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Original Article

Effects of Heat Stress on the Development of GABAergic Neurons in the HPG Axis of Wenchang Chicks

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■Keywords

Heat stress; GABAergic neurons; HPG axis; Wenchang chick.

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ABSTRACT

This study conducted an in-depth investigation on the development of GABAergic neurons and their receptors in HPG axis-related target organs of Wenchang chicks under heat stress. One-day-old healthy Wenchang chicks were randomly divided into control (CK) and heat stress (HS) groups. Chicks in the HS group were placed in a 40±0.5°C climatic chamber for HS treatment from 13:00 to 15:00 daily. By immunohistochemistry and Western blotting, GABA and $GABA_A$ receptor ($GABA_AR$) expression in the hypothalamus of the HS group was significantly higher ($p<0.05$), but $GABA_B$ receptor ($GABA_BR$) expression was significantly lower than that of the CK group ($p<0.05$). Expression of GABA and its two receptors in the pituitary tissues of the HS group was significantly lower than in the CK group ($p<0.05$). Expression of GABA and $GABA_BR$ in ovaries in the HS group was significantly higher, but expression of $GABA_AR$ in the testes of the HS group was lower than that of the CK group ($p<0.05$). In the male chicks, expression of GABA and its two receptors in the hypothalamus, pituitary, and testicular tissues of the HS group was significantly higher than that of the CK group ($p<0.05$). Western blotting showed that the $GABA_AR$ and $GABA_BR$ expression of the HS group was significantly higher than that of the CK group at 3 and 5 weeks of age. Thus, HS caused GABAergic nervous system disorder in the HPG axis of Wenchang chicks and seriously hindered the normal development of GABAergic neurons in chicks, leading to the disorder of the expression of GABA and its receptors in tissues.

INTRODUCTION

Hindered growth and reproductive performance of breeding animals caused by heat stress is one of the serious problems faced by animal husbandry. Given their coverage with feathers and lack of sweat glands, chicks are highly susceptible to heat stress, leading to regulatory disorders of the hypothalamic–pituitary–gonadal (HPG) axis and ultimately resulting in an impaired reproductive system and decreased egg production (Xie *et al.*, 2016). Gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter that plays an important role in the regulation of the HPG axis. It is widely present in the central nervous system and peripheral tissues and is also expressed in the reproductive system of animals. GABAergic neurons can cause gonadotropin-releasing hormone (GnRH) neurons to exhibit excitatory or inhibitory effects by acting on $GABA_A$ receptors ($GABA_AR$) and $GABA_B$ receptors ($GABA_BR$) on the GnRH neuron surface and directly and indirectly impacting pituitary and ovarian functions by affecting the synthesis and release of GnRH in the hypothalamus (Tomaszewska*et al.*, 2003; Zhu, 2016). Therefore, GABA can affect the HPG axis and its target organs by regulating the secretion of related hormones. For example,

heat stress changes the amount of GnRH in the hypothalamus and the secretion of gonadotropin in the pituitary. These changes will further affect the growth and spermatogenesis of male reproductive organs and reduce the success rate of avian reproduction (Chen *et al.*, 2015; Türk *et al.*, 2015). In addition, under heat stress conditions, developmental impairment of HPG axis-related target organs in chicks at different ages is accompanied by abnormal changes in $GABA_A$ and $GABA_BR$ mRNA expression levels. This indicates that regulation of the HPG axis by GABAergic neurons is important for the homeostasis of the body under heat stress conditions (Xie *et al.*, 2016).

The pharmacological properties of GABA have been found to be calming, anxiety-reducing, and antidepressant. GABA can be used as feed additive to regulate immune and reproductive functions and to alleviate the harm of heat stress to animal husbandry (Chen et al., 2002; Leventhal et al., 2003; Chen et al., 2014). For example, one study showed that GABA alleviated the adverse effects of high temperature on the reproductive system of poultry by increasing the serum reproductive hormones, such as follicle-stimulating hormone (FSH) and estradiol (E_2), thereby ensuring the egg laying performance and egg quality of hens in a high-temperature environment (Zhang, 2012) and providing basic information for improving the anti-stress ability of poultry. However, the relationship between heat stress-induced HPG axis dysfunction and GABAergic neurons in chicks remains unclear. This study used immunohistochemistry (IHC) and Western blotting to explore the development of GABA, $GABA_AR$, and $GABA_BR$ in the hypothalamus, pituitary, ovary, and testicular tissues of HPG axis-related target organs in chicks to reveal the mechanism of heat stress on the development of HPG axis GABAergic neurons in chicks.

MATERIALS AND METHODS

Experimental animals and feeding management

One hundred forty-four healthy one-day-old Wenchang chicks (1:1 male-to-female ratio) purchased from Hainan YongJi Live Stock Co., Ltd. (Hainan Province, China), with no significant differences in body weight, were randomly divided into control (CK) and heat stress (HS) groups (n = 72 chicks, including 36 males and 36 females, per group). All chicks were weighed and numbered, followed by housing in a ventilated and regularly disinfected animal room (L:D = 14:10) with free access to feed (purchased from Zhanjiang Yilong Feed Factory, Guangdong Province, China) and sterile water.

Heat stress treatment

Chicks in the HS group were placed in a 40±0.5°C climatic chamber with a relative humidity of 70–80% for 2 h of heat treatment = from 13:00 to 15:00 daily. Simultaneously, chicks in the CK group were placed in a climatic chamber without being heated for 2 h. All chicks were returned to the corresponding cages for routine feeding after heat stress (Chen *et al.*, 2014).

Sample collection and processing

At the end of each week from 1 to 6 weeks of age and after the 2-h heat stress treatment, 6 chicks from each group were randomly selected to immediately dissect the hypothalamus, pituitary, ovary, and testis, followed by weighing. Part of the tissues was rapidly placed in Bouin's solution for 12 h fixation, and another part of the tissues was immediately placed in a cryotube and immersed in liquid nitrogen, followed by preserving in a –80°C freezer after 24 h. Tissues in the fixative were paraffin-embedded and sectioned for immunohistochemistry (IHC). Cryopreserved tissues were used for protein analysis in Western blotting. The experiments were conducted with the approval from the Hainan Normal University Animal Experimentation Ethics Committee.

Measurement of the expression of GABAergic neurons in the HPG axis

Primary anti-GABA antibody (orb6065), anti-$GABA_AR$ antibody (orb10677), and anti-$GABA_BR$ antibody (orb10679) were purchased from Biorbyt Ltd. (Cambridge, United Kingdom). Rabbit IHC detection kits (36312ES75) were purchased from Shanghai Yeasen Biotechnology Co., Ltd. (Shanghai, China) and used in strict accordance with the manufacturer's instructions. After paraffin embedding, the tissues were routinely prepared into 5-μm serial sections, followed by routine immunohistochemical staining,3,3′ -diaminobenzidine (DAB) color development, and hematoxylin nuclear counterstaining. Tissue sections were observed under light microscopy, and positive IHC staining presented as yellowish brown granules. Three tissue sections of each sample were randomly selected to observe the positive IHC staining in 10 fields of view each section under Olympus CX31 microscope (Olympus, Japan). Mshot Image Analysis System v1.0 (Mshot, Guangdong Province, China) was used for photographing and image storage. Image-Pro Plus 6.0 software (Media Cybernetics Corporate, Rockville, MD) was used to measure the integrated optical density (OD) values of GABA-, $GABA_AR$-, and $GABA_BR$-positive cells in the fields of view.

Detection of GABA receptor protein expression in ovary and testis

Ovary and testicular tissues were rinsed with PBS, followed by removing blood using filter paper and adding in RIPA lysate (containing PMSF) at a ratio of 0.2 mg/μL for homogenization with a glass pestle on an ice bath to ensure low-temperature grinding until the tissue cells were completely ruptured. After 30-min lysis on the ice bath, the lysate was transferred into a 1.5-ml centrifuge tube and centrifuged at 12,000 rpm for 5 min at 4°C to collect the supernatant, which was preserved in –20°C for the detection of the protein expression of $GABA_AR$ and $GABA_BR$ in the ovary and testis. The bicinchoninic acid (BCA) assay was used to quantify the total protein extracted from the tissue samples and to calculate the protein concentrations and sample loading volumes in SDS-PAGE. After adding 1× SDS loading buffer to each sample and denaturing the protein for 5 min in boiling water, SDS-PAGE was performed (constant voltage of 200 V for 1.5 h), and the proteins were transferred onto a membrane (constant current of 200 mA for 2 or 2.5 h). Non-fat milk blocking solution was used to block the membrane at room temperature for 1 h, followed by 3 TBST washes (10 min each) and adding 1:1,000 primary antibodies to incubate at room temperature for an hour and overnight at 4°C. After the primary antibody incubation, the membrane was washed in TBST 3 times (10 min each) and incubated with secondary antibodies for 1 h. The membrane was washed again in TBST 3 times (10 min each) and developed using an enhanced chemiluminescent (ECL) substrate kit (Shanghai Yeasen Biotechnology Co., Ltd.). The ImageQuant LAS 4000 biomolecular imager (GE Healthcare Life Sciences, Olympus, Japan) was used for image development and photographing. The Image-Pro Plus 6.0 image analysis system was used to determine the image-integrated OD values of the target protein bands and the internal reference protein bands. The relative protein expression of the target protein was calculated using the ratio of the image-integrated OD value of the target protein band to the image-integrated OD value of the reference protein band (Cheng *et al*., 2013).

Data processing and statistical analysis

All data were processed by Microsoft Excel software (Microsoft Corporation, Redmond, WA) and analyzed by ANOVA with SPSS 20.0 software (IBM SPSS, Chicago, IL). Multiple comparisons were performed by Duncan's multiple range test. The results were presented as mean ± standard deviation (mean ± SEM). $p<0.05$ was considered a statistically significant difference.

RESULTS

Effects of heat stress on GABA and its receptor in the hypothalamus of Wenchang chicks

As shown in Figure 1, the GABA-, $GABA_AR$-, and $GABA_BR$-positive cells were brownish-yellow in color and round or oval. These cells had clear structure, which was randomly distributed in the tissues and mainly distributed in the cell membrane and not the cytoplasm.

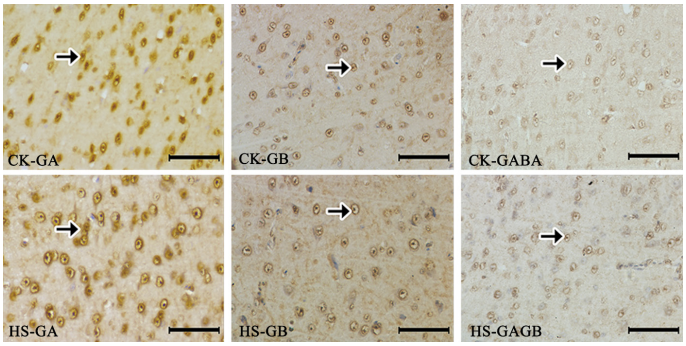

Figure 1 – The immunohistochemical staining of GABA and GABA receptors in hypothalamus of Wenchang (×400, Bar=50 μm). Note: CK, control check; HS, heat stress; GA, $GABA_A$ receptor; GB, $GABA_B$ receptor. The arrowhead is shown as positive reactions.

As shown in Table 1, the densities of GABA and its two receptors in the hypothalamus of female chicks during 1–6 weeks of age increased first and

Table 1 – Effects of heat stress on mean integral optical density of GABA and GABA receptors of hypothalamus in Wenchang female chicks (IOD/μm²).

Item	Group	Age(week)					
		1	2	3	4	5	6
$GABA_A$ receptor	CK	4.93 ± 0.66^{bc}	5.93 ± 1.65^{b}	11.74 ± 1.41^{aA}	11.13 ± 1.67^{aA}	4.35 ± 1.39^{bc}	4.09 ± 1.24^{c}
	HS	5.39 ± 1.51^{b}	6.37 ± 0.3^{ab}	7.42 ± 1.8^{aB}	6.65 ± 1^{abB}	5.62 ± 1.8^{ab}	5.89 ± 1.71^{ab}
$GABA_B$ receptor	CK	3.58 ± 1.03^{dA}	9.5 ± 0.92^{aA}	4.53 ± 0.41^{bc}	4.88 ± 0.62^{bA}	4.5 ± 0.45^{bc}	3.65 ± 0.65^{cd}
	HS	6.01 ± 0.57^{bB}	7.54 ± 1.39^{aB}	4.2 ± 0.3^{c}	3.62 ± 0.78^{cB}	3.91 ± 0.64^{c}	4.52 ± 1.68^{c}
GABA	CK	1.25 ± 0.33^{d}	1.03 ± 0.13^{dB}	2.55 ± 0.43^{cB}	4.29 ± 0.46^{b}	4.01 ± 0.4^{bB}	6.56 ± 0.38^{aA}
	HS	1.22 ± 0.15^{c}	1.26 ± 0.09^{cA}	4.62 ± 0.42^{bA}	4.79 ± 0.62^{b}	7.1 ± 1.24^{aA}	4.18 ± 0.26^{bB}

Note: The uppercase letters for group comparison, the lowercase letters for age comparison, and different letters mean significant difference ($p<0.05$). CK, control check; HS, heat stress.

subsequently decreased. The density of $GABA_AR$-positive cells was the highest at 3 weeks of age. The density of $GABA_AR$-positive cells in the HS group was significantly lower than that in the CK group at 3 to 4 weeks of age (p<0.05). The density of $GABA_BR$-positive cells was the highest at 2 weeks of age, and a significant difference was found between the HS and CK group at 2 and 4 weeks of age (p<0.05). The GABA expression of the HS group was significantly higher than that of the CK group at 2, 3, and 5 weeks of age (p<0.05).

As shown in Table 2, the distribution densities of GABA and its two receptors in the hypothalamus of male chicks from 1 to 6 weeks of age increased first and subsequently decreased, peaking at 3 weeks of age. These distribution densities were significantly higher in the HS group than in the CK group at 3–5 weeks of age (p<0.05). Until 6 weeks of age, the distribution densities of the two receptors in the CK group increased significantly; in contrast, the distribution densities of the two receptors in the HS group reduced significantly. These distribution densities of the positive

Table 2 – Effects of heat stress on mean integral optical density of GABA and GABA receptors of hypothalamus in Wenchang male chicks (IOD/μm²).

Item	Group	Age(week)					
		1	2	3	4	5	6
$GABA_A$ receptor	CK	1.82 ± 0.15^{e}	2.04 ± 0.09^{c}	2.90 ± 0.19^{aB}	2.55 ± 0.08^{bB}	2.23 ± 0.06^{dB}	2.49 ± 0.13^{bA}
	HS	1.90 ± 0.19^{d}	2.10 ± 0.32^{d}	3.53 ± 0.17^{aA}	3.02 ± 0.0^{bA}	2.53 ± 0.09^{cA}	2.08 ± 0.06^{dB}
$GABA_B$ receptor	CK	1.62 ± 0.11^{e}	1.90 ± 0.16^{d}	2.39 ± 0.05^{aB}	2.22 ± 0.07^{bB}	2.05 ± 0.13^{cB}	2.28 ± 0.08^{abA}
	HS	1.67 ± 0.08^{f}	2.08 ± 0.18^{d}	2.80 ± 0.12^{aA}	2.58 ± 0.10^{bA}	2.24 ± 0.08^{cA}	1.88 ± 0.08^{eB}
GABA	CK	1.51 ± 0.16^{e}	1.82 ± 0.19^{d}	2.56 ± 0.17^{aB}	2.43 ± 0.06^{bB}	2.30 ± 0.07^{bc}	2.24 ± 0.08^{cA}
	HS	1.64 ± 0.15^{e}	1.92 ± 0.05^{d}	3.27 ± 0.11^{aA}	2.80 ± 0.14^{bA}	2.35 ± 0.14^{c}	1.95 ± 0.22^{dB}

Note: The uppercase letters for group comparison, the lowercase letters for age comparison, and different letters mean significant difference (p<0.05). CK, control check; HS, heat stress.

cells in the HS group were significantly lower than those in the CK group (p<0.05).

Effects of heat stress on GABA and its receptors in the pituitary of Wenchang chicks

As shown in Figure 2, the GABA- and GABA receptor-positive cells in the pituitary were brownish-yellow in color and round or oval, and some of them were irregular in shape and size, with different staining intensities. The CK group had strongly positive staining of GABA and its two receptors, and the HS group had mainly moderately and weakly positive staining; the numbers of GABA- and GABA receptor-positive cells in the pituitary of the CK group were significantly higher than those in the HS group.

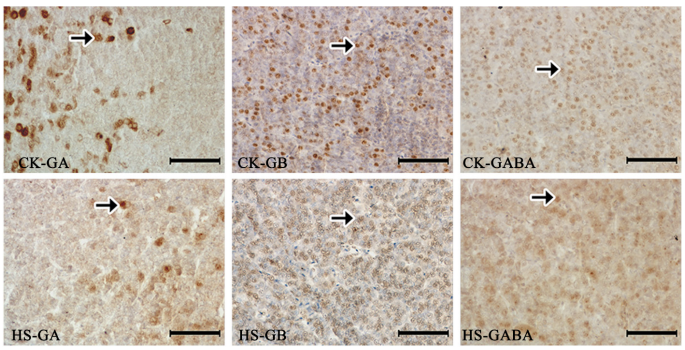

Figure 2 – The immunohistochemical staining of GABA and GABA receptors in adeno-hypophysis of Wenchang chicks (×400□Bar=50 μm). Note: CK, control check; HS, heat stress; GA, $GABA_A$ receptor; GB, $GABA_B$ receptor. The arrowhead is shown as positive reactions.

The distribution densities of GABA and its two receptors in the pituitary gland of chicks from 1 to 6 weeks of age increased first and subsequently decreased. As shown in Table 3, the distribution densities of $GABA_AR$ and $GABA_BR$ in the female chicks of the HS group were significantly lower than those of the CK group at 3–5 weeks of age (p<0.05). Until 6 weeks of age, the distribution densities of the two receptors in the HS group increased significantly, and there were significantly more positively stained cells of the two receptors in the HS group than in the CK group at this time (p<0.05). There were significantly more GABA-positive cells in the HS group than in the CK group at 2 and 4 weeks of age (p<0.05), but fewer at 3 and 5 weeks of age.

At 2–5 weeks of age in the female chicks (Table 4), the distribution densities of GABA- and $GABA_AR$- and $GABA_BR$-positive cells in the pituitary of the HS group were significantly higher than those in the CK group (p<0.05). After 5 weeks of age, the distribution densities of the positive cells tended to be stable in the CK group but maintained a significant decline in the HS group. At 6 weeks of age, the distribution densities of GABA-, $GABA_AR$-, and $GABA_BR$-positive cells in the pituitary of the HS group were significantly lower than those in the CK group (p<0.05).

Table 3 – Effects of heat stress on mean integral optical density of GABA and GABA receptors of adenohypophysis in Wenchang female chicks (IOD/μm²).

Item	Group	Age(week)					
		1	2	3	4	5	6
$GABA_A$ receptor	CK	53.03±8.13^{fA}	113.5±5.47^{c}	166.55±7.97^{aA}	153.69±6.69^{bA}	73.65±7.23^{eA}	88.16±8.76^{dB}
	HS	42.91±9.11^{cB}	104.62±5.76^{b}	129.29±8.91^{aB}	69.53±4.53^{cB}	55.14±4.99^{dB}	135.28±6.34^{aA}
$GABA_B$ receptor	CK	4.55±0.25^{cA}	4.34±0.64^{cB}	10.77±1.81^{bA}	12.78±0.86^{aA}	13.44±0.86^{aA}	10.03±0.82^{b}
	HS	2.95±0.83^{eB}	5.43±0.57^{dA}	8.38±0.62^{cB}	11.73±0.65^{aB}	8.28±1.39^{cB}	10.35±0.64^{b}
GABA	CK	4.48±0.29^{e}	4.79±0.54^{dB}	14.37±1.61^{aA}	6.51±0.5^{cB}	7.19±0.64^{bcA}	7.98±1.14^{b}
	HS	4.06±0.46^{d}	11.76±1.38^{aA}	11.59±0.9^{aB}	8.51±0.5^{bA}	6.24±0.78^{cB}	8.14±0.68^{b}

Note: The uppercase letters for group comparison, the lowercase letters for age comparison, and different letters mean significant difference ($p<0.05$). CK, control check; HS, heat stress.

Table 4 – Effects of heat stress on mean integral optical density of GABA and GABA receptors of adenohypophysis in Wenchang male chicks(IOD/μm²).

Item	Group	Age(week)					
		1	2	3	4	5	6
$GABA_A$ receptor	CK	4.52±0.27^{c}	4.89±0.19^{bcB}	6.11±0.70^{aB}	5.38±0.35^{bB}	5.26±0.08^{bB}	5.20±0.14^{bA}
	HS	4.57±0.48^{d}	5.19±0.27^{cA}	7.31±0.32^{aA}	6.19±0.81^{bA}	5.82±0.48^{bA}	4.59±0.13^{dB}
$GABA_B$ receptor	CK	2.75±0.09^{eB}	2.98±0.08^{dB}	3.88±0.17^{aB}	3.45±0.17^{bB}	3.19±0.29^{c}	3.19±0.14^{cA}
	HS	2.90±0.14^{dA}	3.26±0.17^{cA}	5.02±0.23^{aA}	4.19±0.16^{bA}	3.37±0.10^{c}	2.76±0.16^{dB}
GABA	CK	2.66±0.18^{e}	2.82±0.13^{dB}	4.23±0.14^{aB}	3.95±0.06^{bB}	3.75±0.12^{cB}	3.68±0.15^{cA}
	HS	2.72±0.13^{f}	3.45±0.22^{dA}	5.33±0.49^{aA}	4.53±0.17^{bA}	4.16±0.15^{cA}	3.13±0.14^{eB}

Note: The uppercase letters for group comparison, the lowercase letters for age comparison, and different letters mean significant difference ($p<0.05$). CK, control check; HS, heat stress.

Effects of heat stress on GABA and its receptors in the ovaries of Wenchang chicks

Figure 3 showed that GABA and GABA receptor expression was stained in the cytoplasm and cell membrane as yellow or brown particles in round or ovoid.The GABA and GABA receptor positive cells were mainly distributed in the membrane and cortexof ovary, with a small portion observed at the medulla.

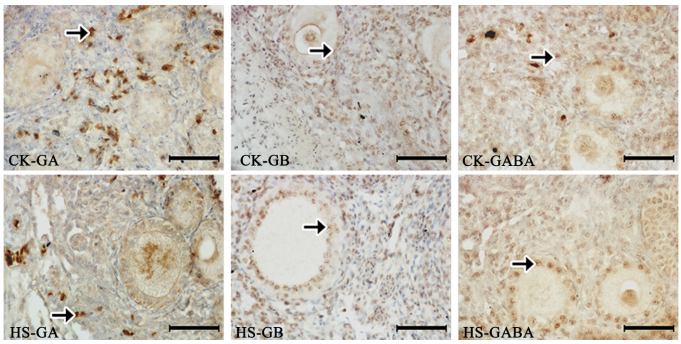

Figure 3 – The immunohistochemical staining of GABA and GABA receptors in ovary of Wenchang chicks (×400, Bar=50 μm). Note: CK, control check; HS, heat stress; GA, $GABA_A$ receptor; GB, $GABA_B$ receptor. The arrowhead is shown as positive reactions.

As shown in Table 5, the distribution densities of $GABA_AR$- and $GABA_BR$-positive cells in the ovaries of the chicks fluctuated during 1–6 weeks of age. The density of $GABA_AR$-positive cells in the ovaries of the HS group was significantly lower than that of the CK group at 1, 2, 4, and 5 weeks of age ($p<0.05$). The density of $GABA_BR$-positive cells in the ovaries of the HS group was significantly higher than that in the CK group at 3, 5, and 6 weeks of age ($p<0.05$). The density of GABA-positive cells in the ovaries of chicks was increased first and subsequently decreased, peaking at 3 weeks of age; compared with the CK group, the density of GABA-positive cells in the ovaries of the HS group was significantly higher ($p<0.05$).

Effects of heat stress on GABA and its receptors in the testes of Wenchang chicks

As shown in Figure 4, GABA- and GABA receptors-positive cells in testes were brownish-yellow in color and round or oval in shape, with irregular sizes and different staining intensities. The positive cells were mainly distributed in the basement membrane, epithelium, and interstitial part of testes.

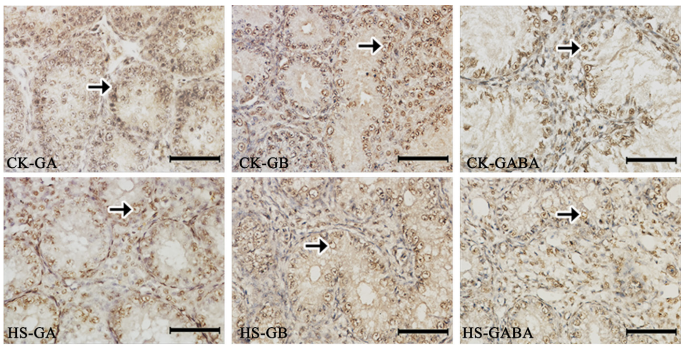

Figure 4 – The immunohistochemical staining of GABA and GABA receptors in testis of Wenchang chicks (×400, Bar=50 μm). Note: CK, control check; HS, heat stress; GA, $GABA_A$ receptor; GB, $GABA_B$ receptor. The arrowhead is shown as positive reactions.

Table 5 – Effects of heat stress on mean integral optical density of GABA and GABA receptors of ovary in Wenchang chicks (IOD/μm²).

Item	Group	Age (Week)					
		1	2	3	4	5	6
$GABA_A$ receptor	CK	79.77±12.16^{bA}	141.55±8.22^{aA}	38.1±7.98^{dB}	61.23±8.79^{cA}	149.67±21.09^{aA}	46.32±6.65^{dB}
	HS	45.83±4.79^{dB}	87.56±6.27^{bB}	89.58±16.14^{bA}	49.64±9.25^{dB}	69.97±17.45^{cB}	110.14±12.13^{aA}
$GABA_B$ receptor	CK	5.24±0.92^{bB}	4.82±0.89^{b}	2.06±0.87^{cB}	8.45±0.78^{aA}	4.98±0.64^{bB}	5.27±0.64^{bB}
	HS	7.92±0.94^{bA}	5.55±0.87^{c}	9.42±1.2^{aA}	6.21±1.01^{cB}	6.37±0.5^{cA}	6.53±0.56^{cA}
GABA	CK	3.94±0.73^{cB}	9.59±0.79^{aB}	10.42±0.58^{aB}	3.98±0.86^{cB}	5.89±1.1^{b}	5.74±0.59^{bB}
	HS	5.28±0.43^{dA}	11.78±0.7^{aA}	11.82±1.12^{aA}	6.85±0.85^{cA}	6.79±0.47^{c}	9.1±0.63^{bA}

Note: The uppercase letters for group comparison, the lowercase letters for age comparison, and different letters mean significant difference ($p<0.05$). CK,control check; HS, heat stress.

As seen in Table 6, the distribution densities of the $GABA_AR$-positive cells in the testes of the HS and CK groups, the $GABA_BR$-positive cells in the testes of the HS group, and the GABA-positive cells in the testes of the CK group increased first and subsequently decreased. The distribution densities of the $GABA_AR$-positive cells in the testes of the CK group and GABA-positive cells in the testes of the HS group decreased and then increased. However, the GABA and its two receptors in the testes of chicks peaked at 3 weeks of age, and the average optical densities of GABA and its receptors in the testes of chicks in the CK group were significantly lower than those of the HS group ($p<0.05$). At 6 weeks of age, the optical densities of $GABA_AR$ and $GABA_BR$ in the testes of the CK group were significantly higher than those of the HS group. Only the GABA-positive expression of the CK group was still significantly lower than that of the HS group at this time ($p<0.05$).

Table 6 – Effects of heat stress on mean integral optical density of GABA and GABA receptors of testis in Wenchang chicks(IOD/μm²).

Item	Group	Age (Week)					
		1	2	3	4	5	6
$GABA_A$ receptor	CK	5.00±0.70^{c}	5.53±0.21^{bA}	6.27±0.10^{aB}	5.90±0.26^{abB}	5.77±0.26^{bB}	5.78±0.28^{bA}
	HS	4.83±0.14^{e}	5.21±0.14^{dB}	7.53±0.21^{aA}	6.82±0.45^{bA}	6.10±0.17^{cA}	4.80±0.28^{eB}
$GABA_B$ receptor	CK	2.65±0.25^{d}	3.16±0.43^{c}	4.45±0.16^{aB}	3.70±0.21^{bB}	3.35±0.05^{cB}	3.80±0.12^{bA}
	HS	2.50±0.11^{d}	2.83±0.11^{d}	5.61±0.71^{aA}	4.81±0.19^{bA}	4.21±0.09^{cA}	2.53±0.10^{dB}
GABA	CK	4.48±0.13^{c}	4.72±0.14^{b}	5.07±0.10^{aB}	4.42±0.16^{cB}	4.47±0.15^{cA}	3.93±0.14^{dB}
	HS	4.20±0.38^{e}	4.56±0.13^{d}	6.40±0.14^{aA}	5.25±0.18^{cA}	4.20±0.15^{eB}	5.53±0.17^{bA}

Note: The uppercase letters for group comparison, the lowercase letters for age comparison, and different letters mean significant difference ($p<0.05$). CK, control check; HS, heat stress.

Protein expression of $GABA_AR$ and $GABA_BR$ in the ovaries and testes of chicks

The protein expression of $GABA_AR$ and $GABA_BR$ in the ovaries and testes of chicks in different groups was detected by Western blotting. The $GABA_AR$ and $GABA_BR$ protein expression in the chick ovaries increased first and subsequently decreased in the CK group; in contrast, the protein expression of $GABA_AR$ decreased first and subsequently increased in the HS group. The protein expression of $GABA_BR$ decreased with age in the ovaries of the HS group. The protein expression of $GABA_AR$ and $GABA_BR$ in the ovaries of the HS group was higher than that in the CK group at 3 and 6 weeks of age but lower than that in the CK group at 4 and 5 weeks of age. These expression patterns were consistent with the IHC results (Figure 5).

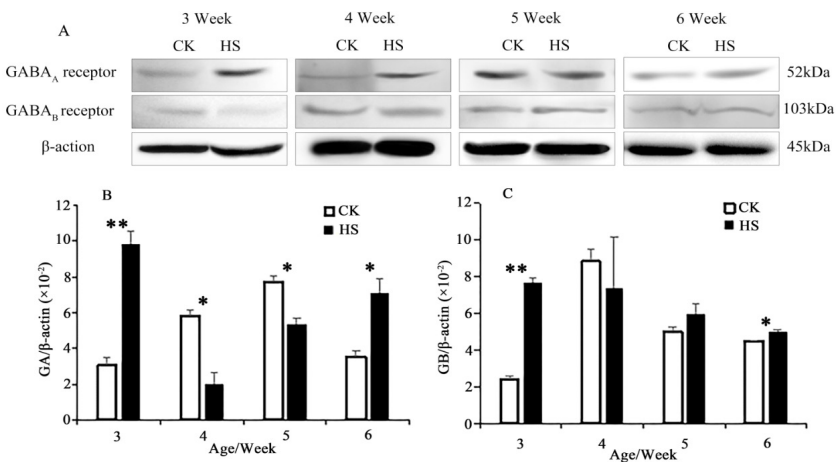

Figure 5 – The expression of $GABA_A$ and $GABA_B$ receptors protein in chicks' ovary. Note: A, $GABA_A$ and $GABA_B$ receptor proteins; B, $GABA_A$ receptor protein; C, $GABA_B$ receptor protein; CK, control check; HS, heat stress.

The protein expression of $GABA_AR$ and $GABA_BR$ in chick testes was relatively low before 5 weeks of age. However, the HS group always showed a decreasing expression trend for both receptors. At 6 weeks of age, the protein expression of $GABA_AR$ and $GABA_BR$ in the chick testes of the CK group was higher than that of the HS group. These results were consistent with the IHC results regarding the expression patterns at different time points (Figure 6).

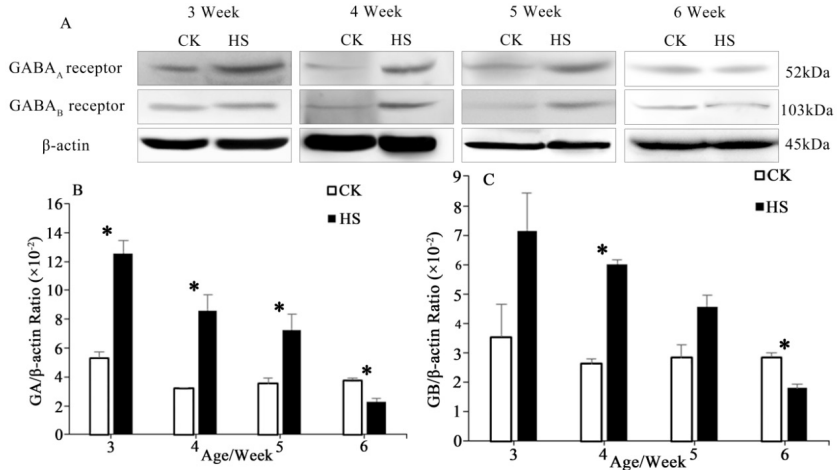

Figure 6 – The expression of $GABA_A$ and $GABA_B$ receptors protein in chicks' testis. Note: A, $GABA_A$ and $GABA_B$ receptor proteins; B, $GABA_A$ receptor protein; C, $GABA_B$ receptor protein; CK, control check; HS, heat stress.

DISCUSSION

As an inhibitory neurotransmitter in the nervous system, GABA inhibits basal metabolism, regulates hormone levels, balances blood electrolytes, enhances antioxidant capacity, and helps overcome and alleviates adverse effects of a high-temperature environment in the poultry body in the summer (Chen *et al.*, 2013; Liang *et al.*, 2016). GABAergic neurons are neuronal cells that not only use GABA as a transmitter to participate in the regulation of metabolism, but also are inseparable from the egg development and sperm capacitation in the body (Watanabe *et al.*, 2002; Geigerseder *et al.*, 2004).

The hypothalamus is the main center for thermoregulation in the body, and the GABAergic nervous system is indispensable for the processes of thermoregulation. It not only accelerates the intracellular oxidation and promotes the thermal dissipation of the body by regulating the secretion of insulin, thyroid hormone, norepinephrine, and other hormones, but also modulates the synthesis and secretion of pituitary hormones by regulating the release of inhibitory hormones from neuroendocrine cells to alleviate the damages caused by heat stress (Tian *et al.*, 2010; Li *et al.*, 2014).

In this study, the protein expression of GABA and its receptors in the hypothalamus of the HS group was slightly stronger than that of the CK group before 2 weeks of age, possibly because of the high-temperature environment, which was beneficial to the early stage of development in the chicks (Racagni*et al.*, 1982). After 2 weeks of age, chicks began to enter the period of accelerated growth. Chicks in the HS group maintained a rapid growth trend, and, to maintain this trend, they required additional metabolic and physiological activities to alleviate the damages of heat stress (Zhou *et al.*, 2015). At this time, the development of female and male chicks was different. At 3 and 5 weeks of age, GABA expression in the hypothalamus tissues of the female chicks of the HS group was significantly higher than that of the GK group. At 3 to 4 weeks of age, GABA expression in the hypothalamus tissues of the male chicks of the HS group was significantly higher than that of the CK group. These results were consistent with our previous report that acute heat stress enhanced the physiological roles of GABA by accumulating GABA (Chen *et al.*, 1999). GABA expression in the HS group was significantly enhanced, presumably to alleviate the effects of heat stress on the hypothalamus of the chicks. Our findings also indicated that after 3 weeks of age, the chicks tried to alleviate the damages caused by heat stress through various means; however, this had no significant effect, and the hypothalamic tissue was obviously damaged and could not secrete more GABA. In addition, GABA is inseparable from GnRH synthesis and secretion in the hypothalamus, further affecting the homeostasis of the body and impeding the normal development of chicks (Hales *et al.*, 2008). Therefore, we believe that heat stress can cause damage to the hypothalamus structure, thereby impeding the growth and development of GABAergic neurons in the hypothalamus of Wenchang chicks.

The pituitary has no distribution of glutamate decarboxylase, and only a small amount of GABA immunopositive nerve endings are present on the lateral and superficial layers of the anterior pituitary. The GABA in these nerve endings most likely originates from the hypothalamus (Oertel *et al.*, 1982; Ohkawa *et al.*, 1983; Lu *et al.*, 2001). In this study, compared with

the CK group, male chicks in the HS group had stronger expression of GABA and its receptors before 5 weeks of age; in contrast, expression of GABA and its receptors decreased in the female chicks, which was similar to their corresponding expression in the hypothalamus. These results indirectly verified the hypothesis that a large amount of GABAergic positive expression in the pituitary gland originated from the hypothalamus. At 6 weeks of age, expression of GABA and its receptors in the male chicks in the HS group was significantly lower than that of the male chicks in the CK group, while the opposite expression pattern was found in the female chicks, indicating that the compensatory mechanism of female chicks corrected the abnormal physiological functions, whereas the male chicks did not recover. Therefore, we believe that heat stress may damage the pituitary tissues more in male chicks than in female chicks. Our previous study showed that heat stress causes significant structural damage to the pituitary and impedes its normal development (Chen et al., 2015). Once the pituitary tissue is damaged, it will lead to insufficient secretion of thyroid hormone, and the metabolic activities and functions in the body will be reduced, which may cause damage to the reproductive system in the severe cases (Santospalacios *et al.*, 2013; Wang *et al.*, 2016).A study has shown that GABA indirectly regulates the level of thyroid-stimulating hormone or affects the 5′-deiodinase activity in the body to further regulate the synthesis and secretion of thyroid hormone, thereby affecting the metabolic balance in the body (Fan *et al.*, 2007). Thus, we believe that high temperature causes damage to the pituitary tissues of chicks, resulting in damage to GABAergic neurons, insufficient GABA secretion, and impaired pituitary function. These impairments will affect the synthesis of gonadotrophins and thyroid-stimulating hormone and the normal development of the body.

A previous study found high concentrations of GABA and GABA synthase in the ovaries and fallopian tubes of rats. Through changes in GABA content during the estrous cycle, the physiological functions of ovaries are regulated (Bowery *et al.*, 1997). This study showed that expression of $GABA_AR$ and $GABA_BR$ in the chick ovaries of the CK group decreased first and subsequently decreased, while the expression of GABA increased first and then decreased, suggesting that a high concentration of GABA may affect the quality of oocytes and increase the apoptotic rate of the ovarian granulosa cells (Yang, 2009). Thus, high GABA concentrations can cause apoptosis of oocytes, whereas GABA receptors may alleviate this apoptosis by regulating the GABA concentration. A study has shown that GABA may inhibit the secretions of E_2 and progesterone from ovarian luteal cells by binding to $GABA_AR$, thereby affecting the growth and development of chicks as well as their reproductive system (Zhang *et al.*, 2000).

In this study, expression of $GABA_AR$ and $GABA_BR$ in the chick ovaries of the HS group increased first and subsequently decreased, peaking at 3 weeks of age. These findings were consistent with the expression pattern of GABA, which greatly increased the binding rate of GABA to $GABA_B$, thereby alleviating the negative effect of HS on ovarian tissues. Several studies have shown that GABA regulates sperm capacitation and acrosome reaction by binding to $GABA_AR$ or $GABA_BR$ (Hu *et al.*, 2002; Puente *et al.*, 2011; Ritta *et al.*, 2004). The GABAergic system in the male rat reproductive system has a direct effect on steroid synthesis and sperm survival and activity. Therefore, a high-temperature environment may cause certain damage to the chick reproductive system by affecting the GABAergic nervous system (Hinoi *et al.*, 2001). This study showed that the expression of the two GABA receptors in the testes of the HS group was significantly lower than that of the CK group at 6 weeks of age. In addition, GABA accumulated and increased in concentration in the testes of the HS group. Studies have shown that high GABA concentrations affect the expression of GABA transporter 1. Abnormal expression of GABA transporter 1 affects the function of the testes in the male reproductive system, causing damage to spermatogenic capacity and reproductive disorders, and leading to infertility (Hu, 2003; Mantz *et al.*, 1995). Our previous report showed that HS destroys the structural integrity and impedes the normal development of chick testicular tissue (Chen et al., 2015). Therefore, high temperature causes damages to testicular tissue, affecting the GABA content and development of GABA receptors and disturbing the stability of the GABAergic nervous system in the testicular tissues, thereby impeding the normal development of the chick´s reproductive system.

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