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Research report

Fed and fasted chicks from lines divergently selected for low or high body weight have differential hypothalamic appetite-associated factor mRNA expression profiles

Jiaqing Yi, Elizabeth R. Gilbert, Paul B. Siegel, Mark A. Cline*

Department of Animal and Poultry Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, United States

HIGHLIGHTS

- Chickens used differ in body weight by 10 fold at selection age.
- Hypothalamic appetite-associated neurotransmitter RNAs were measured in fed and fasted.
- Differential RNA expression may explain differences in food intake and body weight.

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ABSTRACT

We have demonstrated that chicken lines which have undergone intense divergent selection for either low (LWS) or high (HWS) body weight (anorexic and obese containing, respectively) have differential food intake threshold responses to a range of intracerebroventricular injected neurotransmitters. The study reported herein was designed to measure endogenous appetite-associated factor mRNA profiles between these lines in an effort to further understand the molecular mechanisms involved in their differential eating patterns. Whole hypothalamus was collected from 5 day-old chicks that had been fasted for 180 min or had free access to food. Total RNA was isolated, reverse transcribed, and real-time PCR performed. Although mRNAs encoding orexigenic neuropeptides including agouti-related peptide, neuropeptide Y (NPY), prolactin-releasing peptide, and visfatin did not differ in expression between the lines, NPY receptor 5 mRNA was greater in fed LWS than HWS chicks, but fasting decreased the magnitude of difference. Anorexigenic factors including amylin, corticotropin releasing factor (CRF) and ghrelin were not differentially expressed between lines, while mRNA abundance of calcitonin, CRF receptor 1, leptin receptor, neuropeptide S, melanocortin receptor 3, and oxytocin were greater in LWS than HWS chicks. Pro-opiomelanocortin mRNA was lower in LWS than HWS chicks, while fasting decreased its expression in both lines. These results suggest that there are differences in gene expression of appetite-associated factors between LWS and HWS lines that might be associated with their differential food intake and thus contribute to differences in severity of anorexia, body weight, adiposity, and development of obesity.
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1. Introduction

The hypothalamus plays a crucial role in the regulation of appetite by integrating and coordinating multiple nutrient-related signals from both the peripheral and central nervous systems [1,2]. Although most aspects of food intake regulation are conserved between chicks and mammals, some differences apparently arose during divergent evolution. In rats, orexin, motilin, melanin-concentrating hormone (MCH), growth hormone releasing factor, and ghrelin are associated with increased food intake [3–6], whereas prolactin-releasing peptide (PrRP) induces satiety [7]. However, in chicks orexins, motilin, melanin-concentrating hormone and galanin are reported to not affect food intake [8,9] and other factors cause the opposite effect from rats: ghrelin and growth hormone releasing factor inhibit food intake [10], while PrRP increases food intake [11] in chicks.

Through long-term selection (over 56 generations), the Virginia low (LWS) and high (HWS) body weight lines of chickens at selection age (56 days) display a 10-fold difference in body weight with the LWS line comprised of individuals with different severities of anorexia and the HWS all obese [12,13]. The LWS line has a lower threshold to anorexigenic factors, such as

* Corresponding author. Tel.: +1 540 231 4477; fax: +1 540 231 3010.
E-mail address: macline2@vt.edu (M.A. Cline).

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α-melanocyte-stimulating hormone (α-MSH) [14], corticotropin releasing factor (CRF) [15], insulin [16], amylin [17], ghrelin [18], and neuropeptide AF [19]. Based on these results it may be concluded that the LWS line is more sensitive than HWS to anorexigenic factors; however, for others factors such as neuropeptide S [20], calcitonin and calcitonin gene-related peptide [21], the LWS line is less sensitive. For other factors including galanin [22], thresholds in the food intake response are similar between the lines. Regulation of feeding in the lines is hence complex and warrants further investigation to explore the molecular mechanisms underlying differences in feeding behavior.

Electrolytic lesions of the ventromedial hypothalamus led to the development of obesity in the LWS line, but did not affect the magnitude of obesity in the HWS line [23], suggesting that differences in hypothalamic signaling at hatch may modulate feed intake and its cascading effects that result in anorexia and obesity. Hence, in the present study we measured the gene expression profiles of appetite-associated factors in the hypothalamus between the lines during the early post-hatch period in the fed and 3-h fasted state.

2. Materials and methods

2.1. Animals and experimental design

The lines of chickens used in this experiment are from a long-term selection experiment for low or high body weight at 8 weeks of age [24] with details of the selection program reported by refs. [25,26]. The founder population consisted of crosses of 7 inbred lines with the LWS and HWS selected lines maintained as a closed population. Eggs obtained from age contemporary parents from S_{56} generation parental stocks were incubated in the same machine. After hatch, chicks were group caged for 1 d and then transferred to individual cages in a room at 32 ± 1 °C and 50 ± 5% relative humidity. All chicks had free access to a mash diet (21.5% crude protein and 3000 kcal ME/kg) and tap water. The individual cages allowed visual and auditory contact with each other. Chicks were handled twice daily to adapt to handling. All animal protocols were approved by the Institutional Animal Care and Use Committee at Virginia Tech.

Chicks, 5 days post-hatch, were divided into four groups ($n = 10$ for each group): LWS fed, LWS fasted, HWS fed and HWS fasted. The fasting duration was 180 min (to mimic our previous fasting duration) [15,17,21]. Chicks were provided access to drinking water during the fast. Each chick was deeply anesthetized with sodium pentobarbital via cardiopuncture, decapitated, and its brain removed. The whole upside-down brain was snap frozen into liquid nitrogen to the point where the most ventral aspect of the optic lobe was level with the surface of the liquid nitrogen. The brain was left in this position for 11 s. This procedure resulted in brain regions around the hypothalamus freezing and providing firmness necessary to make precise cuts for hypothalamus extraction. Cuts were made visually as per the following anatomy: perpendicular to the midline suture a cut was made at the septopallio-mesencephalic tract and at the third cranial nerves. 2.0 mm parallel to the midline two cuts were made and finally the dorsal cut was made from the anterior commissure to 1.0 mm ventral to the posterior commissure [27]. This block (comprised primarily of the hypothalamus) was immediately stored in RNAlater (Qiagen).

2.2. Reverse transcription and real-time PCR

Hypothalamus was homogenized using 5 mm stainless steel beads (Qiagen, Valencia, CA, USA), Isol Lysis reagent (5-Prime, Gaithersburg, MD, USA) and a Tissue Lyser II (Qiagen) and total RNA was extracted following the manufacturer's instructions (5-Prime). The RNeasy Mini Kit (Qiagen) and RNase-free DNase I (Qiagen) were then used for total RNA purification. The integrity of total RNA samples was evaluated by agarose-formaldehyde gel electrophoresis and concentration and purity assessed by spectrophotometry at 260/280/230 nm with a Nanophotometer Pearl (IMPLEN, Westlake Village, CA, USA).

First-strand cDNA was synthesized in 20 μl reactions from 200 ng of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's instructions. Reactions were performed under the following conditions: 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 min. Primers for real-time PCR were designed with Primer Express 3.0 software (Applied Biosystems) (Table 1) and validated for amplification efficiency before use (95–105%). Real-time PCR reactions were performed in 10 μl reactions contained 5 μl Fast SYBR Green Master Mix (Applied Biosystems), 0.5 μl primers (0.25 μl forward primer and 0.25 μl reverse primer), 1.5 μl nuclease-free water, and 3 μl 10-fold diluted cDNA using a 7500 Fast Real-Time PCR System (Applied Biosystems). The real-time PCR was performed under the following conditions: 95 °C for 20 s and 40 cycles of 90 °C for 3 s plus 60 °C for 30 s. A dissociation step consisting of 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 s was performed at the end of each PCR reaction to ensure amplicon specificity.

2.3. Data analysis

Real-time PCR data were analyzed using the ΔΔCT method with β-actin as the endogenous control and the average of the fed LWS chicks as the calibrator sample. Relative quantities calculated as $2^{-\Delta\Delta CT}$ were used for statistical analysis. The statistical model included the main effect of feeding state, sex, genetic line and their interactions. The mRNA expression of oxytocin, visfatin, leptin receptor and amylin were affected by sex while no interactions involved with sex were significant. Hence, we removed sex in our model for further analysis. Data were analyzed by analysis of variance (ANOVA) using the GLM procedure of SAS 9.3 (SAS institute, Cary, NC, USA). Means were separated using Tukey's test. Data were presented as means ± SE. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Gene expression of orexigenic neuropeptides and some associated receptors in LWS and HWS chicks

Expression profiles of orexigenic neuropeptides and associated receptors in the hypothalamus for LWS and HWS chicks in fed and fasted states are shown in Table 2. The only line by feeding state interaction that was significant was for neuropeptide Y receptor 5 (NPYR5), in which fasting decreased the magnitude of difference in expression between LWS and HWS chicks (Table 3). Neuropeptide Y (NPY), NPY receptor 1 (NPYR1), PrRP, visfatin, and agouti-related peptide (AgRP) mRNA did not differ between lines or feeding states. Visfatin mRNA was greater in males than females. Orexin receptor 2 mRNA was more highly expressed in LWS than HWS chicks and fasting significantly decreased its expression in both lines. Orexin and MCH mRNA were greater in LWS than HWS chicks, but expression did not differ between fed and fasted states. The HWS chicks had greater expression of NPY receptor 2 (NPYR2) than LWS chicks and fasting did not influence its expression. Galanin mRNA did not differ between lines, but was decreased by fasting.

3.2. Gene expression of anorexigenic neuropeptides and some associated receptors in LWS and HWS chicks

The expression profiles of anorexigenic neuropeptides and their receptors are displayed in Tables 4 and 5. Neither line nor