# Effects of varying intensities of heat stress on neuropeptide Y and proopiomelanocortin mRNA expression in rats

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**Abstract.** The aim of the present study was to investigate the effects of varying intensities of heat stress on the mRNA expression levels of neuropeptide Y (NPY), proopiomelanocortin (POMC) and stress hormones in rats. To establish a rat model of heat stress, the temperature and time were adjusted in a specialized heating chamber. Sprague-Dawley (SD) rats were randomly divided into four groups; control (CN; temperature, 24±1˚C); moderate strength 6 h (MS6; temperature, 32±1˚C time, 6 h), moderate strength 24 h (MS24; temperature, 32±1˚C; time, 24 h) and high strength 6 h (HS6; temperature, 38±1˚C; time, 6 h) groups. SD rats were exposed to heat for 14 consecutive days. The levels of heat stress-related factors, including corticotropin-releasing hormone (CRH), cortisol (COR), epinephrine (EPI) and heat shock protein 70 (HSP70), were measured in the rat blood using ELISA. In addition, the weight of the spleen, thymus, hypophysis and hypothalamus were determined. The mRNA expressions levels of NPY and POMC were detected using quantitative PCR. The results showed that the CRH, COR and HSP70 levels were increased in the three heat stress groups compared with the CN group. Notably, the levels of CRH, EPI and HSP70 were increased in the HS6 group compared with the CN and MS6 groups ($P<0.05$). Furthermore, the weights of the hypophysis and hypothalamus in the HS6 group were significantly lower compared with the CN group ($P<0.05$). In addition, NPY and POMC expression levels were downregulated in the MS24 group compared with the CN group. The mRNA expression levels of NPY and POMC were altered in response to different intensities of heat stress. Therefore, their levels were downregulated and upregulated following long-time and moderate-time heat exposure, respectively. The results of the present study suggested that the reduced mRNA expression levels of NPY may be partially responsible for the heat-induced injuries in rats following long-time heat exposure.

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## Introduction

Heat stress response is a complicated process that protects an organism from potential injuries. The response involves the activation of the neuroendocrine axis and the secretion of stress hormones (1). It has been reported that heat stress may elicit a range of coordinated autonomic physical responses to maintain the balance of the organism (2). The organism in turn, possesses a 'thermostat' like function to respond to changes in the environmental temperature, by increasing the body temperature, heart rate and cardiac output and decreasing the organism's activity (3). Stress factors trigger a succession of cascade responses, including the hypothalamic-pituitary-adrenal axis (HPA axis). The HPA axis is a key component of the physiological response to heat stress, and is composed of the paraventricular nucleus (PVN) of the hypothalamus, the hypophysis and the adrenal cortex (4). Therefore, the HPA axis serves a vital role in the stress response (5). Corticotropin-releasing hormone (CRH), which is synthesized and secreted by the neuroendocrine neurons of the hypothalamus, stimulates the release of adrenocorticotropic hormone (ACTH). Glucocorticoids, primarily cortisol (COR), are synthesized in the adrenal cortex following stimulation by ACTH. In contrast, glucocorticoids act on the hypothalamus and pituitary gland by inhibiting the secretion of CRH and ACTH to normalize COR secretion (6).

Neuropeptide Y (NPY) is one of the most abundant polypeptides present in the central nervous system (CNS). NPY can be detected in the hypothalamus, amygdaloid nucleus and hippocampus. The arcuate nucleus of the hypothalamus contains the highest concentration of NPY (7). As a

neurotransmitter, NPY serves an important role in regulating the stress-related behavior and adaptation of the organism to environmental challenges (8,9). In addition, NPY is involved in the central mechanism of regulating psychological and physiological stress, which serves as a protective factor, termed the 'stress factor' (10). It has been shown that CRH and NPY exert opposite effects (11). CRH was primarily discovered in 1955 and was initially detected in the PVN of the hypothalamus (12,13). CRH acts as an important physiological regulator in initiating stress responses and it is the most potent ACTH secretagogue. Several depressive disorders have been attributed to CRH action (14,15).

Proopiomelanocortin (POMC) is a precursor peptide that induces the production of several types of bioactive neuropeptides, such as the opioid peptide, α-melanocyte-stimulating hormone and ACTH (16,17). COR is the primary glucocorticoid hormone and the major end product of the HPA axis (18). COR is secreted in accordance with the host organism's natural circadian rhythm under non-stressful conditions (18). However, in response to stress, COR is released throughout the body (19). Due to a negative feedback mechanism, COR concentration is not increased unconventionally under heat stress conditions (20). ACTH is a derivative of POMC, and it affects COR release, indicating a possible association between ACTH and COR (21).

It has been confirmed that both the HPA axis, but also the sympathetic nervous system (SNS) regulate the normal functioning of the body (18,22). The SNS can promote an abundant release of epinephrine (EPI) into the bloodstream. EPI is a prototypical stress hormone released from the adrenal medulla into the peripheral circulation to maintain the balance of the organism and respond to stressful stimuli (23). In addition, EPI increases blood pressure and glucose concentration in the blood (24). The SNS is more sensitive and responds faster than the HPA axis in stress-responsive biological systems (25). Emerging evidence has suggested that the expression of protective proteins, namely heat shock proteins (HSPs), is upregulated in organisms exposed to high temperatures (26). HSPs belong to a group of highly conserved proteins and act as molecular chaperones (27). HSPs are classified according to their molecular weights and homology in the 110, 90, 70 and 60 kDa classes (28). Several studies have shown that HSP70 is the most abundant, important and inducible protein among the members of the HSP family, and it promotes heat resistance and protects cells from damage (29-31).

Along with continuing climate change, more attention has been paid to the impact of the thermal environment on the organism. However, to the best of our knowledge, there are no studies assessing the effects of different time and temperature exposures on NPY and POMC mRNA expression levels in the hypothalamus and hypophysis. Additionally, whether the combination of the genetic background with stress-related factors affects the response of the organism to heat stress has not yet been elucidated. The aim of the present study was to investigate the changes in POMC and NPY mRNA expression levels. Therefore, the association between the expression of POMC, NPY and stress-related factors under different heat stress conditions was explored using different models. Furthermore, the effects of different intensities of heat stress on the spleen, thymus, hypophysis and hypothalamus weight was also investigated.

## Materials and methods

*Animals and heat exposure groups.* Male Sprague-Dawley (SD) rats weighing 200±20 g, were obtained from the Laboratory Animal Center of Ningxia Medical University (Yinchuan, China). Animals were housed 3-4 per plastic cage with *ad libitum* access to water and food with a 12/12-h light/dark cycle. The cages were cleaned once every 2 days. Following a 1 week habituation period, 60 healthy male SD rats were randomly divided into four groups: Control (CN); moderate strength 6 h (MS6); moderate strength 24 h (MS24); and high strength 6 h (HS6) groups. The rats in the CN group were exposed to a temperature of 24±2˚C for 24 h, and all experimental groups were compared with the same CN group. Rats in the heat exposure groups were placed in an intelligent artificial climate chamber (ZRS-JSW; Hangzhou Pnshar Technology Co., Ltd.). Rats in the MS6 and MS24 groups were exposed to a temperature of 32±1˚C for 6 and 24 h, respectively. Rats in the HS6 group were maintained at 38±1˚C for 6 h. Heat stress was repeated for 14 consecutive days. Heat exposure for 6 and 24 h was considered moderate and long-time exposure, respectively. Finally, heat exposure at 32˚C and 38˚C was considered moderate and high temperature exposure, respectively. All animal experimental procedures were approved by Ningxia Medical University Institutional Review Board (approval no. NXMU-2017-030).

*Blood sample and organ collection.* Following heat exposure, all rats were subjected to intraperitoneal anesthesia with 20% urethane (20 g powdered urethane dissolved in 100 ml deionized water), and blood samples were obtained from the posterior vena cava. Plasma was separated from blood by centrifugation at 4˚C at 4,500 x g for 15 min, and the supernatant was stored at -80˚C for subsequent analyses. The spleen, thymus, hypophysis and hypothalamus were successively collected following intravenous blood collection. Hypophysis and hypothalamus tissues were stored in a refrigerator at -80˚C after liquid nitrogen freezing and were used for gene expression analysis assays.

*Relative organ weights measurement.* Spleen, thymus, hypophysis and hypothalamus were removed and precisely weighed using an electronic analytical balance (JA2003N; Shanghai Yoke Instrument Co., Ltd.). Relative weight was calculated as follows: Relative weight=weight of the organ/weight of the rat.

*Measurement of COR, EPI, CRH and HSP70 in the plasma.* The plasma concentrations of CRH (CSB-E08038r), COR (CSB-E05112r), EPI (CSB-E08678r) and HSP70 (CSB-E08308r) were determined using ELISA (Cusabio Technology LLC). The optical density (OD value) was determined at 450 nm using an universal microplate reader (Bio-Rad Laboratories, Inc.). A standard curve was constructed for each component to determine their concentrations. The concentration values of CRH, COR and HSP70 are expressed in ng/ml and that of EPI in pg/ml.

*RNA isolation and reverse transcription-quantitative (q)PCR.* Total RNA was isolated from the hypophysis (for measurement of POMC) and hypothalamus (for measurement of NPY) using

Table I. Primer sequences, GenBank accession codes and expected product sizes.

| Genes | Sequence, 5'-3' | GenBank accession no. | Base pairs |
|---|---|---|---|
| Proopiomelanocortin | | NM_139326 | 203 |
| Forward | CCTGCTTCAGACCTCCATAGAC | | |
| Reverse | AGCGGAAGTGACCCATGAC | | |
| Neuropeptide Y | | NM_012614 | 107 |
| Forward | GCTCTGCGACACTACATCAATC | | |
| Reverse | GCATTTTCTGTGCTTTCTCTCA | | |
| β-actin | | NM_031144 | 207 |
| Forward | CACCCGCGAGTACAACCTTC | | |
| Reverse | CCCATACCCACCATCACACC | | |

Table II. Relative organ weights.

| Organs | Control | MS6 | MS24 | HS6 |
|---|---|---|---|---|
| Spleen, $x10^{-3}$ g | 2.458±0.265 | 2.350±0.303 | 2.112±0.194[a,b] | 2.293±0.201 |
| Thymus, $x10^{-3}$ g | 2.259±0.478 | 2.225±0.360 | 2.334±0.251 | 2.039±0.288 |
| Hypophysis, $x10^{-5}$ g | 4.182±0.908 | 3.966±0.671 | 4.233±0.633 | 3.510±0.778[a] |
| Hypothalamus, $x10^{-5}$ g | 6.465±1.518 | 7.461±1.213 | 5.702±0.934[b] | 7.708±1.228[a] |

[a]P<0.05 vs. control; [b]P<0.05 vs. MS6 group. MS6, moderate strength 6 h; MS24, moderate strength 24 h; HS6, high strength 6 h.

an RNA extraction kit (Axygen; Corning, Inc.) according to the manufacturer's protocol. RNA integrity was assessed using 2.0% agarose gel electrophoresis. Subsequently, cDNA was synthesized using a cDNA Synthesis kit (Transgen Biotech Co., Ltd.) according to the manufacturer's protocol, and stored at -80˚C for subsequent analysis. The qPCR reactions were performed in a total volume of 25 $\mu$l containing 1 $\mu$l cDNA, 0.5 $\mu$l forward primer (10 $\mu$M), 0.5 $\mu$l reverse primer (10 $\mu$M), 12.5 $\mu$l 2x qPCR SuperMix and 10.5 $\mu$l $ddH_2O$, and amplified on a real-time PCR system (FTC-3000; Funglyn Biotech, Inc.). The qPCR thermocycling conditions were as follows: 94˚C for 5 min (initial denaturation); followed by 36 (for NPY) or 42 (for POMC) cycles of amplification at 94˚C for 30 sec (denaturation), 62˚C for 30 sec (annealing) and 72˚C for 30 sec (extension). The qPCR primer sequences for POMC, NPY and β-actin are listed in Table I. β-actin was used as the loading control. The relative gene expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (32), where $\Delta\Delta Cq=\Delta Cq$ (sample)-$\Delta Cq$ (control) and $\Delta Cq=Cq$ (target gene)-Cq (reference gene).

*Statistical analysis*. Experimental data were analyzed using SPSS version 16.0 (SPSS, Inc.). All results are expressed as the mean ± standard deviation and where compared using a one-way ANOVA followed by a post-hoc SNK-test to compare the differences between two samples. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Relative organ weights*. The relative organ weights are listed in Table II. The measurements showed that the relative weight of the spleen in the MS24 group was significantly lower compared with that in the CN and MS6 groups (CN, P=0.002; MS6, P=0.031; P<0.05). Similarly, the weight of the hypothalamus in the MS24 group was significantly lower than that in the CN group (P=0.002; P<0.05). Compared with the CN group, hypophysis (P=0.049; P<0.05) and hypothalamus (P=0.028; P<0.05) weights were significantly decreased and increased, respectively. In the HS6 group, there was no statistically significant difference in the weight of the thymus amongst the different groups. Although the weights of the spleen, hypophysis and hypothalamus in the MS6 group, hypophysis in the MS24 group and spleen in the HS6 group were lower compared with those in the CN group, no statistically significant differences were observed.

*Plasma concentration of CRH, COR, EPI and HSP70*. Following exposure of rats at an ambient temperature of 38˚C, CRH levels were decreased in the HS6 group compared with those in the CN and MS6 groups (CN, P=0.004; MS6, P<0.001). Additionally, CRH levels were increased in the MS groups, and most notably in the MS24 group (P=0.025; Fig. 1A).

COR concentration was elevated following heat stress in the MS6, MS24 and HS6 groups compared with the CN group (MS6, P=0.042; MS24, P=0.020; HS6, P=0.012). In addition, the COR levels in the HS6 group were higher than those in the MS6 group; however, the differences were not significant. Furthermore, no significant differences were found between the high and moderate temperature exposure groups when exposed for the same duration of time. The increase in COR concentration was similar between the MS6 and MS24 groups (Fig. 1B).

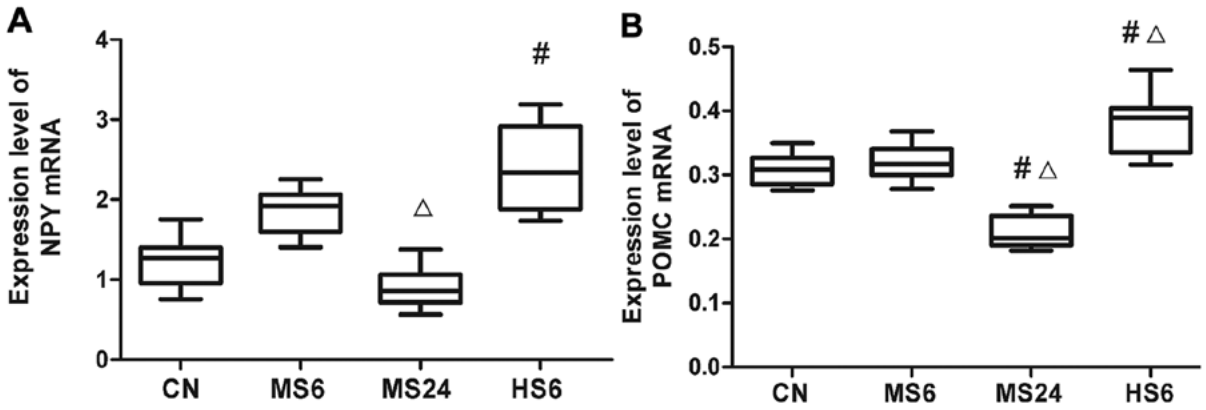


Figure 1. mRNA expression levels of NPY in the hypothalamus and POMC in the hypophysis. (A) NPY and (B) POMC mRNA expression levels in the hypothalamus and hypophysis, respectively. #P<0.05 vs. CN; ΔP<0.05 vs. MS6 group. CN, Control; MS6, moderate strength 6 h; MS24, moderate strength 24 h; HS6, high strength 6 h; CRH, corticotrophin; COR, cortisol; EPI, epinephrine; HSP70, heat shock protein 70; POMC, proopiomelanocortin; NPY, neuropeptide Y.

Regarding EPI plasma levels, its concentration was significantly elevated in the HS6 group (P=0.003) compared with the CN and MS6 groups (P=0.02). In both the MS6 and MS24 groups, EPI was increased compared with the control; however, no significant differences were observed (Fig. 2C).

Finally, HSP70 plasma levels were raised following both moderate and high temperature exposure compared with the control group (MS6, P=0.019; MS24, P=0.005; HS6, P<0.001); however, the difference between the MS6 and MS24 groups was not significant. High temperature exposure induced HSP70 expression in the plasma compared with the moderate temperature exposure under the same exposure time (P=0.008; Fig. 2D).

*mRNA expression levels of NPY and POMC*. NPY mRNA expression levels in the hypothalamus are presented in Fig. 2A. In the MS6 and MS24 groups, NPY mRNA expression was significantly upregulated and downregulated, respectively, compared with that in the MS6 group (P=0.014). In addition, high temperature exposure for 6 h significantly increased NPY expression compared with the CN group (P=0.005).

As shown in Fig. 2B, qPCR analysis showed that POMC mRNA expression levels were significantly increased in the HS6 group compared with the CN (P=0.022) and MS6 (P=0.029) groups; however, there was no statistically significant differences between the CN and MS6 groups. In contrast, POMC expression was significantly reduced in the MS24 group compared with the CN (P=0.005) and MS6 (P=0.002) groups.

## Discussion

The present study investigated the association between stress-related factors and different intensities of heat stress on the expression of POMC and NPY in high-temperature environments. The spleen and thymus are involved in the immune response (33,34). It has been reported that heat stress may cause atrophy of the spleen and thymus to different degrees, which is attributed to the apoptosis of the internal organs (35). In the present study, the effects of different intensities of heat stress on spleen, thymus, hypophysis and hypothalamus weight were first determined. The results showed that the relative spleen weight was significantly decreased in the long-term heat exposure group. In addition, in the long-term heat exposure group, increasing heat stress levels increased the relative hypothalamus weight and decreased the relative weight of the hypophysis. These results indicated that there was an opposite effect of heat stress on the hypothalamus and hypophysis.

Most HSPs are generally stress-inducible as they play a particularly important cytoprotective role in cells exposed to stressful conditions (28). HSP70 is considered the most abundant and widely studied protein and its concentration is significantly altered in response to stressful stimuli (36). The results of the present study demonstrated that the HSP70 plasma levels were elevated in response to heat stress, particularly under exposure to high temperatures. This finding suggested that heat stress-induced damage promoted upregulation of HSP70 in order to protect the organs. Emerging evidence has shown that EPI mediates stress responses by initiating sympathetic nervous system to allow the host organism to resist stress (23). The results of the present study demonstrated that EPI levels were increased in the high temperature exposure group. By contrast, exposure to a moderate temperature did not increase EPI levels, irrespective of the exposure time, suggesting that only high heat stress elevated EPI plasma concentrations.

NPY and CRH neuropeptides are two independent stress factors of the hypothalamus that act by binding to their respective receptors. Currently known NPY receptors have seven kinds, including NPY Y1-Y7. The most abundant Y1 and Y2 receptors are the main regulators of NPY's anti-stress response (37). Under stress, CRH is mainly mediated through its receptors, the main receptors are R1 and R2, and R1 is mainly involved in the beginning of the HPA axis reaction (38). Long term exposure to stress stimuli makes an organism vulnerable to chronic stress, which in turn inhibits the NPY system and downregulates NPY expression, thus attenuating its protective effect. CRH promotes stress-related behaviors, whereas NPY exhibits anti-stress related effects. It has been suggested that under heat stress conditions, NPY responds to the harmful effects of stress by releasing CRH (39). Therefore, the expression levels of NPY and CRH under different heat stress conditions were determined. The results indicated that NPY mRNA expression levels in the CNS could be altered in response to heat stress. As a result, moderate-time heat stress

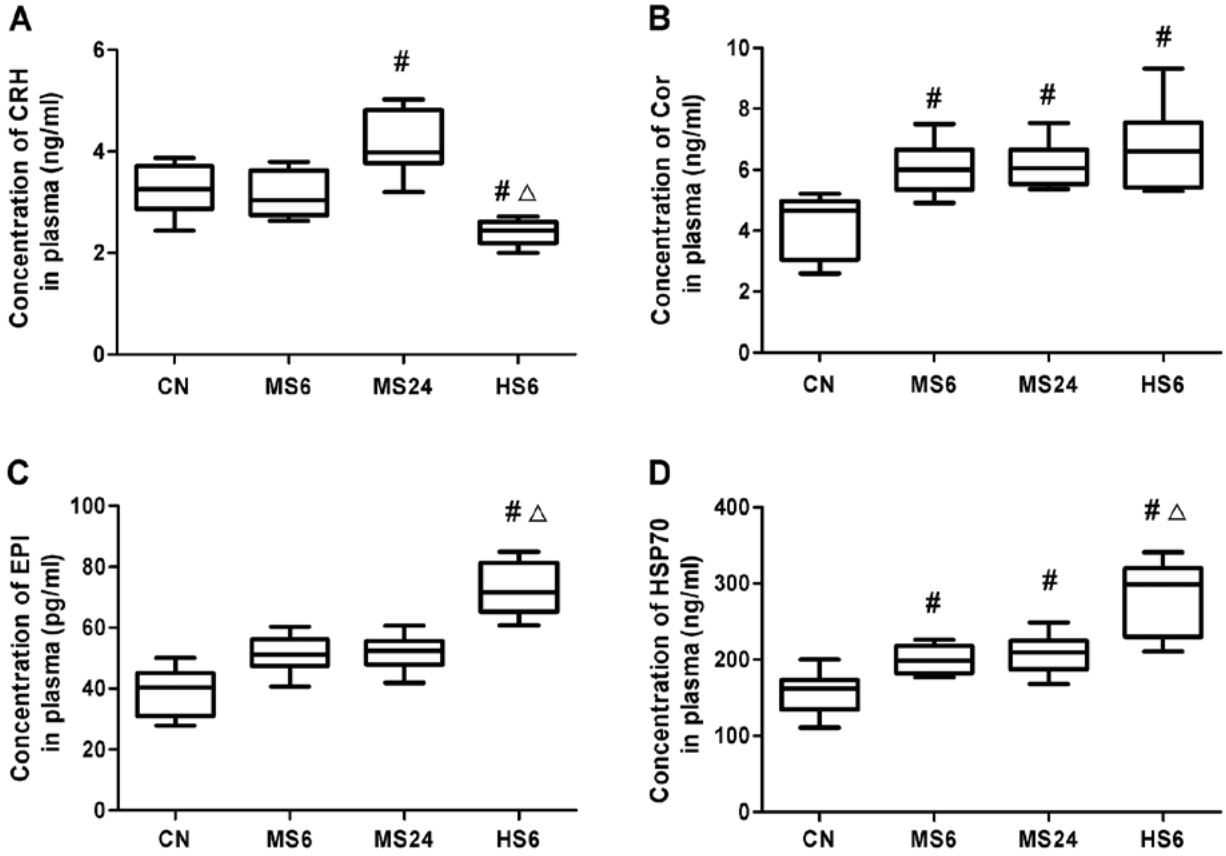


Figure 2. Concentration of different hormones or proteins in the plasma. (A) CRH, (B) COR, (C) EPI and (D) HSP70 levels in the plasma of rats at different temperatures. #P<0.05 vs. CN group; ΔP<0.05 vs. MS6 group. CN, Control; MS6, moderate strength 6 h; MS24, moderate strength 24 h; HS6, high strength 6 h; POMC, proopiomelanocortin; NPY, neuropeptide Y.

exposure upregulated NPY expression to protect the body. At the same time, there was an inverse association between CRH concentration and NPY mRNA expression levels. This finding confirmed the opposing behaviors of CRH and NPY in response to heat stress. Therefore, NPY may moderate the expression and release of CRH, while CRH inhibits NPY expression.

COR is an important stress hormone that is regulated by ACTH and protects the body from stress damage (40). Additionally, ACTH promotes the release of COR, which in turn suppresses the release of ACTH through a negative feedback mechanism to maintain COR balance. ACTH is a derivative of POMC (41). The results of the present study showed that the mRNA expression levels of POMC were also altered in the CNS under heat stress conditions. Furthermore, exposure to high temperatures resulted in upregulation of POMC expression compared with exposure to a moderate temperature for the same duration. COR and POMC were both increased in the moderate-time exposure group, however, POMC expression was decreased when COR concentration increased after the long-time exposure. This finding may be attributed to the association between COR and ACTH; COR levels may not have been high enough in the moderate-time exposure group to inhibit ACTH via the negative feedback mechanism. By contrast, in the case of the long-time exposure group, the levels of COR were high enough to inhibit ACTH in the remaining exposure time in order to maintain the balance of COR concentration. The aforementioned inhibitory effect was accompanied by POMC downregulation.

Overall, the present study investigated the changes in expression of POMC, NPY and heat stress-related factors at different heat stress intensities. Following long-term heat stress exposure, the mRNA expression levels of both NPY and POMC were decreased. Furthermore, the relative weights of the pituitary and hypothalamus were inversely proportional to CRH plasma concentration and NPY gene expression, respectively. In addition, COR concentration was directly proportional to POMC expression in all heat stress groups except the MS24 group. Therefore, the present study suggested that heat damage caused by long-term heat exposure may be involved in NPY and POMC downregulation.

In conclusion, the results of the present study demonstrated that different heat stress intensities modulated NPY and POMC mRNA expression. Therefore, NPY and POMC downregulation may be partially associated with long-time heat exposure-induced injuries.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Authors' contributions

GL conceived and designed the experiments. YG provided theoretical guidance and revised the manuscript. NZ performed the experiments and prepared the manuscript. LM performed the experiments and analyzed the data. XC and LZ provided experimental technical assistance. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

All animal experimental procedures were approved by Ningxia Medical University Institutional Review Board (Yinchuan, China) (approval no. NXMU-2017-030).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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