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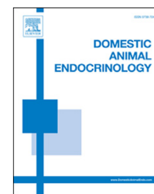

Differential expression of hypothalamic fear- and stress-related genes in broiler chickens showing short or long tonic immobility

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ABSTRACT

The serotonin system and the hypothalamic-pituitary-adrenal axis play important roles in modulating fear and stress-coping characteristics. Tonic immobility (TI) is a fear-related phenotype, and previously we have shown that broiler chickens showing short TI (STI) duration experience better growth performance and higher adaptability to stress. Here, we sought to further elucidate the central mechanisms underlying the phenotypic differences between chickens showing STI and long TI duration, by comparing the hypothalamic expression of genes in the serotonergic system and the hypothalamic-pituitary-adrenal axis under basal and corticosterone-exposed situations. The STI broilers had significantly lower ($P < 0.01$) hypothalamic expression of serotonin reuptake transporter and serotonin receptor 1A. Moreover, 11β-hydroxysteroid dehydrogenase type 2 was expressed significantly lower in STI chickens at the level of both mRNA ($P < 0.01$) and protein ($P < 0.05$). Hypothalamic expression of glucocorticoid receptor (GR) mRNA tended to be higher ($P < 0.059$) in long TI chickens, but the protein content was approximately 2 times higher ($P < 0.01$) in STI chickens. The uncoupled expression of GR mRNA and protein was associated with significantly lower ($P < 0.05$) expression of *gga-miR-181a, gga-miR-211, and gga-miR-22*, which are predicted to target *GR*, in STI chickens. Corticosterone administration reduced the mRNA expression of postsynaptic serotonin receptors, 5-hydroxytryptamine receptor 1B ($P = 0.059$) and 5-hydroxytryptamine receptor 7 ($P < 0.05$), yet significantly increased the protein content of 11β-hydroxysteroid dehydrogenase type 2 ($P < 0.05$). These results suggest that broilers of different TI phenotypes have a distinct pattern of hypothalamic expression of fear- and stress-related genes.

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1. Introduction

Tonic immobility (TI) is an unlearned response characterized by temporal freeze or paralysis state of reduced responsiveness to external stimulation. Tonic immobility is commonly considered as a measure for fear. A long duration of TI is generally considered as an indication for high levels of fearfulness [1]. Birds can naturally be divided into a long (LTI) or short (STI) group, depending on the TI duration. Differences in behavior, reproduction, growth, and welfare between STI and LTI phenotypes have been reported [2–4].

Previous studies have shown that the central serotonergic system plays a crucial role in modulating TI duration. Microinjection of serotonin (5-HT) into different brain areas increases or decreases TI duration in a dose-dependent manner [5,6], whereas fenfluramine- or p-chloroamphetamine–induced 5-HT release is associated with decreased TI duration [7]. The biosynthesis of 5-HT in the brain is controlled by the rate-limiting enzyme

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tryptophan hydroxylase 2 [8], whereas serotonergic neurotransmission is determined by the extracellular 5-HT levels in the synaptic clefts [9]. The extracellular 5-HT levels are regulated by release, reuptake, and metabolism. Activation of 5-HT1A autoreceptors located on the presynaptic membranes inhibits the release of 5-HT in nerve terminals, whereas 5-HT transporter (SERT) reuptakes 5-HT from synaptic clefts into presynaptic neurons to terminate 5-HT action. Most of the 5-HT released to the synaptic spaces is reused by SERT, and the remaining part is inactivated by monoamine oxidase [10]. Disrupted 5-HT system has been reported in humans with psychiatric disorders such as depression [11] or animals that exhibit fear-related behaviors [6]. However, it has not been described whether serotonergic genes are expressed differently between STI and LTI chickens.

Fear-related behavior is closely associated with stress response that is regulated by the hypothalamic-pituitary-adrenal (HPA) axis [12,13]. The stress-induced elevation of glucocorticoid, mainly corticosterone (CORT) in chickens, feeds back to the hypothalamus to inhibit the activity of the HPA axis. This negative feedback is a critical adaptive mechanism that protects the animals from injuries caused by overreactive stress responses. Glucocorticoid receptor (GR) in the hypothalamus mediates the negative feedback of CORT. The intracellular level of CORT available to activate GR is increased by 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), yet decreased by 11β-HSD2 [14]. Activation of the HPA axis increases the TI duration in Japanese quail [15], duck [16], rabbit [17], and rat [18]. Birds of STI and LTI phenotypes display different stress-coping characteristics and HPA axis reactivity as indicated by the serum level of CORT [4,19]. Furthermore, corticotropin-releasing hormone (CRH) secreted from the hypothalamus was implicated to contribute to the differences in HPA axis reactivity between LTI and STI phenotypes in Japanese quails [20]. However, it remains elusive whether the hypothalamic expression of HPA axis genes is different between STI and LTI chickens.

Therefore, the objective of the present study was to delineate the expression pattern of genes involved in the serotonergic system and HPA axis in the hypothalamus of broiler chickens showing a LTI or STI duration, under basal and CORT-exposed situations.

2. Materials and methods

The experimental protocol was approved by the Animal Ethics Committee of Nanjing Agricultural University.

2.1. Animals and management

A total of 468 newly hatched broiler chickens (Ross 308) were wing-banded and housed in an environmentally controlled room according to the feeding standard recommended by the breeding company. The ambient temperature and lighting regime were set as previously described [4]. Standard commercial broiler starter crumble (12.5 ME/kg; 21% CP) and finisher pellet (12.8 ME/kg; 19.5% CP) were provided from 1 to 20 d and 21 to 42 d, respectively. Water was available ad libitum.

2.2. Tonic immobility test

All chickens were subjected to a TI test twice on 10 d and 21 d, respectively, to establish 2 segregated groups of STI and LTI phenotypes. The TI test was performed according to a previously published protocol [21]. In brief, chickens were carried individually to another room devoid of other birds. The chicken was placed on its back on the floor and restrained for 20 s (with 1 hand on the sternum and 1 hand lightly cupping the head of the bird). The experimenter remained silent and virtually motionless in the room, out of the bird's sight. If >10 s elapsed until the bird righted itself, the duration of TI was recorded. If TI was not attained after 3 attempts, a score of 0 s was given. Conversely, if the bird failed to right itself after 10 min, the test was terminated and a maximum score of 600 s was given for TI duration.

Chickens with 2 extremes (shortest and longest) of TI duration were used in the experiment. Eighty chickens showing the shortest TI duration (29.6 ± 2.3 s) and 80 chickens scoring the longest duration (246.2 ± 26.8 s) were classified into the STI and LTI groups, respectively. The remaining chickens with intermediate TI duration were excluded from the study.

2.3. Corticosterone administration

Chickens in the STI and LTI groups were respectively allocated into control and CORT-treated subgroups and reared in four 2×2.7 m^2 pens. From 27 to 42 d, chickens in the CORT groups of both STI and LTI phenotypes (40 per phenotype) were supplied water supplemented with 5 mg/L corticosterone (C2505; Sigma, St. Louis, MO, USA), whereas chickens in the control groups were supplied water supplemented with an equivalent volume of the solvent (absolute ethanol).

After 42 d, all chickens were slaughtered by fast decapitation that is accepted as an ethical type of euthanasia for the chicken. Hypothalamic samples were dissected, frozen in liquid nitrogen, and stored at −70°C.

2.4. Quantitation of mRNA by real-time PCR

Total RNA was extracted from the hypothalamus with the TRIzol total RNA Kit (15596026; Invitrogen, Carlsbad, CA, USA) and treated with 10 U of DNase I (RNase Free, D2215; TaKaRa Bio Inc, Otsu, Japan) to eliminate possible contamination of genomic DNA. Two micrograms of total RNA was reverse transcribed with AMV reverse transcriptase (M5101; Promega, Madison, WI, USA), and 2 μL of diluted cDNA (1:10) was used for PCR. All the primers, as listed in Table 1, were synthesized by Invitrogen (Shanghai, China). Real-time PCR was performed in Mx3000P (Stratagene, La Jolla, CA, USA). Chicken β-actin was selected as the reference gene, for β-actin mRNA abundance did not differ among groups. The amplification efficiencies of β-actin and all the target genes were within the acceptable range of approximately 88% to 113%, as recommended in the method of using $2^{-\Delta\Delta Ct}$ to analyze the real-time PCR data [22], and the mRNA levels were expressed as the fold change relative to the mean value of the STI control group.

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