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Embryonic exposure to corticosterone modifies aggressive behavior through alterations of the hypothalamic pituitary adrenal axis and the serotonergic system in the chicken

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ABSTRACT

Exposure to excess glucocorticoids (GCs) during embryonic development influences offspring phenotypes and behaviors and induces epigenetic modifications of the genes in the hypothalamic–pituitary–adrenal (HPA) axis and in the serotonergic system in mammals. Whether prenatal corticosterone (CORT) exposure causes similar effects in avian species is less clear. In this study, we injected low (0.2 μg) and high (1 μg) doses of CORT into developing embryos on day 11 of incubation (E11) and tested the changes in aggressive behavior and hypothalamic gene expression on posthatch chickens of different ages. *In ovo* administration of high dose CORT significantly suppressed the growth rate from 3 weeks of age and increased the frequency of aggressive behaviors, and the dosage was associated with elevated plasma CORT concentrations and significantly downregulated hypothalamic expression of arginine vasotocin (AVT) and corticotropin-releasing hormone (CRH). The hypothalamic content of glucocorticoid receptor (GR) protein was significantly decreased in the high dose group ($p < 0.05$), whereas no changes were observed for GR mRNA. High dose CORT exposure significantly increased platelet serotonin (5-HT) uptake, decreased whole blood 5-HT concentration ($p < 0.05$), downregulated hypothalamic tryptophan hydroxylase 1 (TPH1) mRNA and upregulated 5-HT receptor 1A (5-HTR1A) and monoamine oxidase A (MAO-A) mRNA, but not monoamine oxidase B (MAO-B). High dose CORT also significantly increased DNA methylation of the hypothalamic GR and CRH gene promoters ($p < 0.05$). Our findings suggest that embryonic exposure to CORT programs aggressive behavior in the chicken through alterations of the HPA axis and the serotonergic system, which may involve modifications in DNA methylation.

Introduction

Phenotypic variations among individuals are determined by their genetic background and are influenced by various environmental factors operating during the vulnerable period of embryonic and neonatal development (Frazer et al., 2009). Maternal influence is one of the non-genetic factors that play a critical role in this variability (Guibert et al., 2010). Maternal stress during embryonic development affects the offspring phenotypes, physiology and behaviors (Del Giudice, 2012). In avian species, mothers can prenatally influence offspring phenotypes through the modulation of egg hormonal levels (Groothuis et al., 2005). Corticosterone (CORT) has been reported to be present in chicken eggs (Rettenbacher et al., 2009), and its concentration is influenced by several factors including the breed (Ahmed et al., 2013; Navara and Pinson, 2010) and physiological status of the hen (Saino et al., 2005).

Excess exposure of embryos and fetuses to maternal glucocorticoids (GCs) is accomplished by the placenta in mammals (Seckl, 2004) or by egg deposition in avian species (Saino et al., 2005). Embryonic exposure to maternal GCs has been found to have short- and long-term consequences. In the short term, CORT exposure influenced hatch weight (Janczak et al., 2006) and growth rate (Hayward and Wingfield, 2004). The long term consequences include the re-programming of the hypothalamic pituitary adrenal (HPA) axis which regulates stress responses and fear-related aggressive behaviors (Seckl and Meaney, 2004).

Previous studies have shown that the central serotonin (5-HT) system plays a crucial role in modulating aggression (Kravitz, 2000) and elevated serotonergic activity usually predisposes reduced aggression (Summers and Winberg, 2006). The biosynthesis of 5-HT in the brain is controlled by the rate-limiting enzyme tryptophan hydroxylase (TPH) (Grahame-Smith, 1964). TPH1 is the predominant isoform responsible for 5-HT synthesis in both central and periphery tissues (Cote et al., 2003; Gutknecht et al., 2009), while TPH2 was found only in the brain (Walther et al., 2003). Serotonergic activity is determined by the extracellular 5-HT levels that are regulated by 5-HT release, reuptake and metabolism. Activation of 5-HT1A autoreceptor (5-HTR1A) located on the presynaptic membranes inhibits the release of serotonin, while the 5-HT released to the synaptic spaces is either taken up by the serotonin transporter back to the presynaptic neurons, or inactivated by monoamine oxidase (MAO) (Popova, 2006).

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In mammals, prenatal stressors are reported for re-programming the HPA axis and the 5-HT system (Harris and Seckl, 2011; Lupien et al., 2009). Dysregulation of both systems has been associated with mental health in general and with mood disorders in particular (Belmaker and Agam, 2008). Low levels of blood 5-HT has been associated with altered physiological functions, including the HPA axis (Leonard, 2006) and aggressive behavior (Booij et al., 2010). In humans, early life stress-induced aggression is associated with disrupted HPA axis activity and reduced functioning of the 5-HT system in adulthood (Veenema, 2009). In avian species, the majority of studies investigating artificial elevations in egg yolk CORT focused on growth and behaviors (Henriksen et al., 2011). The effects of *in ovo* injections of CORT on aggressive behaviors and its association with the HPA axis and the 5-HT system remain unclear.

Epigenetic modifications are reversible and mitotically heritable alterations in genomic expression that occur independently of changes in the gene sequence (Vialou et al., 2013). Chronic stress can induce heritable changes in gene expression patterns through DNA methylation or through histone modifications in humans (Kim et al., 2009). Prenatal exposure to synthetic GCs increased global DNA methylation in many organs of newborn guinea pigs (Crudo et al., 2012). In avian species, only one study reported the effects of social isolation and restraint in early life on corticotropin releasing hormone receptor-1 and early growth response gene expression in the chicken hypothalamus (Goerlich et al., 2012). Whether early life experiences cause epigenetic modifications on the gene promoters has not been investigated in chickens.

We used a model of *in ovo* injection of CORT during incubation to test our hypotheses that aggressive behavior and plasma CORT concentration may be changed in *in ovo* CORT-treated chickens, and that these phenotypic changes may be associated with alterations in the hypothalamic expression of genes in the HPA axis and the 5-HT system. Moreover, embryonic CORT exposure may modulate the CpG methylation status on the promoters of related genes.

Materials and methods

Egg incubation and CORT injection

Two hundred and ten fertilized chicken eggs (63.6 ± 0.43 g) were selected from the eggs laid by hens one month after onset of lay in a breeding company (Wen's, Guangdong, China) and randomly divided into three groups (70 in each group). The time line of the experiment is shown in Fig. 1A. CORT (Sigma-Aldrich, USA) was dissolved in absolute alcohol, rather than the oil that affected embryonic development of chickens in our previous trials, and diluted in PBS to produce doses of 0.2 μg and 1 μg in a volume of 100 μL solution containing minimal amount of alcohol. The doses were determined according to the previous publications (Haussmann et al., 2012; Heiblum et al., 2001; Janczak et al., 2006), taking into consideration the CORT concentrations detected in the yolk (3–4 ng/g) and the albumen (0.5 ng/g) (Ahmed et al., 2013). On embryonic day 11 (E11), the eggs were removed from incubator and injected with PBS (control) and a 0.2 μg (low) or a 1 μg (high) dose of CORT under septic conditions. Eggs were injected randomly by advancing a Hamilton syringe into a hole in the middle of the long axis until the yolk membrane was penetrated (approximately 20 mm below the surface). The incubation conditions were set according to our previous publication (Su et al., 2012). The chicks were hatched inside the incubator and were left to dry completely (up to 12 h) before they were removed. Upon removal from the incubator, the chicks were placed in the brooders in groups with fresh water and food provided *ad libitum*. The growth performance was recorded from hatching to 10 weeks of age. A behavior test was performed on posthatch day 28 (D28). On D42, blood samples were collected for the plasma CORT measurements. On D105, all the chickens were killed by rapid decapitation, an ethical type of euthanasia for the chicken. Blood samples were collected for the measurement of whole blood 5-HT and platelet 5-HT uptake and hypothalami were dissected and kept at −80 °C for further analysis. For all the tests and measurements, except for growth rate, six birds per group were used. Different batches of six birds were used for different measurements in order to minimize the stress caused by different manipulations.

The experiment procedures were approved by the Animal Ethics Committee of Nanjing Agricultural University.

Behavior test

The behavior test was performed on D28 as described previously (Kitaysky et al., 2003). Briefly, 6 chickens (3 males and 3 females) from each group were placed in an experimental arena (similar in size and structure to the nests where the chickens were raised) established in a room familiar to the chickens. The room was visually and acoustically isolated from the aviary of other chickens. For visual identification, chickens were marked with different colors (red, green, blue) on different locations (head, back and tail). Neither the colors nor their locations affected the behaviors of chickens in the present study. Aggression was defined as a chick pecking, grabbing, twisting skin on the head or nape of another chick. A person who was blind to the experimental design performed all the behavioral observations and analyses.

Plasma CORT assay

The birds used for taking blood samples were trained prior to the sampling to get used to handling. Blood sample (approximately 1 mL) was taken from the jugular vein and duplicate plasma samples (2 × 50 μL) were used for CORT assay. The plasma CORT concentration was measured with a commercial enzyme immunoassay kit (500655, Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions. The calculated detection limit of the assay was 27 pg/mL and all the determinations fell within the range of detection. The intra-assay coefficient of variation was 5%. The cross-reactivity of the antibody was 11% with 11-dehydrocorticosterone, 7% with 11-deoxycorticosterone, 0.31% with progesterone, 0.17% with cortisol, 0.06% with aldosterone, 0.03% with testosterone, 0.02% with pregnenolone, 0.01% with 5α-DHT and less than 0.01% with other steroids.

Whole blood 5-HT

The whole blood 5-HT was measured according to a previous publication (Bolhuis et al., 2009). Briefly, 1 mL of blood was pipetted into 50 mL tubes; then, 2 mL of 0.9% NaCl, 1 mL of 3% ascorbic acid and 5 mL of PBS were added to the tubes, followed by the addition of 20 mL of n-butanol. The tubes were shaken for 5 min and centrifuged at 895 g for 15 min. A portion of the butanol layer (15 mL) was transferred into a fresh tube containing 2 mL of 0.1 mol/L HCl and 25 mL of cyclohexane. The tubes were shaken for 20 s and centrifuged for 4 min at 46 *g*. The butanol-cyclohexane layer was removed, and 1 mL of the acidic phase was pipetted into another tube containing 0.3 mL of 12 mol/L HCl and vortexed for 3 s. The fluorescence was determined in a fluorescence spectrophotometer at 283 nm and 540 nm. A standard curve was prepared by taking 0.1, 0.2, 0.3, 0.4 and 0.5 mL of 275.5 μmol/L serotonin hydrochloride dissolved in Krebs–Ringer-phosphate buffer, filled to a volume of 1 mL with 0.9% NaCl, and the procedure described above was followed.

Platelet 5-HT uptake

The platelet 5-HT uptake was determined on the same day of sampling. Approximately 3–4 mL of whole blood was used to prepare the platelet rich plasma (PRP) by low speed centrifugation for 1 min at 378 *g*. Within 2 h after blood collection, the PRP samples were analyzed for 5-HT uptake as described previously (Fisar et al., 2008). Briefly, the