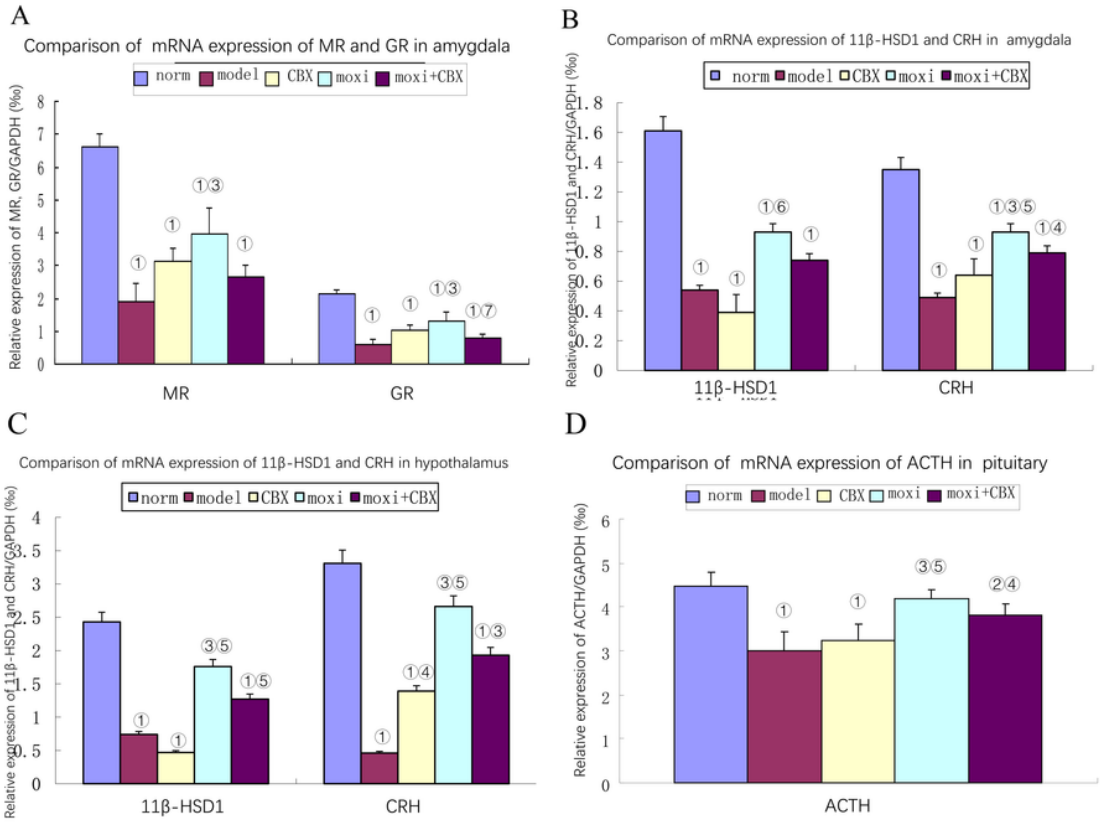

Figure 2

Comparison of the relative mRNA expressions of MR, GR, 11β-HSD1, CRH and ACTH. Compiled results in a bar graph for the relative mRNA expression (A) of MR and GR / GAPDH in amygdala, (B) of 11β-HSD1 and CRH / GAPDH in amygdala, (C) of 11β-HSD1 and CRH / GAPDH in hypothalamus, (D) of ACTH / GAPDH in pituitary. ▯P < 0.01, ▯P < 0.05 versus norm▯▯P < 0.01, ▯P < 0.05 versus model▯▯P < 0.01, ▯P < 0.05 versus CBX▯▯P < 0.01, ▯P < 0.05 versus moxi. Data are shown as the mean±standard error of the mean (1-way analysis of variance and Student-Newman-Keuls post hoc test, n = 6 rats/group). norm: normal group; model: model group; CBX: carbenoxolone intraperitoneal injection group; moxi: moxibustion group; moxi + CBX: moxibustion plus carbenoxolone intraperitoneal injection group. MR: mineralocorticoid receptor; GR: glucocorticoid receptor; 11β-HSD1: 11 beta-hydroxysteroid dehydrogenase type 1; CRH: corticotropin-releasing hormone; ACTH: adrenocorticotrophic hormone; GAPDH: glyceraldehyde-3- phosphate dehydrogenase.

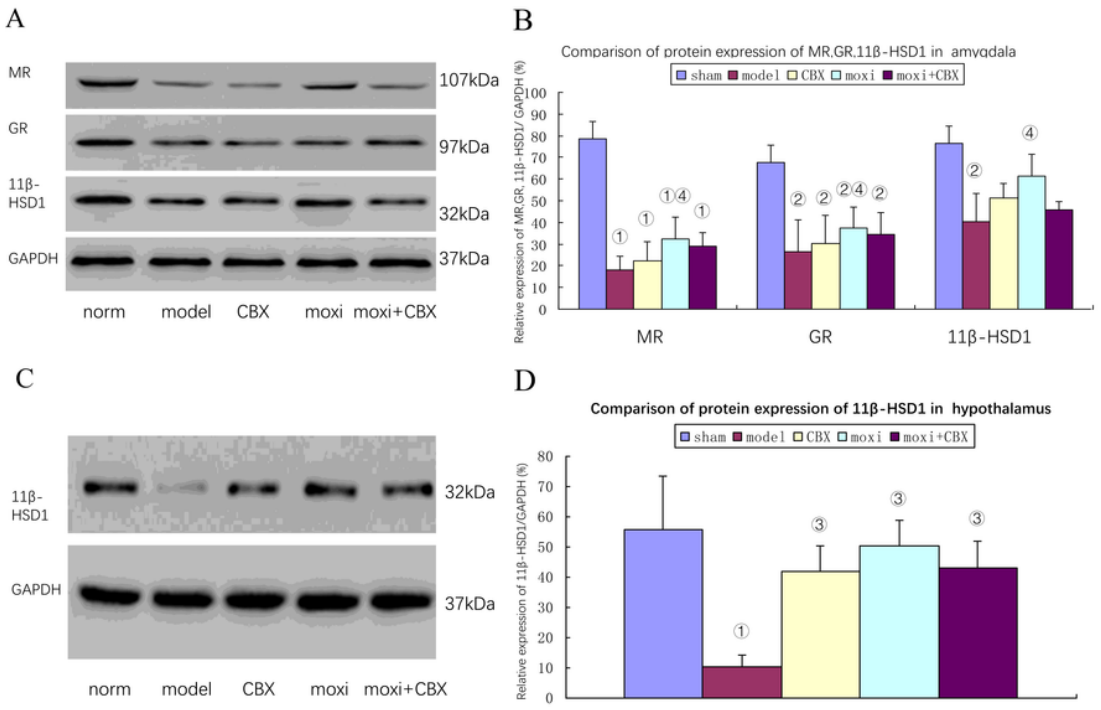

Figure 3

MR, GR and 11β-HSD1 activation in the amygdala and hypothalamus following treatment. (A) Western blotting bands for MR, GR, 11β-HSD1 and GAPDH expression in the amygdala. (B) Compiled results in a bar graph for the ratio of MR, GR and 11β-HSD1 / GAPDH expression. (C) Western blotting bands for 11β-HSD1 and GAPDH expression in hypothalamus. (D) Compiled results in a bar graph for the ratio of 11β-HSD1 / GAPDH expression. ①P < 0.01, ②P < 0.05 versus norm③④P < 0.01, ④P < 0.05 versus model. Data are shown as the mean±standard error of the mean (1-way analysis of variance and Student-Newman-Keuls post hoc test, n = 4 rats/group). norm: normal group; model: model group; CBX: carbenoxolone intraperitoneal injection group; moxi: moxibustion group; moxi + CBX: moxibustion plus carbenoxolone intraperitoneal injection group. MR: mineralocorticoid receptor; GR: glucocorticoid receptor; 11β-HSD1: 11 beta-hydroxysteroid dehydrogenase type 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

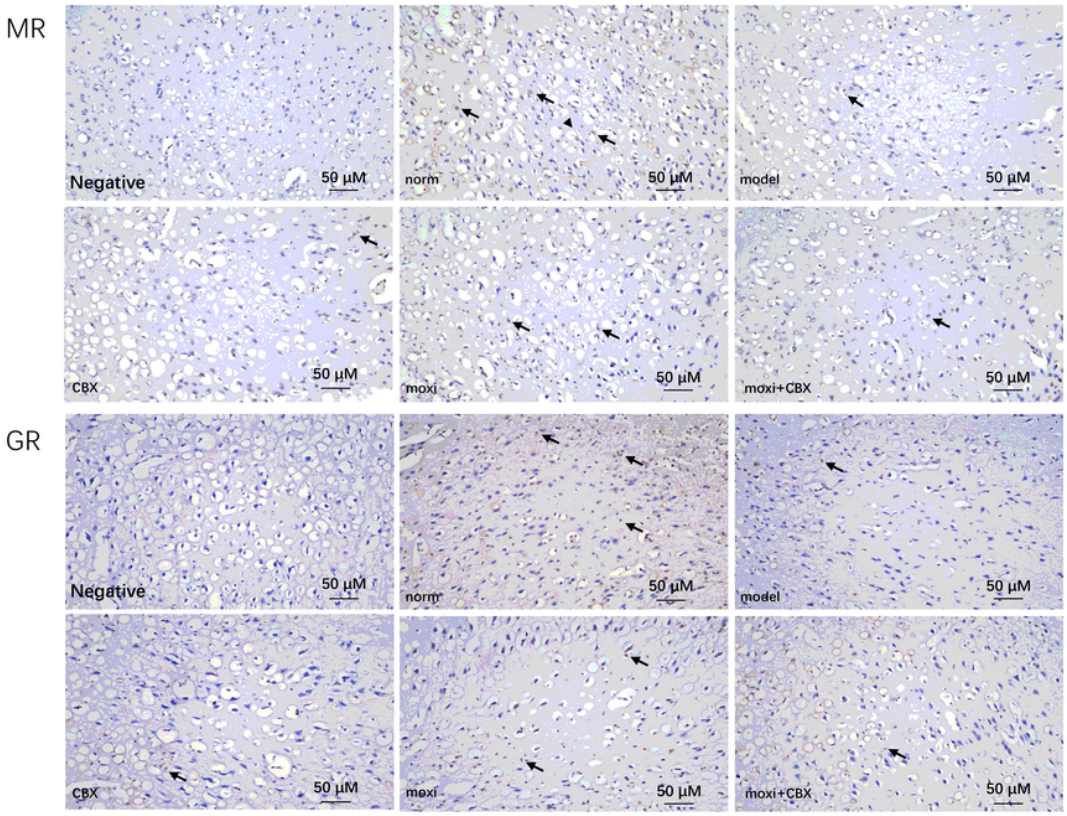

Figure 4

mRNA expression of MR and GR in the amygdala following treatment (in situ hybridization, × 200). Bar = 50 μm. norm: normal group; model: model group; CBX: carbenoxolone intraperitoneal injection group; moxi: moxibustion group; moxi + CBX: moxibustion plus carbenoxolone intraperitoneal injection group. MR: mineralocorticoid receptor; GR: glucocorticoid receptor.

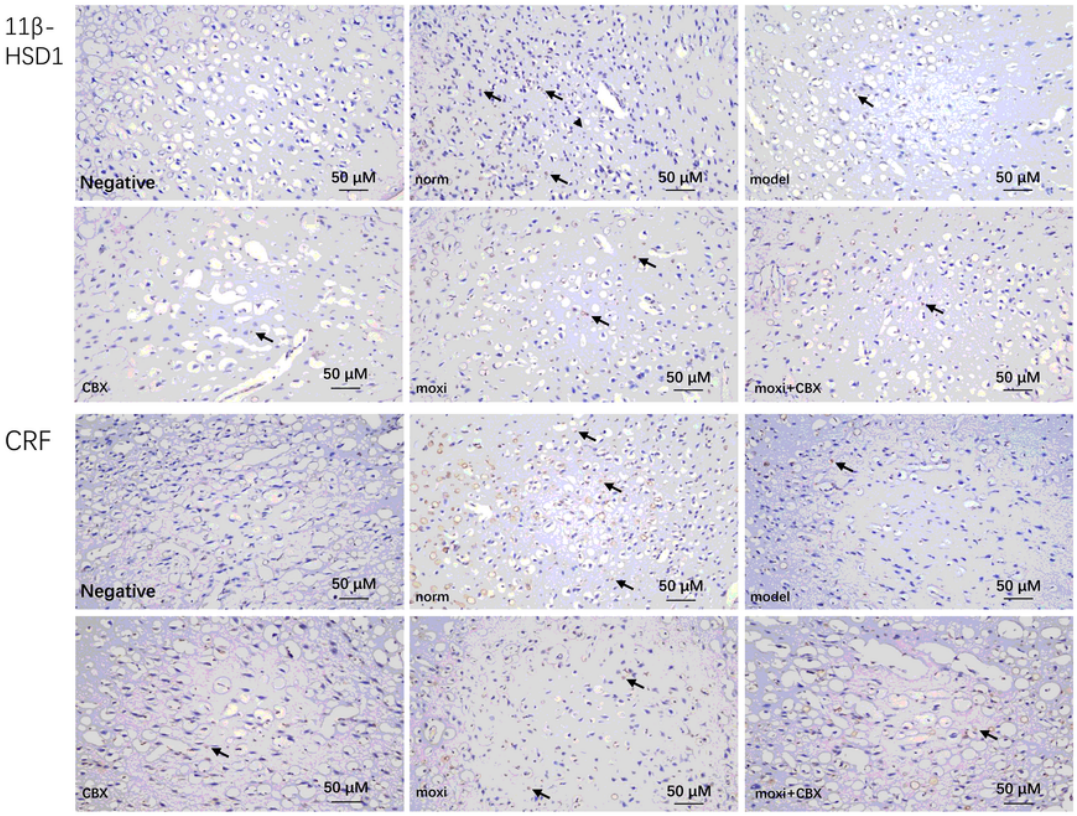

Figure 5

mRNA expression of 11β-HSD1 and CRF in the amygdala following treatment (in situ hybridization, × 200). Bar = 50 μm. norm: normal group; model: model group; CBX: carbenoxolone intraperitoneal injection group; moxi: moxibustion group; moxi + CBX: moxibustion plus carbenoxolone intraperitoneal injection group. 11β-HSD1: 11 beta- hydroxysteroid dehydrogenase type 1; CRF: corticotropin releasing factor.

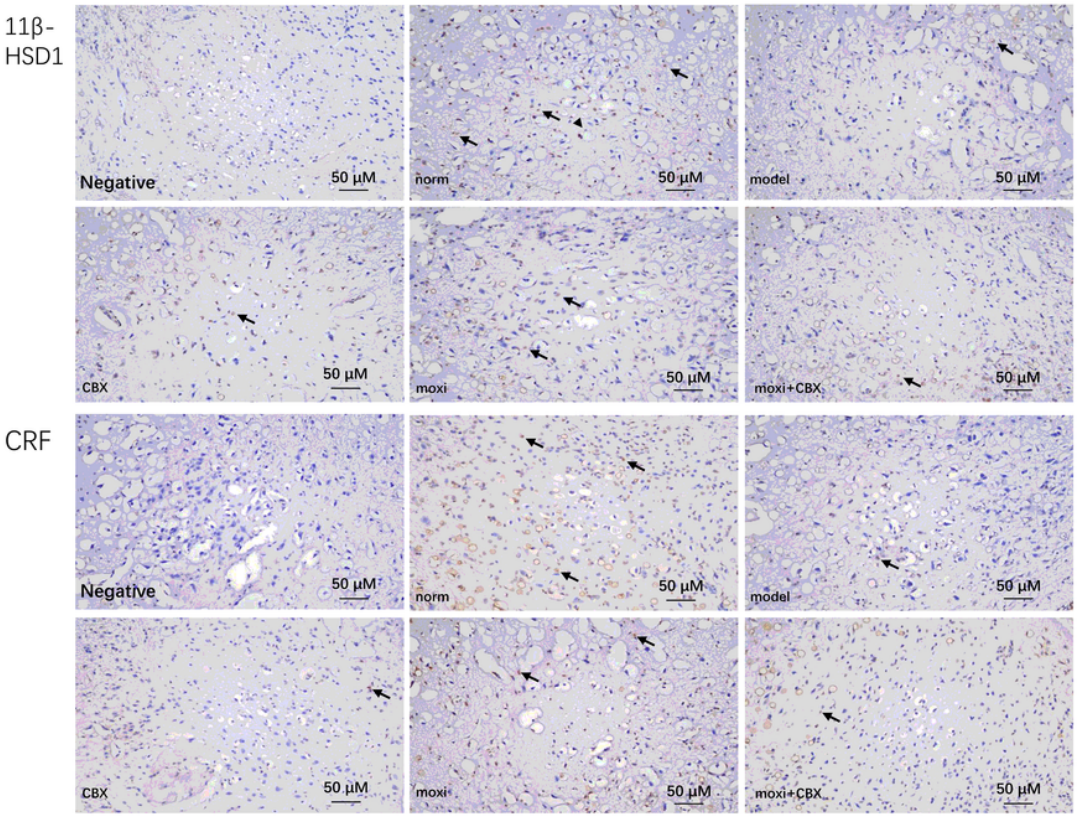

Figure 6

mRNA expression of 11β-HSD1 and CRF in the hypothalamus following treatment (in situ hybridization, × 200). Bar = 50 μm. norm: normal group; model: model group; CBX: carbenoxolone intraperitoneal injection group; moxi: moxibustion group; moxi + CBX: moxibustion plus carbenoxolone intraperitoneal injection group. 11β-HSD1: 11 beta- hydroxysteroid dehydrogenase type 1; CRF: corticotropin releasing factor.

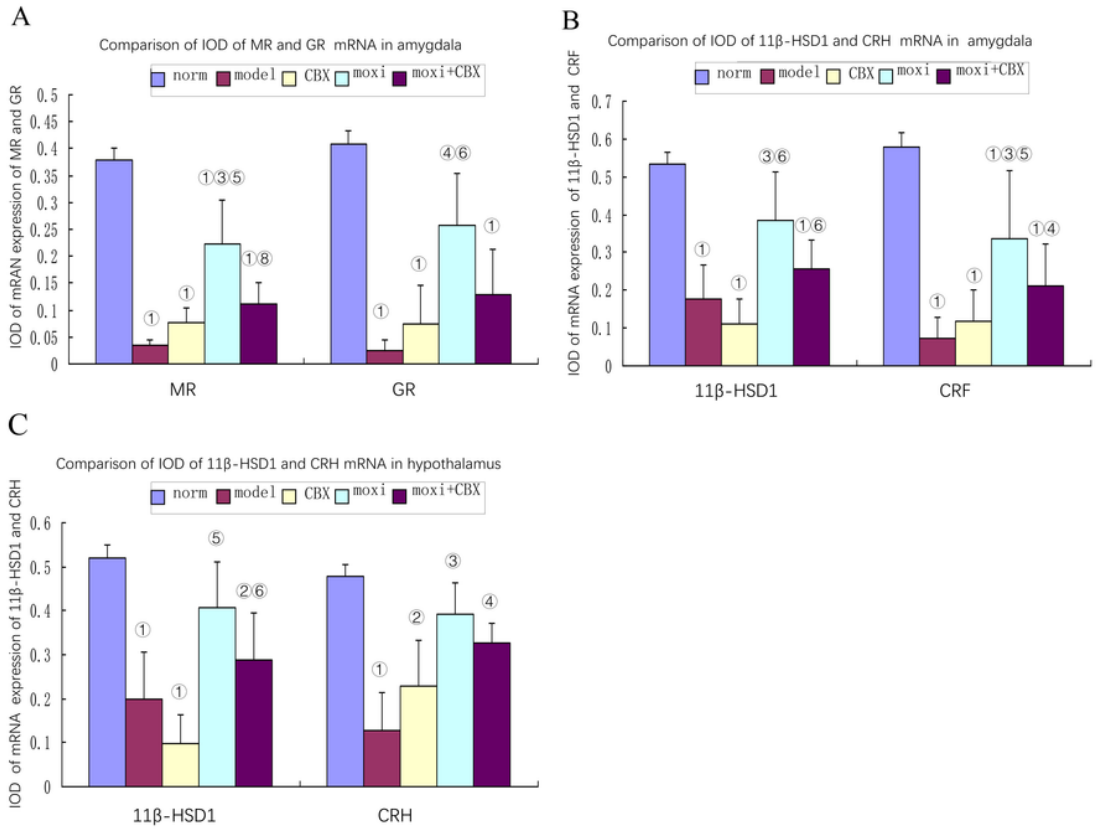

Figure 7

Compiled results in a bar graph for IOD values of MR, GR, 11β-HSD1 and CRF mRNA-positive cells. Compiled results in a bar graph for IOD values (A) of MR and GR in the amygdala, (B) of 11β-HSD1 and CRF in the amygdala, (C) of 11β-HSD1 and CRF in the hypothalamus. ▯P < 0.01, ▯P < 0.05 versus norm▯▯P < 0.01, ▯P < 0.05 versus model▯▯P < 0.01, ▯P < 0.05 versus CBX▯▯P < 0.01, ▯P < 0.05 versus moxi. Data are shown as the mean±standard error of the mean (1-way analysis of variance and Student-Newman-Keuls post hoc test, n = 6 rats/group). norm: normal group; model: model group; CBX: carbenoxolone intraperitoneal injection group; moxi: moxibustion group; moxi + CBX: moxibustion plus carbenoxolone intraperitoneal injection group. MR: mineralocorticoid receptor; GR: glucocorticoid receptor; 11β-HSD1: 11 beta-hydroxysteroid dehydrogenase