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Fasting of 3-day-old chicks leads to changes in histone H3 methylation status

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ABSTRACT

Unfavorable nutritional conditions during early developmental periods may cause neuronal network remodeling in the hypothalamus, which influences subsequent adaptability to those same stressful conditions. Alterations in hypothalamic plasticity as a result of neuronal remodeling are achieved by variations in the repertoire of proteins expressed via gene transcriptional activation or repression, both of which are modulated by histone methylation status. This study demonstrates that fasting had a stimulatory effect on dimethylation and trimethylation of histone 3 at lysine 27 (H3K27) in preoptic/anterior hypothalamus (PO/AH) of 3-day-old chicks. The expression of enhancer of zeste 2 (EZH2), a H3K27-specific histone methyltransferase (HMT), was significantly increased by fasting in the paraventricular nucleus (PVN) and PO/AH, which is consistent with the upregulation of H3K27 dimethylation and trimethylation. Furthermore, in the PVN, corticotrophin-releasing hormone (CRH) mRNA expression was significantly inhibited, while mRNA expressions of thyrotropin-releasing hormone (TRH) and type 2 deiodinase (D2) were significantly stimulated by fasting. These findings highlight the potential role of H3K27 methylation status in early feed stress responses in chicks and may be indicative of an epigenetic mechanism for later adaptation to feed intake stress.

1. Introduction

Encountering unfavorable environmental conditions during early developmental periods by any organism, including birds, may cause the phenotype to deviate from normal ontogenetic development, which is known as developmental plasticity [1]. Exposure to either environmental or feed stressors during critical periods of development causes changes in hypothalamic neuronal networks, and can modulate stress responses. Overfeeding during critical periods of hypothalamic development leads to alterations of the methylation pattern and consequently, the regulatory "set point" of a gene promoter that is critical for body weight regulation [2]. Little is currently known about the molecular mechanisms governing this feed stress adaptation in chicks, but like those in mammals, they involve neuronal differentiation and alteration in the repertoire of protein expression [3].

Metabolism changes during early developmental periods might imply rapid changes in neuronal plasticity, which involve alterations in gene transcription. Epigenetic regulation of transcription, primarily by covalent modifications of amino acid residues in DNA-wrapped histones as well as changes in the methylation status of DNA, has been shown to be a mechanism for inducing variations in phenotype by differences in nutrition during early life [4]. Histone acetylation and phosphorylation, which correlate with active gene transcription, have been implicated in behavioral plasticity and neuroadaptations to stress [5–7]. However, the role of histone methylation in plasticity changes in neuronal networks responding to stressors is still not clarified [8]. Generally, histone methylation correlates with either transcriptional activation or repression, depending on the histone and the amino acid being methylated [9]. Several families of histone methyltransferases (HMTs) have been identified that catalyze the methylation of specific arginines or lysines in histones H3 and H4 [10]. Unlike histone acetyltransferases (HATs), HMTs are more specific and usually modify a single lysine or arginine in a single histone [11]. The specificity of HMTs makes it possible to evaluate the direct upstream factor for methylation at a specific lysine in a single histone.

In the present study, we investigated the role of epigenetic modifications in fasting stress adaptation by evaluating post-translational dimethyl and trimethyl modification of histone 3 at lysine 27 (H3K27). The expression of enhancer of zeste 2 (EZH2), a H3K27-specific HMT, was tested to support the contribution of H3K27 methylation to feed-control establishment. Furthermore, mRNA expression of stress-related factors corticotrophin-releasing hormone (CRH) and arginine vasotocin (AVT) and metabolism-regulating factor thyrotropin-releasing hormone (TRH) was also examined to bridge epigenetic modifications and fasting adaptation.

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2. Materials and methods

2.1. Bird housing

One-day-old Cobb male broiler chicks were obtained from George's Hatchery (Harrisonburg, VA, USA), and raised in battery cages in social groups. They were given continuous artificial illumination and *ad libitum* access to water and a commercial starter diet. Temperature was maintained at 32 ± 1 °C for all the battery cages. All experimental procedures were approved by the Virginia Tech Animal Care and Use Committee.

2.2. Fasting treatment and tissue sampling

At 3 days post hatch, the test chicks were randomly divided into three groups of twelve chicks each and subjected to the following treatments: fed *ad libitum* (control, fasted for 0 h), fasted for 6 h and fasted for 24 h. All chicks had free access to water throughout the experimental period. At the end of each treatment, chicks were sacrificed by decapitation. Immediately after decapitation, the whole brain was removed and placed in a cool dissection plate with the ventral surface facing up. The brain was cut mid-sagitally into two hemispheres to expose midline structures. The preoptic/anterior hypothalamus (PO/AH) was dissected out by a frontal cut along the optic chiasm and a transverse cut below the tractus septomesencephalicuson (TSM) on both sides. The two cuts were made along the presented arrow (Fig. 1A). The paraventricular nucleus (PVN) was rapidly microdissected under a magni-focuser (Edroy Products Company, Inc., Nyack, NY, USA), using the TSM and commissura anterior (CA) as landmarks. The cuts were made as shown in Fig. 1B.

The PO/AH and PVN were dissected and immediately frozen in liquid nitrogen and subsequently stored at −80 °C until mRNA and protein analysis. To confirm the discrete neuroanatomical location of epigenetic alterations as a result of feed stress induction in the PO/AH or PVN, an additional brain area, the frontal part of the brain (FB), was dissected and evaluated. Dissection, handling and storage of these tissues were similar to other tissues.

2.3. RNA isolation and real-time polymerase chain reaction (PCR)

Sample tissues were individually homogenized in Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and total RNA was extracted according to the manufacturer's instructions. The protein fractions were stored at −80 °C as phenol–ethanol supernatants awaiting subsequent analysis. Total RNA concentration was then quantified by measuring the absorbance at 260 nm. Fifty micrograms of total RNA was treated with DNase-I (Promega, Madison, WI, USA) to eliminate contaminating genomic DNA. Purified DNA-free RNA was dissolved in RNase-free water and immediately used as templates in reverse transcription. Two micrograms of total RNA was incubated at 42 °C for 1 h in a 25 μL mixture containing 200 U Moloney Murine Leukemia Virus reverse transcriptase (M-MLV) (Promega), 1 × M-MLV reaction buffer (Promega), 25 U RNase inhibitor (Promega), 4 μg Oligo dT primer (Eurofins MWG Operon, Huntsville, AL, USA) and 0.5 mM dNTP (Promega, Madison, WI, USA). The reaction was terminated by heating at 95 °C for 5 min and quickly cooling on ice. The cDNA (RT products) were aliquoted and stored at −20 °C.

Real-time RT-PCR was performed in the iCycler iQ Multi-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Two microliters of 4-fold diluted RT product was used for PCR in a final volume of 25 μL containing 12.5 μL 2× iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and 0.5 μM primers mix (Table 1). Chicken β-actin mRNA was used as a reference gene for normalization purposes. The expression of β-actin mRNA showed no marked difference between treatments. The thermal cycling conditions were: initial denaturation (10 min at 95 °C), then a two-step amplification program (15 s at 95 °C, 60 s at 60 °C) was repeated 50 times. The PCR efficiency of each sample was determined using Realtime PCR Miner (*http://www.miner.ewindup.info/*) according to the instructions of the author [13]. The average efficiencies of tested genes were all higher than 90%. The standard deviation of efficiencies from different samples was lower than 1% and the coefficient of variation of efficiencies was less than 5%. Average efficiency of all tested samples was used for analysis. The real-time RT-PCR data were analyzed by the relative quantification ($\Delta\Delta C_T$) method [14]. All mRNA expression levels were expressed as fold-change compared to the control group for each chick.

2.4. Protein isolation and western blotting

Proteins were extracted from stored phenol-ethanol supernatants according to the manufacturer's instruction (Molecular Research Center, Inc., Cincinnati, OH, USA). Protein extracts were separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The blots were blocked in 5% bovine serum albumin (BSA) dissolved in Tris-buffered saline with Tween-20 (TBST, 20 mmol Tris, pH 7.4, 150 mmole NaCl, and 0.1% Tween-20) for 3 h at 4 °C and then briefly washed two times for 5 min each in TBST. The blots were incubated overnight at 4 °C with primary antibodies against EZH2 (1:500), Tri-Methyl-Histone H3 (Lys27) (H3K27me3) (1:1000), Di-Methyl-Histone H3 (Lys9) (H3K27me2) (1:1000) and β-actin (1:1000) in TBST with 1% BSA. All antibodies were from Cell Signaling Technology (Danvers, MA, USA). The blots were washed twice for 5 min each and then three times for 10 min each. Blots were

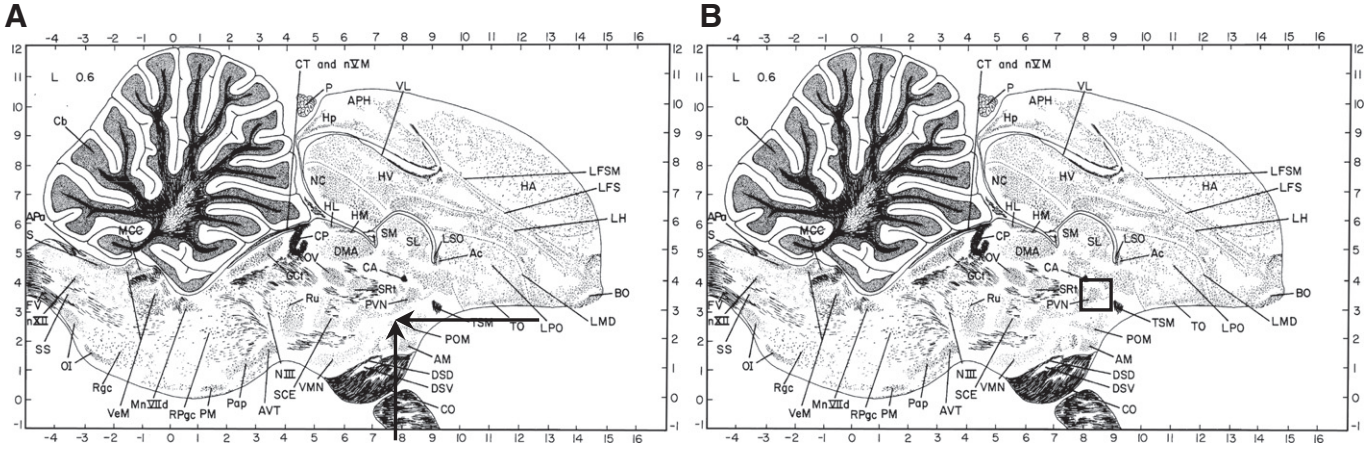

Fig. 1. Dissection guides for preoptic/anterior hypothalamus (PO/AH) (A) and paraventricular nucleus (PVN) (B) in chickens. The figure was adapted from Kuenzel and Masson [12].