Hypothalamic Regulation of Food Intake in Obese and Anorexic Avian Models

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Abstract

Chickens from lines that have been divergently selected for either low (LWS) or high (HWS) body weight at 56 days of age for more than 57 generations serve as unique models to study eating disorders. The LWS have different severities of anorexia while all HWS become obese. Over the past decade our groups has demonstrated that these lines have differential food intake threshold responses to a range of intracerebroventricular (ICV) injected neurotransmitters. The major brain region regulating homeostatic regulation of appetite is the hypothalamus, and hence this dissertation was focused on understanding how the hypothalamus is different between LWS and HWS lines. Experiments 1 and 2 were performed as follows: whole hypothalamus as well as individual hypothalamic nuclei, respectively, were collected from 5 day-old chicks that had been fasted for 180 min or had free access to food. The hypothalamic nuclei included those primarily associated with appetite including the lateral hypothalamus, paraventricular nucleus (PVN), ventromedial hypothalamus, dorsomedial nucleus, and arcuate nucleus (ARC). Total RNA was isolated, reverse transcribed, and real time PCR performed. Hypothalamic expression of anorexigenic factors was greater in LWS than HWS, those factors including calcitonin, corticotropin-releasing factor receptor 1, leptin receptor, neuropeptide S, melanocortin receptor 3 (MC3R), and mesotocin. The gene expression data from individual hypothalamic nuclei revealed that mesotocin from the PVN may play an important role in the inhibition of appetite in the LWS. Experiment 3 was then designed to evaluate the effects of stress on food intake: besides the differences

in hypothalamic gene expression between the lines, they also have different feeding responses when stressed: ICV injection of neuropeptide Y (0.2 nmol, NPY) did not increase food intake in LWS on day 5 after stress exposure. Experiment 4 was thus designed to study the molecular mechanisms underlying conditional feeding responses to exogenous NPY after stress in the LWS. The melanocortin system (AgRP and MC3R) changed in the hypothalamus after stress in the LWS, and hence may be responsible for the loss of responsiveness to exogenous NPY in stressed LWS. Experiment 5 was designed to evaluate whether hypothalamic differences exist at the protein level: label-free liquid chromatography coupled to tandem-mass spectrometry was used to measure the abundance of proteins in the hypothalamus. Hypothalamus was obtained from fed and 180 minute-fasted 5 day-old male LWS and HWS chicks. Proteins involved in energy metabolism were different between the lines. Differences were also found in proteins involved in GABA synthesis and uptake as well as protein ubiquitination. In conclusion, these results suggest that different feeding behaviors of LWS and HWS may be due to differences in gene and protein expression in the hypothalamus.

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Chapter 1

Introduction and literature review

Hypothalamic control of feed intake

Obesity is the result of a disruption in energy balance, where energy intake chronically exceeds energy expenditure and leads to the accumulation of fat in the body. In recent decades, obesity has become one of the major threats to human health worldwide. Obesity not only increases the risk of developing a number of life-threatening health problems such as diabetes [1], hypertension [1, 2], gallstones [3], heart disease [4], and stroke [5, 6]; it also poses considerable economic burdens on society. For example, the total estimated cost of obesity (including medical cost and reduced productivity) was $245 billion in the United States in 2012 [7].

Conversely, anorexia, which has been shown to be associated with self-starvation from severe fear of weight gain as well as acute or chronic infections, inflammation, and trauma followed by severe weight loss [8-10], is one of the most difficult psychiatric disorders to treat, one of the reasons being that its etiology is still not well understood [11]. Animal models used to study mechanisms involved in the regulation of food intake and anorexia include the anorexic mouse (anx/anx mouse) with a phenotype characterized by reduced food intake, and the low body weight-selected line of chickens (LWS), which exert natural anorexia as a correlated response to having undergone more than 50 generation selection for body weight [12-14].

Because of the above-mentioned impacts of obesity and anorexia on society worldwide, an understanding of appetite regulation and energy homeostatic control becomes increasingly important to facilitate the development of preventative and therapeutic strategies. Indeed,

significant achievements have been made in the past century regarding the advancement of knowledge on the neuronal control of energy intake and expenditure.

Appetite regulation is a complex system including both the central nervous system and the periphery. The hypothalamus, which consists of several distinct nuclei, plays a crucial role in the regulation of appetite by integrating and coordinating multiple nutrient-related signals from both the peripheral and central nervous system [15]. Hence, a better understanding about how the hypothalamus coordinates information to control energy homeostasis is becoming critical to combat the epidemic of obesity.

The lesion experiments performed during the early part of the 20th century revealed the role of the hypothalamus in feeding control, specifically that the lateral part of the hypothalamus serves as a 'feeding center' while the ventromedial part of the hypothalamus acts as a 'satiety center [16]. Overall, research has shown that the arcuate nucleus (ARC), the lateral hypothalamic area (LHA), the paraventricular nucleus (PVN), the ventromedial nucleus (VMH), and the dorsomedial nucleus (DMN) play important roles in energy balance regulation and associated signaling pathways are partly understood. These hypothalamic nuclei are interconnected with one another to form regulatory networks that integrate signals related to energy intake and energy expenditure. From an anatomical perspective, Schwartz [17] divided the hypothalamic nuclei involved in the regulation of appetite and food intake into two distinct types, the first order neurons in the ARC, and second order neurons (PVN and LHA) which are richly projected onto by axons from ARC [18]. The connection and interaction of individual hypothalamic nuclei is more complicated than described above and more detailed information is shown below.

Arcuate nucleus

Located at the base of the hypothalamus around the third ventricle, the ARC is in direct

contact with blood-borne circulating molecules such as glucose, insulin, leptin or ghrelin due to the fact that the ARC has a weak blood-brain barrier that allows these factors to enter from the vascular system. Morphologically, the ARC extends from the posterior borders of the optic chiasm to the mammillary bodies in rats [19]. The ARC receives extensive neuronal input from other hypothalamic regions: the strongest inputs from the periventricular, paraventricular, and median preoptic nucleus; weaker inputs from orexin-expressing neurons from the LH [20].

Two distinct neuronal populations from the ARC are involved in the regulation of appetite and food intake. Neuropeptide Y (NPY) and agouti-related peptide (AgRP), which are co-localized in the ARC [21], both exert orexigenic effects, while cocaine- and amphetamine-regulated transcript (CART) and pro-opiomelanocortin (POMC), which are also co-localized in distinct but adjacent subsets of neurons within the ARC [22], encode proteins that are modified to function as anorexigenic neuropeptides. The NPY/AgRP-expressing neurons project heavily to the PVN and LH [23, 24].

The ARC is essential in the regulation of energy homeostasis. As mentioned above, the ARC contains two subsets of neurons that have opposite effects on feeding. One group of neurons co-express NPY and AgRP. Both of these neuropeptides play important roles in energy balance. The intracerebroventricular (ICV) injection of NPY or AgRP has been demonstrated to increase food intake in mammals [25, 26]. In response to fasting, NPY and AgRP mRNA expression were increased in the ARC [27]. The orexigenic effect of NPY is well conserved in chickens as reported by Kuenzel et al., (1987) [28]. However, controversy exists for whether there is an effect of AgRP on appetite regulation in chickens. Tachibana et al., (2001) reported that ICV injection of AgRP increased food intake in layer-type chicks but not broiler chicks [29].

However, ICV injection of AgRP could attenuate the anorexigenic effect of α-melanocyte stimulating hormone (α-MSH) in broiler chicks [29].

The other major group of neurons in the ARC contain POMC and CART. The POMC encodes the precursor protein that can be cleaved into melanocortins (α-MSH, β-MSH, γ-MSH, and adrenocorticotrophic hormone (ACTH)) and β-endorphin [30]. The central melanocortin system also includes the ARC NPY/AgRP expressing neurons, brainstem POMC-expressing neurons as well as as downstream targets of POMC- and AgRP-containing neurons expressing melanocortin receptors 3 and 4 (MC3R and MC4R) [30]. The central melanocortin system is one of the well-defined neuronal pathways involved in the regulation of energy homeostasis. In the central nervous system, the melanocortins are agonists of both MC3R and MC4R, while AgRP functions as an antagonist for both receptors. McMinn et al., (2000) reported that ICV injection of α-MSH decreased food intake in rats [31]. In chickens, α-MSH also functions as a strong anorexigenic neuropeptide [32].

Paraventricular nucleus

The paraventricular nucleus (PVN) consists of densely clustered heterogeneous nuclei that are located on both sides of the roof of the third ventricle [33, 34]. The PVN contains magnocellular neurosecretory cells that project to the posterior pituitary for the secretion of oxytocin and vasopressin in mammals and parvocellular neurosecretory cells secreting CRF and thyroid-releasing hormone [35].

The PVN has the ability to detect and integrate information from released from the ARC, such as NPY, AgRP, and melanocortins [36]. Administration of NPY directly into the PVN produced a dose-dependent increases in food intake [37]. Food intake was inhibited after the administration of a melanocortin agonist into the PVN through MC4R [38] [39]. However, the

administration of AgRP, which is the endogenous antagonist of MC4R [40], into the PVN, increases food intake [39]. Other peptides that have been tested for effects in the regulation of food intake after injection into the PVN include cholecystokinin (CCK), corticotropin-releasing factor (CRF), norepinephrine (NE), galanin, leptin, and orexin [41-44] [45, 46]. Furthermore, the expression of c-Fos, which is an early gene marker for neuronal activation, was augmented in the PVN neurons in response to the injection of both orexigenic and anorexigenic neuropeptides [47-49]. Thus, the PVN is a crucial site in the regulation of food intake.

Dorsomedial nucleus

Regarding the disruption of feeding, the lesioning of the DMH produces less accentuated effects than lesions of the VMH [50]. The DMH, as with other areas in the hypothalamus, can be activated by microinjection of orexigenic peptides, such as galanin and GABA [51] [52]. DMH projections can be traced to the VMH and PVN [including the parvocellular (PaPC) and magnocellular (PaMC) PVN] [50, 53, 54]. Although ARC efferents containing NPY project to DMN, NPY concentration did not increase in response to fasting in the DMN [55].

Ventromedial nucleus

Lesioning of the VMH in mammals produced rapid hyperphagia and increased body weight that lasted for a long time [56, 57]. The VMH is also linked with other hypothalamic nuclei that are involved in appetite regulation. The VMH receives projections containing NPY, β-endorphin, and CART from the ARC [58-61]. The VMH also projects to the DMH and PVN, hence supporting that lesioning of the VMH may disrupt information flow to the PVN and DMH for the release of orexigenic signals, which leads to up-regulated food intake [50, 53]. The injection of NPY, GABA, and β-endorphin directly into the VMH stimulated food intake while the injection of leptin reduced feeding [44, 51, 52, 62-64].

Lateral hypothalamic area

In rats, the LHA is a contiguous area dorsal and lateral to the VMH, spreading from the lateral preoptic area to the mesencephalic tegmentum, and serves as a passage for the medial forebrain bundle and other fibers that connect the forebrain and midbrain structures with each other and several hypothalamic sites [19]. The LHA has long been considered the "feeding center". Lesioning of the LHA produced temporary weight loss [56, 65].

Two subsets of neuronal populations have been demonstrated to localize in the LH: melanocyte-concentrating hormone (MCH)-expressing neurons and orexin-expressing neurons. Abbott et al., (2003) reported the effect of ICV injection of MCH into various hypothalamic nuclei on food intake in rats and found that MCH increased food intake when injected into the ARC, PVN, and DMN [66]. Two subtypes of orexin have been identified: orexin-A and –B [67]. Both orexin-A and –B have stimulatory effects on food intake in mammals [67]. In chickens, however, neither ICV injection of MCH nor orexin increases food intake [68, 69].

Neuropeptide Y

Neuropeptide Y (NPY), a 36 amino acid peptide that was first isolated from the pig brain as reported in 1982 [70], is one the most abundant neuropeptides in the brain [71]. Since its discovery, the functions of NPY have been intensely studied and identified, including roles in the regulation of food intake and energy expenditure, locomotion, circadian regulation, learning, and memory [72-75].

NPY is part of the NPY family, which also contains peptide YY and pancreatic polypeptide [70]. NPY exerts its effect through binding to NPY receptors, namely Y1 to Y6, which belong to the G-protein–coupled family of receptors [76].

NPY has been widely studied for its role in the regulation of appetite and energy homeostasis. In the rat brain, NPY is widely distributed and is most concentrated in the PVN and ARC in the hypothalamus [77]. The effects of NPY in the regulation of feeding behavior are thought to be regulated through Y1, Y2, Y4, and Y5 receptors in rats [78]. NPY is one of the most potent orexigenic neuropeptides. Central injection of NPY strongly induces feeding in rodents [79, 80]. The effect of NPY on food intake in chickens was first reported by Kuenzel et al. [28].

In chicken brain, NPY immunoreactive fibers can be seen in a wide range of different areas, including the lobus parolfactorius; hyperstriatum, neostriatum, paleostriatum, and archistriatum; hippocampal and parahippocampal areas; dorsolateral corticoid area; piriform cortex; two thalamic areas contiguous to the 1. rotundus; 2. dorsolateralis anterior thalami, pars lateralis, and pars magnocellularis; 3. Periventricularis hypothalami; 4. paraventricularis magnocellularis; region lateralis hypothalami; 5. infundibuli; inner zone of the median eminence; dorsal and lateral portions of the 6. opticus basalis; 7. raphes; and 8. reticularis paramedianus, but were the most abundant in the hypothalamus [81].

The chicken NPY receptors Y1 and Y5, which display 80-83% and 64-72% identity in amino acid sequence compared with their mammalian (rat) orthologues, respectively, and are highly expressed in the ARC, are involved in the regulation of food intake [82]. The NPY Y2 receptor in chickens showed 75-80% identity to the mammalian Y2 receptor and abundant expression in the hippocampus [83]. The chicken NPY Y4 receptor displays 57-60% amino acid identity with the Y4 receptor in mammals and its expression was detected in the hippocampus, brainstem and cerebellum [84]. The chicken NPY receptor Y6 mRNA is expressed in the

hypothalamus, gastrointestinal tract and adipose tissue, while the Y7 mRNA was only expressed in the adrenal [85]. The function of these two receptors still needs to be explored.

Neuroendocrine regulation of feed intake in chickens under stress

Although many aspects regarding regulation of food intake in chicks are similar to that in mammals such as rats, chicks have unique responses that are different from mammals. In rats, the orexigenic peptides that are reported to increase food intake include orexin, motilin, melanin-concentrating hormone, growth hormone releasing factor, galanin, and ghrelin [25, 86-90], while prolactin-releasing peptide (PrRP) decreases food intake [91]. However, except for NPY which showed similar orexigenic effects in chicks [28], orexins, motilin, melanin-concentrating hormone and galanin do not increase food intake in chicks [68, 69]. Moreover, ghrelin and growth hormone releasing factor exert opposite effects in chicks, whereby they inhibit food intake [92], while PrRP increases food intake [93]. Ghrelin is mainly produced in the stomach, and has also been detected in the ARC in rats and mice [87, 94, 95]. Similar to rats, chicken ghrelin mRNA is mainly expressed in the proventriculus and is absent in the gizzard, and low levels of its expression can be detected in the ARC. In rats, the orexigenic effect of ghrelin is mediated by activating orexigenic neuropeptides like NPY, AgRP and orexin [96, 97]. In addition, plasma ghrelin concentrations are increased by fasting and decreased by re-feeding [98, 99]. However, ICV injection of ghrelin activates the hypothalamo-pituitary-adrenal (HPA) axis resulting in increased plasma corticosterone, and the ghrelin-induced anorexigenic effect is attenuated by astressin, which is a CRF receptor antagonist [100].

In order to cope with stress, an intricate series of adaptive responses are initiated to coordinate various systems in the body to fight against the detrimental effects caused by stress. The HPA axis is one of the main effector systems involved in the regulation of the stress

response. Many studies have demonstrated the negative effect of stress on feed intake [101-104]. In general, it is estimated that feed intake is reduced 1.5% for every 1 °C increase in environmental temperature in laying hens [105]. Similar to chickens, beef cattle, dairy cow, and swine have also been reported to decrease their feed intake in response to stress [106-108]. Among all organ systems, the central nervous system, especially the hypothalamus, plays a pivotal role in the regulation of feed intake under stress by integrating and coordinating the actions of a variety of neuropeptides initiated by stress.

The neurohypophysial hormone arginine vasotocin (AVT) is an antidiuretic hormone in non-mammalian vertebrates, which is similar to vasopressin in mammals with only one amino acid substitution [109, 110]. In the avian brain, major areas containing AVT neurons include the supraoptic nucleus, lateral hypothalamic area, PVN, bed nucleus of the stria terminalis medial division, and nucleus accumbens [111]. Four AVT receptor subtypes have been identified in chickens, the AVT type 1 to 4 receptors (AVT1R to AVT4R, respectively) [112-114]. AVT is released during dehydration and promotes the reabsorption of water in the kidney. AVT is also involved in the response to heat stress [115, 116]. For example, plasma concentrations of AVT increased after exposure to 32 °C for 90 min [116] and 37 °C for 60 min in domestic fowl [117]. The action of AVT released from the magnocellular subdivision in the PVN binding to AVT2R in the pituitary is considered to be the major pathway of AVT involved in the stress response [111]. Both ICV and peripheral injection of AVT has been shown to decrease feed intake in chickens [115, 118, 119]. However, compared with CRF, another neuropeptide released from the parvocellular subdivision in the PVN in response to stress, AVT showed less potency in inducing corticosterone (CORT) release when administered into the CNS [118], whereas peripheral

administration of AVT was more efficacious at stimulating the release of CORT [119]. Figure 1-1 shows the anatomical action of AVT and CRF in chickens.

The CORT is one of the major steroid hormones that is secreted by the avian adrenal cortex [120]. Acute heat stress stimulates the release of CORT from adrenal glands [121] and increases plasma concentration of CORT in chickens [122, 123]. The release of CORT from the adrenal cortex is mediated by the activation of the HPA axis, in which the production of CRF from the paraventricular nucleus (PVN) in the hypothalamus stimulates the release of ACTH in the pituitary gland and then ACTH moves through the circulatory system to reach its major target tissue, the adrenal cortex, thereby leading to the production and release of CORT. Along with the activation of the HPA axis, the production of CRF, ACTH, and CORT are all also involved in appetite regulation.

The avian neuropeptide CRF, a 41-amino acid peptide, is highly conserved between birds and mammals (identical amino acid sequence) [124]. Two CRF receptor subtypes named CRF receptor 1 (CRFR1) and CRF receptor 2 (CRFR2), which are G-protein coupled receptors, have been identified in chickens [125, 126]. Compared with mammalian CRF receptors, chicken CRFR1 shows 87-88% identity in amino acids sequence to mammal CRFR1 [125] while the amino acid sequence of chicken CRFR2 exhibits 78-80% identity to mammalian CRFR2 [126]. CRF is widely distributed in the CNS. The major distribution of cell bodies that express CRF include the lateral bed nucleus of the stria terminalis, the nucleus accumbens, PVN, and lateral hypothalamic area in the hypothalamus [111]. CRF is one of the most potent anorexigenic neuropeptides that reduces food intake in both chickens and mammals [127-129]. Intracerebroventricular injection of CRF decreased feed intake and delayed pecking rhythm within 15 min post-injection in broilers [130]. Furuse et al., (1997) reported that ICV injection of

CRF activated the HPA axis and increased plasma CORT [128]. In addition to heat stress as described above, other stressors, such as immobilization [131], low temperature [132] and isolation [133] also stimulate HPA axis in chickens.

The release of CRF from parvocellular neurons in the PVN activates pro-opiomelanocortin (POMC) in the anterior pituitary ACTH-producing cells through the CRFR1 in chickens, thereby mediating the release of ACTH into peripheral blood where it reaches its target receptor in the adrenal cortex, thus regulating the release of glucocorticoid hormones [134]. ACTH is cleaved from POMC by prohormone convertase PC 1 in the corticotropic cells of the anterior pituitary lobe [135]. Central injection of ACTH (1-24) significantly decreased food intake in rats [136]. Our group has also demonstrated the effect of exogenous ACTH on feed intake in broilers [137]. ICV injection of ACTH (1 nmol) significantly reduced feed intake but did not affect water intake in neonatal chickens.

The adrenocortical response to "heat stress", which leads to a rapid elevation of circulating glucocorticoid levels, acts as a major physiological mechanism allowing chickens to successfully cope with higher environmental temperature [121]. CORT, the major stress hormone in avians, allows for the re-distribution of body energy, increases cardiovascular activity, suppresses the immune system, and inhibits a wide range of costly anabolic processes in the body including digestion, growth, and reproduction in order to partition energy to cope with stress. In order to study the physiological effects of CORT in chickens, researchers added CORT into either water or feed to reduce physical or physiological stress [138, 139]. Lin et al., (2004) demonstrated that CORT in feed was associated with elevated plasma CORT and decreased feed intake [138].

Other neuroendocrine changes caused by heat stress may be also involved in the regulation of feed intake under heat stress. For example, Song et al., reported that heat stress significantly increased the mRNA levels of ghrelin and cocaine- and amphetamine-regulated transcript (CART) in the hypothalamus of laying hens [140]. Ghrelin, which increases feed intake in mammals [141, 142], has the opposite effects on feed intake in chickens [92, 143]. CART has also been demonstrated to decrease feed intake in chickens [144]. Hence, heat stress induced decreases in feed intake in chickens may partly be related to the increase in production and release of ghrelin and CART in the hypothalamus. Thyroid hormones also play important roles in adaptation to heat stress due to actions on the regulation of metabolic rate in chickens [145-147]. In response to high environmental temperature, thyroid hormone secretion is depressed [148, 149]. Triiodothyronine (T3) has been demonstrated to increase feed intake in rats when injected peripherally or centrally [150-152]. However, no research has been conducted to examine the effect of T3 on feed intake in chickens. The suppressed secretion of thyroid hormones by high environmental temperature may be related to decrease feed intake in chickens.

Conclusions

The hypothalamus plays an important role in the regulation of energy intake and expenditure by integrating signals from both periphery and CNS. The individual hypothalamic nuclei, such as ARC, DMN, LHA, PVN, and VMH, are involved in appetite regulation. Hypothalamic neuropeptides play a major role in the regulation of feeding behavior. For example, NPY is one of the most potent orexigenic neuropeptides in both chickens and mammals. The HPA axis not only functions in animals stress response, but also play a role in appetite regulation.

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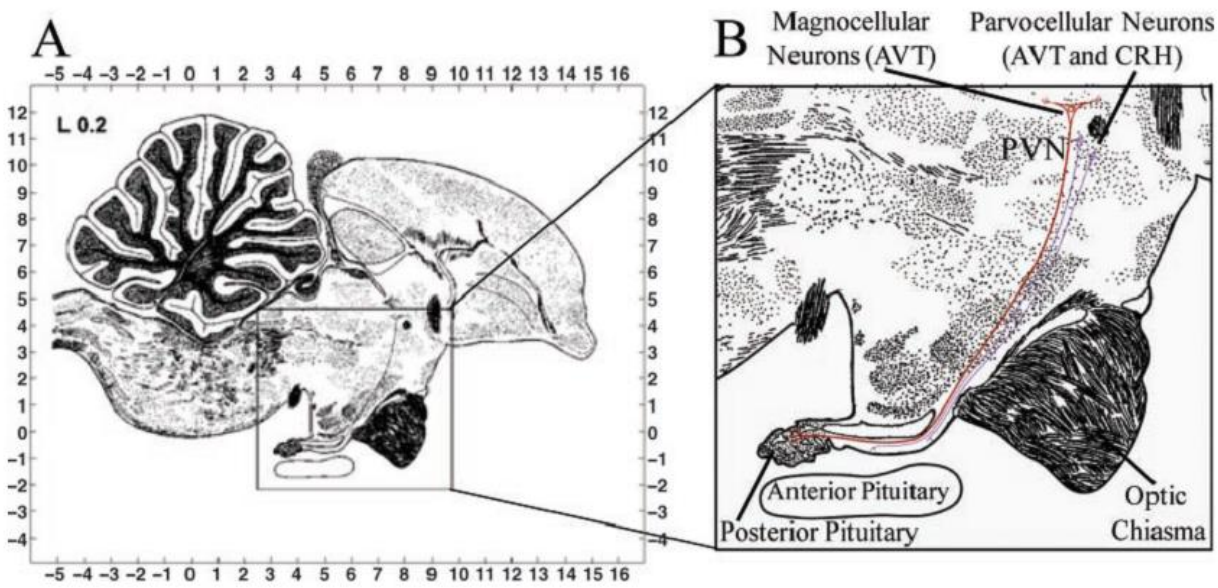

Figure 1-1. A. Sagittal view of chicken brain; B. The boxed-in area shows the diencephalic region containing neuroendocrine components of the hypothalamic-pituitary-adrenal axis. (B) Magnocellular neurons containing arginine vasotocin (AVT) project to the posterior pituitary. Parvocellular neurons containing either corticotropin-releasing hormone (CRH) or AVT project to the median eminence (ME). Neurosecretions from ME are transported to the anterior pituitary. PVN = paraventricular nucleus. From Kuenzel and Jurkevich et al., (2010)

Chapter 2

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

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 similar between fasted LWS and HWS. 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.

Keywords: anorexia, appetite, chick, hypothalamus, obesity

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 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-hour fasted state.

Materials and methods

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 [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 3,000 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 seconds. 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 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).

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) followed by 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 2-1) 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 15s was performed at the end of each PCR reaction to ensure amplicon specificity.

Data analysis

Real-time PCR data were analyzed using the ΔΔCT method with β-actin as the endogenous control and the fed LWS chicks as the calibrator sample. Relative quantities calculated as 2- ΔΔCT 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, synaptotagmin I, leptin receptor and amylin were affected by sex while no interactions involed 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 as significant as $P < 0.05$.

Results

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 2-2. 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 2-5). Neuropeptide Y (NPY), NPY receptor 1 (NPYR1), PrRP, visfatin, and agouti-related peptide (AgRP) mRNA did not differ between lines or feeding state. 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.

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 3 and 4. Neither lines nor feeding state affected mRNA abundance of melanocortin receptor 4 (MC4R), cocaine and amphetamine regulated transcript (CART), ghrelin receptor (growth hormone secretagogue receptor, GHSR) or amylin. Amylin mRNA was greater in males than females. In LWS chicks, mRNA expression was greater for calcitonin, neuropeptide S (NPS), oxytocin, CRF receptor 1 (CRFR1), leptin receptor (LEPR), and melanocortin receptor 3 (MC3R) than in HWS chicks. Oxytocin and LEPR mRNA was greater in males than females. Expression levels of mRNA for NPS and MC3R were reduced in chicks fasted for 180 min, but not affected by line. Expression of pro-opiomelanocortin (POMC) and interleukin-1β receptor (IL-1βR) mRNA were greater in HWS than LWS chicks, while fasting decreased POMC mRNA expression but had no effect on IL-1βR mRNA. Fasting increased the expression of CRF and CRF receptor 2 (CRFR2), whereas no differences were observed for abundance of CRF and

CRFR2 between the two lines. Statistically, there was a significant interaction for the expression of GHSR between line and feeding state but the Tukey's test did not detect differences among groups (Table 2-6).

DDC expression in the hypothalamus

DDC mRNA was greater in LWS chicks than HWS chicks, and was up-regulated by fasting.

Discussion

In the present study, we measured the mRNA abundance of a range of hypothalamic appetite-related factors in fed and 3-hour fasted states in 5 day post-hatch LWS and HWS lines of chickens. Two feeding states were employed as it was anticipated that fasting would enhance differential expression profiles between the lines, and for our previous work with anorexigenic factors the same 180 min fast was used to accentuate line differences. Thus, we may use the result here to start to explain the molecular mechanisms associated with differential food intake responses for both the anorexigenic and orexigenic factors that we have evaluated in these lines. At selection age (56 days post-hatch) the difference in body weight is 10 fold (about 2,000 g vs 173 g for male HWS and LWS respectively), accomplished through 56 generations of selection. The majority of appetite-associated studies in these lines have been conducted when chicks are 5 days post-hatch. This age was chosen because it is advantageous to study hypophagia in the LWS line which is comprised of individuals with different magnitudes of anorexia. About 5 to 20% of LWS chicks do not survive the first week after hatch because they never initiate feeding [30]. Therefore, the most extreme form of anorexia can only be studied prior to day 6 post-hatch. On the contrary, at hatch the HWS chicks thrive. It should be noted that at hatch the HWS line is not obese, but rather this line develops obesity later in life, by 56 days. Thus, the early post-hatch

period allows for the study of anorexia in the LWS and appetite regulation in HWS before the onset of obesity.

Although NPY mRNA did not differ between LWS and HWS chicks, HWS chicks had greater expression of NPYR2 mRNA than LWS, while LWS chicks expressed more NPYR5 than HWS in the hypothalamus. Expression of NPYR5 was greater in LWS than HWS under both feeding conditions, but the difference was accentuated in the fed state, implying that hypophagia (fasting in HWS) up-regulates its expression. Compared with our previous study, the expression of NPYR5 was consistent, whereas the NPYR2 expression difference between the lines was the opposite [31]. The difference may due to the differences in experimental design, in which the fasting time was different (3 h in the current study vs 4 h fasting in the previous study) and also animal models in the previous study were treated with insulin. Greater NPYR5 gene expression in LWS chicks is intriguing in that these birds exhibit anorexia and we reported that this line does not respond to exogenous NPY with increased food intake under all conditions [32]. The NPYR2 and NPYR5 may be involved in the regulation of feeding behavior in chickens [33]. The activation of NPYR2 by NPY (13-36) resulted in an increase in food intake only at 30 min post injection, demonstrating the weak involvement of NPYR2 in the regulation of food intake, while the activation of NPYR5 potently increased food intake in chickens [33]. The relatively higher expression of NPYR5 in LWS as compared to HWS may be a compensatory strategy in response to their relatively low appetite.

Central administration of galanin significantly increased food intake in rats [34]. However, galanin's effect on food intake is different between broiler chicks and our lines. Central injection of galanin did not stimulate food intake in neonatal chicks in one study [8], while injection increased food intake in LWS and HWS chicks with similar thresholds [22]. In the present study,

no difference was observed in galanin expression between LWS and HWS chicks further supporting that long term selection for body weight did not affect galanin's mechanism of inducing hunger.

The expression profile of orexin and MCH in the present study was different between LWS and HWS chicks, where HWS expressed more orexin while LWS expressed more MCH. However, neither of these neuropeptides affect food intake in chickens after central injection [8, 9]. Because expression was different may imply that these factors are important in regulation of food intake pathways but themselves are not capable of affecting it independently.

Some mRNAs for anorexigenic neuropeptides and associated receptors, including calcitonin, NPS, oxytocin, CRFR1, LEPR, and MC3R, were greater in LWS chicks than HWS chicks. The greater abundance of these factors in the LWS line may contribute to the differential thresholds that we have observed after exogenous anorexigenic neuropeptide injection. For example, we demonstrated that the LWS line has a latency to reduce food intake while in the HWS line anorexia was not dose-dependent following NPS injection [20]. The two fold increase in NPS mRNA in LWS compared to HWS suggests that NPS plays a role in anorexia in LWS. These data support that the differences we previously observed were due to the synergistic effect of endogenous NPS and exogenous injected NPS on food intake in LWS. Fasting significantly decreased the expression of NPS, which is expected as food deprivation attenuates anorexigenic tone in order to increase food intake, and supports that NPS is an innate regulator of food intake in chickens.

The LWS chicks expressed more MC3R than HWS chicks in hypothalamus and brainstem on day 0 and day 56, which is consistent with the present study [35]. The expression of LEPR and MC3R was also consistent with a previous study, in which LEPR and MC3R

mRNA were greater in LWS than HWS at the age of 4 days post-hatch [36]. The MC3R is closely related to the control of food intake and knockout of this receptor induces overeating and obesity in mice [37]. It is important in birds too as activation of MC3R attenuated food intake in both fasted broiler and layer chickens [38]. We reported that LWS chicks have a lower threshold for anorexic effects of central stimulation by α-MSH [14] which is likely caused by increased MC3R availability. Thus, anorexia in the LWS line may be via a melanocortin-dependant pathway.

The POMC is the precursor of several other appetite-related neuropeptides, such as anorexigenic melanocortins, most importantly α-MSH and ACTH, and the orexigenic β-endorphin. In the current study, the HWS chicks expressed more POMC mRNA than LWS chicks, which is consistent with our previous study [31]. Compared with other anorexigenic neuropeptides, POMC mRNA was not more highly expressed in LWS chicks. This may be because POMC is the precursor of both orexigenic and anorexigenic neuropeptides. However, as expected, fasting significantly reduced POMC mRNA in both lines, consistent with other studies [39].

The satiety hormone leptin was first discovered in mice [40] and its function in relation to appetite regulation has been widely studied [41, 42]. Although the chicken LEPR gene was reported [43], there is still a struggle to clone chicken leptin and its existence in the chicken genome is questioned [44]. Despite this controversy in chickens, leptin directly targets the LEPR in the hypothalamus to regulate food intake in mammals [45]. It was reported that LWS chicks decreased food intake dose dependently after central injection of leptin, whereas no response was observed in HWS [46]. In the current study, we found that LWS chicks express more LEPR than HWS chicks in the hypothalamus which may explain our past food intake response. The co-

existence of LEPR and melanocortin in POMC-secreting neurons in the hypothalamus suggests a possible interaction between leptin and α-MSH signaling in rats [47]. Activation of melanocortin receptors (MC3R or MC4R) potentiated leptin-induced STAT3 activation [48]. The higher expression of both MC3R and LEPR in LWS chicks may help potentiate the leptin-induced food decrease in LWS chicks and could possibly explain why LWS chicks are more sensitive to leptin than HWS chicks. LEPR also co-expresses with NPY expressing neurons in the ARC and the activation of LEPR inhibits the expression of NPY [49]. Although the expression of NPY did not differ between LWS and HWS chicks, the higher expression of LEPR and the higher sensitivity of LWS chicks to exogenously injected leptin may indicate the existence of an endogenous ligand for LEPR, which has not yet been identified, that plays a role in the regulation of feed intake and is associated with the anorexia of LWS.

L-aromatic amino acid decarboxylase (DDC) is responsible for the conversion of L-dopa and L-5-hydroxytryptophan to dopamine and serotonin (5-HT), respectively [28], both of which are implicated in food intake regulation [29]. LWS chicks are less sensitive to exogenous 5-HT than HWS [50], and there were differences in hypothalamic expression of genes associated with receptor subunits for 5-HT and dopamine between 4-day old LWS and HWS [36]. In the present study, DDC expression was greater in LWS than HWS and mRNA was up-regulated during the fasting state, consistent with a recent study where DDC was greater in 5-day old LWS than HWS after 4 hours of fasting [31]. These same differences were observed in 90-day old chickens and single-nucleotide polymorphisms were detected in the DDC gene that may help explain differences in expression between the lines [51]. Collectively, results suggest that the serotonergic and dopaminergic systems were affected by long-term selection for low and high body weight in LWS and HWS, respectively.

In sum, we have demonstrated that the differences in feeding behavior between LWS and HWS chicks may due to different gene expression profiles of various neuropeptides in the hypothalamus. Although most of the orexigenic neuropeptides in the hypothalamus did not differ between LWS and HWS chicks, LWS chicks expressed higher levels of anorexigenic neuropeptides, which may directly contribute to the reduced appetite in LWS chicks. In general, line differences were independent of feeding status and transcript abundance was influenced similarly in LWS and HWS by 180 min of food withdrawal. Although differences may reflect mechanisms underlying differences in feeding behavior between the lines, it is important to note that transcript abundance may not necessarily mirror protein abundance, and due to post-translational modifications and other post-transcriptional regulatory mechanisms, transcripts may not reflect abundance and activity of the bioactive peptide. Due to the heterogeneous nature of the hypothalamus, it is also possible that mRNA abundance in the whole hypothalamus dilutes or masks the differences that could be occurring in individual nuclei. Thus, further studies on the molecular mechanisms underlying appetite regulation should also explore nuclei-specific abundance and activity of the functional neuropeptides.

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Table 2-1. Primers used for real-time PCR

Gene	Accession No.	Sequences (forward/reverse)
β-actin	NM_205518.1	GTCCACCGCAAATGCTTCTAA / TGCGCATTTATGGGTTTTGTT
Oxytocin	XM_001231491.3	TGGCTCTCTCCTCAGCTTGTTAT / GGCACGGCACGCTTACC
Orexin	NM_204185.2	CCAGGAGCACGCTGAGAAG / CCCATCTCAGTAAAAGCTCTTTGC
ORXR2	NM_001024584.1	TGCGCTACCTCTGGAAGGA / GCGATCAGCGCCCATTC
MCH	NM_001195795.1	GTGGGCAGAAAGCAACTACCTT / TCAGTGTCAGCTGGAAAAGCA
MC3R	XM_004947236.1	GCCTCCCTTTACGTTCACATGT / GCTGCGATGCGCTTCAC
MC4R	NM_001031514.1	CCTCGGGAGGCTGCTATGA / GATGCCCAGAGTCACAAACACTT
AgRP	XM_004950992.1	GGTTCTTCAACGCCTTCTGCTA / TTCTTGCCACATGGGAAGGT
CART	XM_003643097.2	GCTGGAGAAGCTGAAGAGCAA / GGCACCTGCCCGAACTT
POMC	NM_001031098.1	GCCAGACCCCGCTGATG / CTTGTAGGCGCTTTTGACGAT
NPY	NM_205473.1	CATGCAGGGCACCATGAG / CAGCGACAAGGCGAAAGTC
NPYR1	NM_001031535.1	TAGCCATGTCCACCATGCA / GGGCTTGCCTGCTTTAGAGA
NPYR2	NM_001031128.1	TGCCTACACCCGCATATGG / GTTCCCTGCCCCAGGACTA
NPYR5	NM_001031130.1	GGCTGGCTTTGTGGGAAA / TTGTCTTCTGCTTGCGTTTTGT
CRF	NM_001123031.1	TCAGCACCAGAGCCATCACA / GCTCTATAAAAATAAAGAGGTGACATCAGA
CRFR1	NM_204321.1	CTGCTGTCCTTGCTGGGAAT / ATCCTCCCCCGGATTGAC
CRFR2	NM_204454.1	GGATCAAATACAACACCACAAAAAAT / GGCCCATGTCCCATTGC
PrRP	NM_001082419.1	GAGCGCTCCATGGAAATCAG / ATGCCACGGCCGGTGTAC
Amylin	NM_205397.1	GCTAGGTGCAAGCGTGGAA / GCACGCCTGCGTTAGTGA
Ghrelin	NM_001001131.1	GAAGCACTGCCTAACGAAGACA / GGATGCTGAGAAGGAGAATTCCT
GHSR	AB095994.1	TCTGCGAGCGAAGGTGATC / AGACGGCCCAGAGGATGAG
Visfatin	NM_001030728.1	CCGGTAGCTGATCCAAACAAA / CCAGCAGGTGTCCTATGCAA
NPS	XM_004942355.1	GTGGGCAGGAGCGAAGAG / CCACACCGTTGCGAAAGG
LEPR	NM_204323.1	GCAAGACCCTCTCCCTTATCTCT / TCTGTGAAAGCATCATCCTGATCT
IL-1βR	NM_205485.1	CATGCCAGAATCCATCAAATATGT / CCGAGAAGTCCCCTGTCCAT
Galanin	NM_001159678.1	CGAATTTCTGACTTACTTGCATCTTAA / AAAGGTTTGTTTCCTCTGGTGAAG
DDC	XM_004935144.1	TGGAATCCACCCACGTCAA / TCGGTCGCCAGCTGTGA
Calcitonin	NM_001271966.1	CGAGTGACGCTCAGTGATTACG / GAACTCTTTCACCAGCGCATT

Primers were designed with Primer Express 3.0 (Applied Biosystems) for orexin receptor 2 (ORXR2), melanin concentrating hormone (MCH), melanocortin receptor 3 and 4 (MC3R and MC4R), agouti-related peptide (AgRP), cocaine and amphetamine regulated transcript (CART), pro-opiomelanocortin (POMC), neuropeptide Y (NPY), neuropeptide Y receptor sub-types 1, 2, and 5 (NPYR1, NPYR2, and NPYR5), corticotropin-releasing factor (CRF), corticotropin-releasing factor receptor sub-types 1 and 2 (CRFR1 and CRFR2), prolactin releasing peptide (PrRP), growth hormone secretagogue receptor (GHSR), neuropeptide S (NPS), LEPR (leptin receptor), interleukin-1β receptor (IL-1βR), DDC (L-aromatic amino acid decarboxylase).

Table 2-2. The effect of different feeding states on hypothalamic orexigenic neuropeptide and receptor mRNA

Variables	NPY	NPYR1	NPYR2	NPYR5	PrRP
Line					
LWS	1.05 ± 0.07	0.94 ± 0.03	0.94 ± 0.03	0.95 ± 0.03	0.99 ± 0.06
HWS	0.99 ± 0.07	1.02± 0.03	1.10 ± 0.07	0.72 ± 0.03	1.05 ± 0.09
P value	0.61	0.06	0.03	<0.0001	0.58
Feeding status					
Fed	0.99 ± 0.06	1.01 ± 0.03	1.02 ± 0.02	0.85 ± 0.04	0.98 ± 0.06
Fasted	1.05 ± 0.08	0.95 ± 0.03	1.01 ± 0.08	0.83 ± 0.03	1.07 ± 0.09
P value	0.53	0.14	0.86	0.51	0.39
Two-way interaction					
P value	0.76	0.14	0.09	0.01	0.11

Table 2-2. Continued

Variables	Visfatin	AgRP	Orexin	ORXR2	MCH	Galanin
Line						
LWS	1.02 ± 0.04	1.08 ± 0.10	0.99 ± 0.05	0.92 ± 0.03	0.97 ± 0.05	0.91 ± 0.05
HWS	0.98 ± 0.04	0.98 ± 0.14	1.29 ± 0.06	0.70 ± 0.03	0.81 ± 0.04	0.83 ± 0.04
P value	0.41	0.59	0.0006	<0.0001	0.01	0.17
Feeding status						
Fed	1.00 ± 0.04	1.01 ± 0.09	1.15 ± 0.06	0.88 ± 0.04	0.95 ± 0.05	0.95 ± 0.04
Fasted	1.00 ± 0.03	1.04 ± 0.15	1.13 ± 0.07	0.74 ± 0.03	0.83 ± 0.05	0.79 ± 0.04
P value	0.97	0.89	0.83	0.0006	0.06	0.02
Two-way interaction						
P value	0.73	0.96	0.66	0.51	0.70	0.43

Relative quantity values were analyzed by ANOVA and the model included the main effects of genetic line, feeding status, and the interaction between them. Data in table are expressed as means ± standard error (n=10) for main effect of genetic line and feeding status. Also shown are corresponding P values for main effects and two-way interaction between feeding status and genetic line. Genes include neuropeptide Y (NPY), NPY receptor sub-types 1, 2, and 5 (NPYR1, NPYR2, and NPYR5), prolactin releasing peptide (PrRP), agouti-related peptide (AgRP), orexin receptor 2, and melanin concentrating hormone (MCH).

Table 2-3. The effect of different feeding states on hypothalamic anorexigenic neuropeptide and receptor mRNA

Variables	POMC	IL1BR	CRF	CRFR1	CRFR2	Amylin	DDC
Line							
LWS	0.86 ± 0.07	1.00 ± 0.05	1.18 ± 0.08	0.98 ± 0.03	1.14 ± 0.06	0.96 ± 0.05	1.21 ± 0.08
HWS	1.07 ± 0.06	1.15 ± 0.04	1.02 ± 0.07	0.85 ± 0.02	1.06 ± 0.06	0.93 ± 0.04	0.98 ± 0.04
P value	0.01	0.03	0.09	0.0005	0.31	0.56	0.01
Feeding status							
Fed	1.12 ± 0.06	1.10 ± 0.06	0.94 ± 0.04	0.93 ± 0.02	0.96 ± 0.07	1.00 ± 0.04	0.98 ± 0.06
Fasted	0.81 ± 0.06	1.05 ± 0.04	1.25 ± 0.08	0.90 ± 0.03	1.24 ± 0.04	0.89 ± 0.04	1.20 ± 0.07
P value	0.0002	0.43	0.0016	0.30	0.0007	0.053	0.009
Two-way interaction							
P value	0.77	0.79	0.86	0.61	0.16	0.98	0.17

Relative quantity values were analyzed by ANOVA and the model included the main effects of genetic line, feeding status, and the interaction between them. Data in table are expressed as means ± standard error (n=10) with corresponding *P* values for main effects and the two-way interaction between feeding status and genetic line. Genes include pro-opiomelanocortin (POMC), interleukin-1β receptor (IL-1βR), corticotropin-releasing factor (CRF), CRF receptor sub-types 1 and 2 (CRFR1 and CRFR2), and DDC (L-aromatic amino acid decarboxylase).

Table 2-4. The effect of different feeding states on hypothalamic anorexigenic neuropeptide and receptor mRNA

Variables	MC3R	MC4R	Ghrelin	GHSR	CART	Calcitonin	NPS	Oxytocin	LEPR
Line									
LWS	0.92 ± 0.03	0.97 ± 0.04	0.97 ± 0.05	0.95 ± 0.04	1.06 ± 0.07	1.07 ± 0.05	0.92 ± 0.05	0.92 ± 0.05	0.97 ± 0.04
HWS	0.69 ± 0.03	0.99 ± 0.04	0.95 ± 0.05	0.96 ± 0.03	0.97 ± 0.10	0.67 ± 0.03	0.45 ± 0.03	0.63 ± 0.03	0.83 ± 0.03
P value	<0.0001	0.74	0.82	0.83	0.44	<.0001	<.0001	<0.0001	0.007
Feeding status									
Fed	0.87 ± 0.04	1.01 ± 0.04	1.00 ± 0.05	0.96 ± 0.04	0.90 ± 0.05	0.85 ± 0.05	0.75 ± 0.07	0.82 ± 0.06	0.92 ± 0.05
Fasted	0.74 ± 0.03	0.95 ± 0.04	0.93 ± 0.05	0.94 ± 0.03	1.12 ± 0.10	0.90 ± 0.07	0.62 ± 0.05	0.73 ± 0.04	0.88 ± 0.03
P value	0.004	0.32	0.36	0.67	0.052	0.42	0.01	0.13	0.52
Two-way interaction									
P value	0.41	0.86	0.61	0.02	0.20	0.47	0.16	0.09	0.19

Relative quantity values were analyzed by ANOVA and the model included the main effects of genetic line, feeding status, and the interaction between them. Data in table are expressed as means ± standard error (n=10) with corresponding P values for main effects and the two-way interaction between feeding status and genetic line. Genes include melanocortin receptor 3 and 4 (MC3R and MC4R), growth hormone secretagogue receptor (GHSR), cocaine and amphetamine regulated transcript (CART), neuropeptide S (NPS), and leptin receptor (LEPR).

Table 2-5. The interaction between genetic line and feeding status on the mRNA abundance of NPYR5

	Fed	Fasted
LWS	1.00 ± 0.04^{a}	0.89 ± 0.03^{a}
HWS	0.69 ± 0.03^{b}	0.75 ± 0.04^{b}

Relative quantity of hypothalamic neuropeptide Y receptor 5 (NPYR5) mRNA in fed or 3-hour fasted chicks from lines divergently selected for low (LWS) or high (HWS) body weight. There was an interaction between feeding status and genetic line ($P = 0.01$). Values represent means ± standard error (n=10). Values with different superscripts are different from each other ($P < 0.05$; Tukey's test).

Table 2-6. The interaction between genetic line and feeding status on the expression of GHSR mRNA

	Fed	Fasted
LWS	1.01 ± 0.05	0.88 ± 0.04
HWS	0.91 ± 0.05	1.00 ± 0.04

Relative quantity of hypothalamic growth hormone secretagogue receptor (GHSR) mRNA in fed or 3-hour fasted chicks from lines divergently selected for low (LWS) or high (HWS) body weight. There was an interaction between feeding status and genetic line ($P = 0.02$). Values represent means ± standard error (n=10). No differences were observed among groups ($P > 0.05$; Tukey's test).

Chapter 3

Differential expression of appetite-associated factor mRNA in hypothalamic nuclei avian models of anorexia and obesity

Abstract: Chickens from lines that have been divergently selected for either low (LWS) or high (HWS) body weight at 56 days of age for more than 57 generations serve as unique models to study eating disorders. The LWS have different severities of anorexia while all HWS become obese. We reported that these lines differ in mRNA expression profiles of appetite-associated factors in the whole hypothalamus. Here we measured gene expression of these factors in distinct appetite-associated nuclei in the hypothalamus of LWS and HWS. Individual appetite-related nuclei, which include the lateral hypothalamus, paraventricular nucleus (PVN), ventromedial hypothalamus, dorsomedial nucleus, and arcuate nucleus (ARC), were collected from chicks that had been either fasted or provided continuous access to food. Several appetite-associated mRNAs were differentially expressed between the lines and among hypothalamic nuclei. Fasting increased NPY expression in the ARC of LWS but not HWS chicks. The expression of agouti-related peptide and NPY in the ARC was only greater in LWS than HWS chicks during fasting. There was more neuropeptide Y receptor 5 mRNA in the PVN of fed LWS than HWS, and fasting decreased its expression in the PVN of LWS but not HWS chicks. The mRNA quantities of corticotropin -releasing factor receptor 2 and mesotocin were greater in fasted LWS chicks than HWS chicks in all nuclei. The LWS chicks also expressed more melanocortin receptor 3 mRNA than HWS in all nuclei in which it was detected in the fasted but not fed state. These results suggest that different feeding behaviors of LWS and HWS chicks may be due to differences in gene expression of appetite-associated factors in specific appetite-related nuclei. That fasting accentuated these differences suggests that they are appetite regulation-related. This also serves as the first comprehensive screening of appetite associated factor mRNA across distinct hypothalamic nuclei in an avian species.

Keywords: anorexia, appetite, chicks, hypothalamic nuclei, obesity

Introduction

The hypothalamus, which consists of several distinct nuclei, plays a crucial role in the regulation of appetite by integrating and coordinating multiple nutrient-related signals from both peripheral and central nervous systems [1, 2]. The lateral hypothalamus (LH), paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), dorsomedial nucleus (DMN), and arcuate

nucleus (ARC) are critically involved in appetite regulation [3-6]. Two subsets of neuronal populations that have opposite effects on feeding behavior are localized in the ARC. They include orexigenic neuropeptide Y (NPY)/agouti-related peptide (AgRP) expressing neurons and anorexigenic pro-opiomelanocortin (POMC)/cocaine and amphetamine regulated transcript (CART) expressing neurons [7, 8]. The hypothalamic melonocortin system in the ARC is perhaps the best-characterized neuronal pathway involved in the regulation of energy intake and expenditure [9]. The melanocortins (α-, β- , and γ-melanocyte stimulating hormones) and AgRP are agonists and an antagonist of melanocortin receptors 3 and 4 (MC3R and MC4R), respectively [9]. The PVN contains magnocellular neurosecretory cells that express oxytocin and vasopressin in mammals, and parvocellular neurosecretory cells that secrete corticotropin -releasing factor (CRF) and thyroid-releasing hormone [2]. Corticotropin -releasing factor is one of the most potent anorexigenic neuropeptides in chickens [10]. In the LH, two subsets of neuronal populations have been identified: melanocyte-concentrating hormone (MCH)-expressing neurons and orexin-expressing neurons [11, 12]. Abbott et al. (2003) reported that intracerebroventricular injection of MCH into the ARC, PVN, or DMN increased food intake [13]. Two subtypes of orexin have been identified, orexin-A and -B [11], and while both have a stimulatory effect on food intake in rats [11], neither ICV injection of MCH nor orexin increases food intake in chickens [14, 15].

Through long-term selection (over 56 consecutive generations), the Virginia low (LWS) and high (HWS) body weight lines of chickens at selection age (56 days) display a more than 10-fold difference in body weight with LWS comprised of individuals with different severities of anorexia and HWS all becoming obese [16, 17]. During the past decade, we have reported differences between LWS and HWS in their appetite-associated responses (including hypothalamic nuclei activation) to centrally-injected neuropeptides. For example, LWS has a lower threshold in the food intake response to anorexigenic factors, such as α- melanocyte-stimulating hormone (MSH) [18], corticotropin -releasing factor (CRF) [19], insulin [20], amylin [21], ghrelin [22], and neuropeptide AF [23] compared to HWS. In contrast, HWS have a lower threshold response to the injection of neuropeptide S (NPS) [24] and calcitonin and calcitonin gene-related peptide [25] than LWS, while for both ghrelin [22] and galanin [26] thresholds of sensitivity are similar in the lines. For two factors there is a complete loss of response in one line: LWS does not respond to neuropeptide Y (NPY) with increased food intake after the

combination of cold exposure and food deprivation [27] and the HWS does not decrease food intake in response to leptin [28].

In order to elucidate the molecular mechanisms underlying differences in appetite regulation between LWS and HWS chicks, we measured the hypothalamic mRNA abundance of a range of appetite-related factors during the fed and fasted states [29]. Even though the amounts of most of the orexigenic factors were not different between LWS and HWS chicks, LWS chicks expressed greater quantities of mRNA encoding anorexigenic neuropeptides and associated receptors, such as CRF receptor 1, calcitonin, leptin receptor, melanocortin receptor 3 (MC3R), NPS, and mesotocin [29]. Further knowledge on the neurocircuitry in the hypothalamus related to feeding behavior can be acquired by studying gene expression in individual nuclei, including those described above. Thus, in the present study the mRNA expression profiles of appetite-associated factors were measured in individual nuclei, including the ARC, DMN, LH, PVN, and VMH, to further understand how appetite is regulated in LWS and HWS chicks.

Materials and methods

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 56 days of age [30-32]. The founder population consisted of crosses of 7 inbred lines, with the LWS and HWS selected lines maintained as closed populations. Eggs obtained from age contemporary parents from S56 generation parental lines 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 3,000 kcal ME/kg) and tap water [33]. 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 randomly divided into four groups (n = 20 for each group): LWS fed, LWS fasted, HWS fed and HWS fasted. The fasting duration was 180 min in order to mimic our previous fasting durations [19, 21, 25]. Chicks had access to drinking water during the fast. Each chick was deeply anesthetized with sodium pentobarbital via cardiopuncture and then perfused via the carotid artery with 2.5 mL of RNA stabilizing buffer (16.7 mM sodium citrate, 13.3 mM EDTA, and 3.5 M ammonium sulfate; pH=5.2). Within 30

min of perfusion, brains were sectioned in a cryostat at -10 °C into 500 µm thick coronal sections that were collected in the direction from rostral to caudal: LH, PVN, and VMH were collected at plate 8.0, 7.4, and 6.8 respectively, and DMN and ARC were collected at plate 5.4 based on anatomy described by Kuenzel and Masson, 1988 [34]. Punches were collected on a metal block housed on dry ice, using sterile disposable biopsy instruments (1 mm, Braintree Scientific Inc., Braintree, MA). The punches were immediately submerged in RNA lysis buffer with 1% beta-mercaptoethanol (Norgen Biotek), vortexed, snap-frozen in liquid nitrogen, and stored at -80 °C. The remaining brain section was photographed and punch accuracy verified via the overlays containing the respective nuclei boundaries.

Total RNA isolation, reverse transcription, and real-time PCR

The punches were vortexed vigorously for 30 s and incubated at room temperature for 5 min before adding 70% molecular biology-grade ethanol, and total RNA was isolated using the Total RNA Purification Micro Kit and Rnase-Free DNase I kit (Norgen Biotek), following the manufacturer's instructions. The concentration and purity of total RNA were assessed by spectrophotometry at 260/280/230 nm with a Nanophotometer Pearl (IMPLEN, Westlake Village, CA, USA). RNA integrity was verified using Experion RNA StdSens Chips (Bio-Rad).

First-strand cDNA was synthesized in 20 µl reactions from 100 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. A test was conducted to confirm that the genes we selected to measure were expressed in distinct nuclei. Five pooled RNA samples from each nucleus were used for reverse transcription in reactions with or without (negative control) reverse transcriptase. 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 of 5 µM forward primer and 0.25 µl of 5 µM reverse primer), 1.5 µl nuclease-free water, and 3 µl 5-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 15s was performed at the end of each PCR reaction to ensure amplicon specificity.

Where CT values were greater than 35 and did not differ between cDNA generated from standard and negative control reverse transcription reactions, genes were described as being negligibly expressed.

Data analysis

Real-time PCR data were analyzed using the ΔΔCT method [35] with β-actin as the endogenous control and the average of the fed LWS chicks as the calibrator sample. Relative quantities calculated as 2- ΔΔCT were used for statistical analysis. The statistical model included the main effect of genetic line, hypothalamic nuclei, feeding state, and their interactions. Since feeding only affected the expression of leptin receptor, ghrelin, neuropeptide Y (NPY), and NPY receptors 1, 2, and 5, feeding state was included in the analysis of only these genes while it was removed from the model for all other genes and secondary ANOVAs were performed for those genes within feeding state. Data were analyzed by analysis of variance (ANOVA) using JMP 11 pro (SAS institute, Cary, NC, USA). Means were separated using Tukey's test when the interaction was significant. Differences were considered significant at $P < 0.05$. Data are presented as means ± SE.

Results

Because of the limited amount of template cDNA available from each total RNA isolation, not all target genes were evaluated in all nuclei. Genes that we selected to measure were chosen based on a report of the site-specific expression of hypothalamic neuropeptides and their receptors in different nuclei in mammals [36], and based on our previous hypothalamic gene expression studies with the body weight chicken lines [29]. Gene expression data are summarized in Tables 2, 3, and 4. Within each gene and nuclei, "NM" indicates that the gene was not measured in that particular nuclei while "NE" indicates that the gene was negligibly expressed based on the criteria described in the methods.

The expression of orexigenic neuropeptides and related receptors mRNA in hypothalamic nuclei

Significant interactions among genetic line, hypothalamic nuclei, and feeding were observed for the expression of NPY, NPYR1, and NPYR5 (Table 2). Fasting increased the expression of NPY in the ARC of LWS but not HWS chicks (Figure 1A). In the fasted state, LWS chicks expressed more NPY mRNA than HWS in the ARC. There was more NPYR5 in the PVN of LWS than HWS chicks, but only in the fed state (Figure 1C). Fasting decreased the

expression of NPYR5 in the PVN of LWS, but not HWS. In fasted LWS, NPY mRNA was greater in the ARC than other nuclei (Figure 1A). The mRNA expression of agouti-related peptide (AgRP) was greater in LWS than HWS in the fasted state while no difference was detected in the fed state (Table 3). Orexin mRNA was more abundant in LWS than HWS (across all nuclei) in both the fed and fasted state and its appetite-associated receptor orexin receptor 2 was greater in LWS than HWS, but only in the fed state (Table 3).

The expression of anorexigenic neuropeptides and related receptors mRNA in hypothalamic nuclei

The expression of LEPR was greater in HWS than LWS (Table 2). There was an interaction between hypothalamic nuclei and feeding state on the expression of ghrelin and LEPR (Figure 2F and 2G). Fasting decreased the expression of ghrelin in the PVN but not ARC while the expression of LEPR was decreased in the ARC in response to fasting. The expression of CART in the PVN was greater in HWS than LWS during both the fed and fasted states (Table 3). In HWS, the PVN expressed more CART mRNA than other nuclei in both the fed and fasted states (Figure 2A). The expression of c-Fos was greater in LWS than HWS in the LH in the fed state, and was greater in the LH than other nuclei in fed LWS (Figure 2B). When fasted however, c-Fos expression was similar among nuclei and between lines. Expression of CRF was greater in the PVN than DMN or LH in both the fed and fasted states, but in the former, the nuclei distribution was line-dependent, namely expression did not differ among nuclei in LWS, whereas in HWS expression of CRF was greater in the PVN than either LH or DMN (Figure 2C). The expression of CRFR1 was greater in HWS than LWS chicks in the ARC in the fasted state (Figure 2D). In contrast, expression of CRFR2 was greater in the LWS than HWS in all nuclei under both feeding conditions. In fed chicks, expression of DDC was greater in the DMN than all other nuclei in LWS and greater in DMN and ARC compared to other nuclei in HWS (Figure 2E). When fasted, MC4R mRNA was greater in HWS than LWS chicks in the PVN (Figure 2H), while mesotocin mRNA was two-fold greater in LWS than HWS in the PVN (Figure 2I). The expression of POMC was greater in the ARC of HWS than LWS in both the fed and fasted states. We also measured the expression of leptin, which was recently identified in chickens [37]. We designed the leptin primers based on the cDNA sequence published by Serouussi et al. (2015) [37]. However, the test study showed that CT values were relatively high, with no difference in CT between cDNAs from standard and negative control reverse transcription reactions in all

hypothalamic nuclei. Hence we concluded that leptin was negligibly expressed in the nuclei that we collected.

Discussion

There are differences in gene expression of appetite-associated factors in the whole hypothalamus of fed and fasted LWS and HWS [29]. Here, the focus was on specific hypothalamic regions that are directly associated with appetite regulation, with a short-term fasted imposed in order to accentuate differences that are likely to be appetite-related. We used a punch biopsy technique in concert with real time PCR to measure gene expression in distinct hypothalamic nuclei. This technique have advantages over in situ hybridization and immunohistochemistry in that it can be used systematically to demonstrate the distribution of various neuropeptides simultaneously thus reducing the effects of replication as there are many factors which influence appetite, and is also more quantitative than histological methods [38]. Here we collected five distinct appetite-associated hypothalamic nuclei, which enabled us to quantify many appetite-related factors simultaneously and determine the relative abundance in different nuclei and between the lines. Concomitantly, mRNA was measured in individual nuclei from individual chicks using Kuenzel's chicken brain atlas as a guide [34]. Although this placed a constraint on the number of genes that could be measured because of the RNA yield from each nuclei, it provided the strength of using chick as the experimental unit. As complete pedigrees are available on individuals that can be traced back to the original founder population, the effects of genetic background can be more clearly delineated.

Neuropeptide Y is one of the most potent orexigenic factors in both mammals and chickens [39, 40]. We have consistently observed differences in hypothalamic expression of NPY between the lines at different ages and under different physiological conditions [27, 41]. In the current study, there was greater NPY expression in the ARC of LWS than HWS during fasting. NPY mRNA was greater in the ARC than LH and PVN in fasted LWS chicks. Fasting has been reported to increase NPY mRNA expression in the hypothalamus [8], and the ARC is the primary site of NPY synthesis in the hypothalamus. In the LWS chicks, fasting increased NPY mRNA in the ARC and the ARC was the region where NPY mRNA was mostly expressed. Using in situ hybridization to detect NPY mRNA distribution in chickens, Wang et al. (2001) reported that the ARC contained many intensely labeled neurons while the PVN and VMH were moderately labeled [42]. Therefore the relative abundance of NPY in different nuclei reported herein is

consistent with Wang et al. (2001) [42]. That the fasted anorexic LWS expressed more NPY than fasted HWS in the ARC and that their response to exogenous NPY is conditional supports the thesis that while NPY signaling is present in LWS its effects may be overridden by other factors under certain conditions [27, 43]. The increased expression of NPY mRNA may serve as a mechanism to attempt to compensate for the reduced energy balance in LWS. Moreover, greater expression of NPY mRNA in LWS does not necessarily reflect greater amounts of the biologically active peptide and it is possible that there is decreased translation or differences in processing of the bioactive peptide; to pursue these hypotheses was beyond the scope of current study.

Factor associated with reducing food intake were also differentially expressed in this study. The expression of CRFR2 and mesotocin were greater in LWS than HWS chicks in the fasted state. Although CRF mRNA was not different between lines, its nuclei distribution was consistent with that reported in the literature, with enrichment of the transcript in the PVN [44]. Urocortin III, which is a member of the CRF peptide family and has a particular specificity for CRFR2, was also not different between LWS and HWS. Ogino et al. (2014) reported that ICV injection of UCN III decreased food intake in chickens [45]. Although no difference was observed in the expression of UCN III between LWS and HWS, the greater expression of CRFR2 in fasted LWS may be associated with their reduced food intake.

Mesotocin is a neurohypophysis hormone in chickens and is the orthologous gene to oxytocin in mammals. Mesotocin differs from oxytocin by a single amino acid. The hypothalamic expression of mesotocin is predominantly localized in the parvocellular and magnocellular subgroups of the PVN in chicken [46]. Mesotocin acts through the oxytocin receptor and central injection of mesotocin inhibited appetite in chickens [47], which is consistent with effects of oxytocin on food intake in mammals [48]. That the expression of mesotocin was approximately 2-fold greater in LWS than HWS in the fasted state is consistent with our previous studies on whole hypothalamus [29]. Mesotocin was selected as a target gene in this study because our previous research identified mesotocin (referred to as oxytocin in the previous studies) from RNA sequencing of the hypothalamus as one of the more differentially expressed genes between 90 day-old fasted LWS and HWS. In mammals, the primary site of transcript expression in the hypothalamus is the PVN, which is consistent with our observations where amplification in PVN samples was of a magnitude greater than actin (CT < 20 cycles).

There was a two-way interaction of genetic line and hypothalamic nuclei on c-Fos expression in the fed state, in which expression was greater in the LH of LWS than HWS. Expression of c-Fos is a commonly used marker for neuronal activity [49] and the greater expression of c-Fos mRNA in LWS suggest a hyperactive LH in this line. The LH is regarded as the classical 'hunger center' as lesioning of the LH led animals to starve to death in mammals[50]. Electrical stimulation of the LH increased food intake in cats [51]. Two neuronal appetite-associated populations are known to be localized in the LH: MCH-expressing neurons and orexin-expressing neurons and thay both increase food intake in mammals [52, 53]. However, neither have been reported to increase food intake in chickens [14, 15]. There might be other neuronal populations within the LH that interact with other hypothalamic nuclei to regulate appetite in chickens, and there are likely other genes that we did not measure in this study that encode factors associated with appetite-related pathways that involve the LH.

Food intake has been well known to be regulated by the autonomic nervous system (ANS) [54]. The lesioning of the VMH increased food intake and decreased sympathetic nervous system tone while LH lesioning led to a decrease in food intake and increase in sympathetic activity [55]. Yasuda et al. (2005) reported that injection of orexin B into the third ventricle of rats increased brown adipose tissue sympathetic nerve activity [56], which demonstrated that orexin plays a role in the sympathetic outflow to regulate energy balance. Zhang et al. (2014) reported that the LWS chicks had increased fatty acid oxidation efficiency in abdominal fat and increased rates of oxidation in abdominal fat compared with HWS chicks [57]. In the current study, LWS chicks had greater expression of c-Fos and orexin in the LH in the fed state, which demonstrates that they have greater orexin neuronal activity which may explain the higher efficiency of fatty acid oxidation. Using double-virus transneuronal labeling, Jansen et al. (1995) reported that part of oxytocin immunoreactive neurons (less than 10% of double infected neurons) in the PVN innervate the sympathetic nervous system [58]. As mentioned above, the expression of mesotocin was greater in fasted LWS than HWS. The increased expression of mesotocin in the PVN may thus lead to an increase in the activity of the sympathetic nervous system which could partially explain the low food intake in LWS. Kuo et al. (2000) reported that LWS have greater sympathetic tone [59], which supports our current findings.

In conclusion, the results show that gene expression of appetite-associated factors differ between LWS and HWS in hypothalamic nuclei. Moreover these differences were influenced by

the feeding state, with 3 hours of fasting accentuating differences between the lines. There were also differences in nuclei distribution of the measured factors. These differences were consistent with the mammalian literature and also provide new information on the relative abundance of various factors in different hypothalamic nuclei in an avian species. Genes differentially expressed between the lines were involved in several appetite-associated neural pathways, such as NPY (NPY and NPYR5) and AgRP (AgRP, MC3R, and MC4R). The greater expression of CRFR2, MC3R, and mesotocin may contribute to the hypophagia and anorexia of LWS chicks. These findings may thus enrich our understanding of eating disorders in higher organisms.

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Table 3-1. Primers used for real-time PCR.

Gene	Accession No.	Sequences (forward/reverse)
β-actin	NM_205518.1	GTCCACCGCAAATGCTTCTAA / TGCGCATTTATGGGTTTTGTT
AgRP	XM_004950992.1	GGTTCTTCAACGCCTTCTGCTA / TTCTTGCCACATGGGAAGGT
CART	XM_003643097.2	GCTGGAGAAGCTGAAGAGCAA / GGCACCTGCCCGAACTT
c-fos	NM_205508.1	TGTTCCTGGCAATATCGTGTTC / CTTTCCCCCCCACGTAAGA
CRF	NM_001123031.1	TCAGCACCAGAGCCATCACA / GCTCTATAAAAATAAAGAGGTGACATCAGA
CRFR1	NM_204321.1	CTGCTGTCCTTGCTGGGAAT / ATCCTCCCCCGGATTGAC
CRFR2	NM_204454.1	GGATCAAATACAACACCACAAAAAAT / GGCCCATGTCCCATTGC
DDC	XM_004935144.1	TGGAATCCACCCACGTCAA / TCGGTCGCCAGCTGTGA
Galanin	NM_001159678.1	CGAATTTCTGACTTACTTGCATCTTAA / AAAGGTTTGTTTCCTCTGGTGAAG
Ghrelin	NM_001001131.1	GAAGCACTGCCTAACGAAGACA / GGATGCTGAGAAGGAGAATTCCT
GHSR	AB095994.1	TCTGCGAGCGAAGGTGATC / AGACGGCCCAGAGGATGAG
LEPR	NM_204323.1	GCAAGACCCTCTCCCTTATCTCT / TCTGTGAAAGCATCATCCTGATCT
MCH	NM_001195795.1	GTGGGCAGAAAGCAACTACCTT / TCAGTGTCAGCTGGAAAAGCA
MC3R	XM_004947236.1	GCCTCCCTTTACGTTCACATGT / GCTGCGATGCGCTTCAC
MC4R	NM_001031514.1	CCTCGGGAGGCTGCTATGA / GATGCCCAGAGTCACAAACACTT
NPY	NM_205473.1	CATGCAGGGCACCATGAG / CAGCGACAAGGCGAAAGTC
NPYR1	NM_001031535.1	TAGCCATGTCCACCATGCA / GGGCTTGCCTGCTTTAGAGA
NPYR2	NM_001031128.1	TGCCTACACCCGCATATGG / GTTCCCTGCCCCAGGACTA
NPYR5	NM_001031130.1	GGCTGGCTTTGTGGGAAA / TTGTCTTCTGCTTGCGTTTTGT
Orexin	NM_204185.2	CCAGGAGCACGCTGAGAAG / CCCATCTCAGTAAAAGCTCTTTGC
ORXR2	NM_001024584.1	TGCGCTACCTCTGGAAGGA / GCGATCAGCGCCCATTC
mesotocin	XM_001231491.3	TGGCTCTCTCCTCAGCTTGTTAT / GGCACGGCACGCTTACC
OXTR	NM_001031569.1	TGTGCTGGACGCCCTTCT / TCCTGCGGAGCGTTGGT
POMC	NM_001031098.1	GCCAGACCCCGCTGATG / CTTGTAGGCGCTTTTGACGAT

Primers were designed with Primer Express 3.0 (Applied Biosystems). Abbreviations: agouti-related peptide (AgRP), arginine vasotocin (AVT), cocaine and amphetamine regulated transcript (CART), corticotropin-releasing factor (CRF), corticotropin-releasing factor receptor sub-types 1 and 2 (CRFR1 and CRFR2, respectively), dopamine decarboxylase (DDC), growth hormone secretagogue receptor (GHSR), leptin receptor (LEPR), melanin concentrating hormone (MCH), melanocortin receptors 3 and 4 (MC3R and MC4R, respectively), neuropeptide Y (NPY), neuropeptide Y receptor sub-types 1, 2, and 5 (NPYR1, NPYR2, and NPYR5, respectively), orexin receptor 2 (ORXR2), oxytocin receptor (OXTR), pro-opiomelanocortin (POMC).

Table 3-2. The expression of orexigenic neuropeptides and receptors mRNA in hypothalamic nuclei

	Variables	AgRP	Galanin	MCH	NPY	NPYR1
Fed	Line					
	LWS	1.55 ± 0.50	1.15 ± 0.13	1.19 ± 0.25	1.25 ± 0.18	0.88 ± 0.07
	HWS	0.95 ± 0.21	0.94 ± 0.09	1.30 ± 0.21	1.51 ± 0.20	0.91 ± 0.08
	P value	0.21	0.17	0.74	0.24	0.33
	Distribution					
	ARC	1.18 ± 0.23	1.12 ± 0.12	ND	3.75 ± 0.24^{c}	ND
	DMN	ND	ND	ND	0.94 ± 0.12^{ab}	1.04 ± 0.10^{b}
	LH	ND	ND	1.25 ± 0.16	ND	0.16 ± 0.01^{a}
	PVN	ND	0.94 ± 0.10	ND	0.95 ± 0.13^{b}	1.21 ± 0.07^{b}
	VMH	ND	ND	ND	0.45 ± 0.04^{a}	1.00 ± 0.05^{b}
	P value		0.28		<0.0001	<0.0001
	Interaction					
	P Value		0.28		0.77	0.15
Fasted	Line					
	LWS	1.62 ± 0.42	0.85 ± 0.11	1.15 ± 0.18	3.96 ± 0.99	0.74 ± 0.07
	HWS	0.65 ± 0.13	0.93 ± 0.12	0.95 ± 0.14	1.91 ± 0.35	0.85 ± 0.09
	P value	0.04	0.86	0.39	0.002	0.02
	Distribution					
	ARC	1.14 ± 0.23	1.09 ± 0.12^{b}	ND	8.64 ± 1.40^{b}	ND
	DMN	ND	ND	ND	0.82 ± 0.13^{a}	1.05 ± 0.12^{b}
	LH	ND	ND	1.04 ± 0.11	ND	0.21 ± 0.02^{a}
	PVN	ND	0.67 ± 0.08^{a}	ND	0.77 ± 0.14^{a}	1.19 ± 0.07^{b}
	VMH	ND	ND	ND	0.55 ± 0.08^{a}	1.08 ± 0.10^{b}
	P value		0.01		<0.0001	<0.0001
	Interaction					
	P value		0.33		<0.0001	0.007

Relative quantity values were analyzed by ANOVA within each feeding state and the model included the main effects of genetic line, hypothalamic nuclei, and the interaction between them. Data in table are expressed as means ± standard error with corresponding P values for main effects and the two-way interaction between genetic line and hypothalamic nuclei. Genes include agouti-related peptide (AgRP), melanin-concentrating hormone (MCH), neuropeptide Y and its receptors 1, 2, and 5 (NPY and NPYR1, 2, and 5), orexin receptor 2 (ORXR2). ND indicates that the respective mRNA not determined in those nuclei.

Table 3-2. Continued.

	Variables	NPYR2	NPYR5	Orexin	ORXR2
Fed	Line				
	LWS	1.53 ± 0.44	0.69 ± 0.10	2.67 ± 0.93	1.01 ± 0.05
	HWS	0.88 ± 0.16	0.34 ± 0.04	0.35 ± 0.11	0.77 ± 0.05
	P value	0.13	0.0003	0.01	0.001
	Distribution				
	ARC	1.13 ± 0.20	0.44 ± 0.03[b]	ND	ND
	DMN	ND	ND	ND	ND
	LH	ND	0.09 ± 0.01[a]	1.36 ± 0.47	0.90 ± 0.05
	PVN	ND	0.82 ± 0.11[c]	ND	ND
	VMH	ND	ND	ND	0.89 ± 0.05
	P value		<0.0001		0.34
	Interaction				
	P Value		0.0004		0.07
Fasted	Line				
	LWS	0.78 ± 0.17	0.41 ± 0.04	2.10 ± 0.67	0.82 ± 0.05
	HWS	0.71 ± 0.07	0.26 ± 0.03	0.34 ± 0.10	0.79 ± 0.04
	P value	0.70	<0.0001	0.01	0.26
	Distribution				
	ARC	0.75 ± 0.09	0.48 ± 0.04[b]	ND	ND
	DMN	ND	ND	ND	ND
	LH	ND	0.11 ± 0.01[a]	1.22 ± 0.37	0.83 ± 0.04
	PVN	ND	0.50 ± 0.05[b]	ND	ND
	VMH	ND	ND	ND	0.76 ± 0.06
	P value		<0.0001		0.20
	Interaction				
	P value		0.02		0.02

Table 3-3. The expression of anorexigenic neuropeptides and receptors mRNA in hypothalamic nuclei

	Variables	CART	c-fos	CRF	CRFR1	CRFR2
Fed	Line					
	LWS	0.56 ± 0.09	1.1 3± 0.14	1.14 ± 0.21	1.35 ± 0.12	3.00 ± 0.41
	HWS	1.62 ± 0.33	0.95 ± 0.06	1.09 ± 0.20	1.47 ± 0.15	3.01 ± 0.39
	P value	0.0004	0.006	0.89	0.98	0.56
	Distribution					
	ARC	0.62 ± 0.06^{a}	1.19 ± 0.10^{b}	ND	2.71 ± 0.34^{b}	2.48 ± 0.13^{b}
	DMN	ND	1.02 ± 0.07^{b}	0.94 ± 0.23^{a}	2.15 ± 0.10^{b}	8.27 ± 0.80^{c}
	LH	0.14 ± 0.02^{a}	1.70 ± 0.41^{c}	0.50 ± 0.11^{a}	0.58 ± 0.11^{a}	1.31 ± 0.19^{ab}
	PVN	2.88 ± 0.44^{b}	0.99 ± 0.08^{ab}	1.76 ± 0.29^{b}	0.99 ± 0.04^{a}	0.94 ± 0.06^{a}
	VMH	0.11 ± 0.01^{a}	0.48 ± 0.04^{a}	ND	0.73 ± 0.06^{a}	2.20 ± 0.21^{ab}
	P value	<0.0001	<0.0001	0.001	<0.0001	<0.0001
	Interaction					
	P Value	<0.0001	0.0002	0.01	0.13	0.99
Fasted	Line					
	LWS	0.63 ± 0.09	1.11 ± 0.11	1.24 ± 0.23	1.33 ± 0.13	2.44 ± 0.31
	HWS	0.99 ± 0.29	1.19 ± 0.15	0.95 ± 0.21	1.61 ± 0.21	2.13 ± 0.27
	P value	0.005	0.50	0.86	0.27	0.03
	Distribution					
	ARC	0.74 ± 0.11^{a}	1.19 ± 0.18	ND	3.23 ± 0.31^{c}	2.67 ± 0.24^{b}
	DMN	ND	1.39 ± 0.23	0.60 ± 0.13^{a}	2.11 ± 0.21^{b}	6.43 ± 0.94^{c}
	LH	0.20 ± 0.03^{a}	1.14 ± 0.23	0.81 ± 0.23^{a}	0.72 ± 0.14^{a}	1.26 ± 0.20^{a}
	PVN	2.17 ± 0.49^{b}	1.28 ± 0.16	1.84 ± 0.29^{b}	0.90 ± 0.04^{a}	1.13 ± 0.13^{a}
	VMH	0.22 ± 0.09^{a}	0.76 ± 0.12	ND	0.76 ± 0.08^{a}	2.27 ± 0.46^{ab}
	P value	<0.0001	0.36	0.002	<0.0001	<0.0001
	Interaction					
	P value	<0.0001	0.74	0.25	0.003	0.11

Relative quantity values were analyzed by ANOVA within each feeding state and the model included the main effects of genetic line, hypothalamic nuclei, and the interaction between them. Data in table are expressed as means ± standard error with corresponding P values for main effects and the two-way interaction between genetic line and hypothalamic nuclei. Genes include cocaine and amphetamine regulated transcript (CART), corticotropin-releasing factor (CRF), CRF receptor sub-types 1 and 2 (CRFR1 and CRFR2), L-aromatic amino acid decarboxylase (DDC), growth hormone secretagogue receptor (GHSR), leptin receptor (LEPR), melanocortin receptor 3 and 4 (MC3R and MC4R), oxytocin receptor (OXTR), pro-opiomelanocortin (POMC), and urocortin III (UCN III). ND indicates that the respective mRNA not determined in those nuclei.

Table 3-3. Continued.

	Variables	DDC	Ghrelin	GHSR	LEPR	MC3R
Fed	Line					
	LWS	0.97 ± 0.17	1.04 ± 0.04	1.40 ± 0.10	1.32 ± 0.12	1.99 ± 0.27
	HWS	1.13 ± 0.0.16	0.98 ± 0.05	1.36 ± 0.10	1.74 ± 0.08	1.84 ± 0.19
	P value	0.92	0.35	0.08	0.10	0.37
	Distribution					
	ARC	1.72 ± 0.25^{b}	1.08 ± 0.05	2.07 ± 0.09^{b}	2.04 ± 0.15^{b}	2.61 ± 0.20^{b}
	DMN	2.58±0.31^{c}	ND	ND	ND	1.16 ± 0.13^{a}
	LH	0.34±0.09^{a}	ND	ND	ND	ND
	PVN	0.44±0.06^{a}	0.95 ± 0.05	0.98 ± 0.05^{a}	ND	ND
	VMH	0.18±0.02^{a}	ND	1.13 ± 0.07^{a}	1.08 ± 0.07^{a}	ND
	P value	<0.0001	0.08	<0.0001	<0.0001	<0.0001
	Interaction					
	P Value	0.04	0.20	0.89	0.23	0.58
Fasted	Line					
	LWS	0.71 ± 0.0.11	0.93 ± 0.05	1.42 ± 0.14	1.74 ± 0.26	2.50 ± 0.31
	HWS	0.71 ± 0.0.14	0.89 ± 0.07	1.51 ± 0.10	2.55 ± 0.27	1.69 ± 0.18
	P value	0.61	0.37	0.99	0.04	0.03
	Distribution					
	ARC	1.04±0.21^{b}	1.07 ± 0.06^{b}	2.14 ± 0.16^{b}	2.80 ± 0.24^{b}	2.64 ± 0.22^{b}
	DMN	2.11±0.37^{c}	ND	ND	ND	1.12 ± 0.13^{a}
	LH	0.27±0.05^{a}	ND	ND	ND	ND
	PVN	0.48±0.06^{ab}	0.75 ± 0.05^{a}	1.07 ± 0.08^{a}	ND	ND
	VMH	0.27±0.06^{a}	ND	1.07 ± 0.10^{a}	1.09 ± 0.14^{a}	ND
	P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	Interaction					
	P value	0.99	0.54	0.23	0.55	0.57

Table 3-3. Continued.

	Variables	MC4R	Mesotocin	OXTR	POMC	UCN III
Fed	Line					
	LWS	0.83 ± 0.09	0.34±0.09	1.96 ± 0.25	0.81 ± 0.13	0.75 ± 0.11
	HWS	1.09 ± 0.12	0.25±0.06	2.43 ± 0.26	2.10 ± 0.11	1.00 ± 0.17
	P value	0.005	0.34	0.54	<0.0001	0.25
	Distribution					
	ARC	0.53 ± 0.05^{a}	0.009 ± 0.002^{a}	3.85 ± 0.31^{b}	1.49 ± 0.17	ND
	DMN	0.66 ± 0.05^{a}	0.0005 ± 0.0001^{a}	3.81 ± 0.49^{b}	ND	ND
	LH	ND	0.08 ± 0.02^{a}	0.96 ± 0.21^{a}	ND	ND
	PVN	1.64 ± 0.15^{b}	1.16 ± 0.13^{b}	1.49 ± 0.23^{a}	ND	0.89 ± 0.10
	VMH	ND	0.003 ± 0.0007^{a}	0.73 ± 0.06^{a}	ND	ND
	P value	<0.0001	<0.0001	<0.0001		
	Interaction					
	P Value	0.002	0.51	0.053		
Fasted	Line					
	LWS	0.86 ± 0.12	0.51 ± 0.13	1.88 ± 0.25	0.74 ± 0.12	0.81 ± 0.19
	HWS	0.97 ± 0.12	0.22 ± 0.07	2.21 ± 0.30	2.13 ± 0.31	0.72 ± 0.24
	P value	0.26	0.07	0.73	0.0004	0.78
	Distribution					
	ARC	0.59 ± 0.05^{a}	0.01 ± 0.003^{a}	3.91 ± 0.56^{c}	1.43 ± 0.21	ND
	DMN	0.67 ± 0.07^{a}	0.0005 ± 0.0002^{a}	2.60 ± 0.49^{bc}	ND	ND
	LH	ND	0.04 ± 0.009^{a}	1.00 ± 0.12^{a}	ND	ND
	PVN	1.39 ± 0.18^{b}	1.59 ± 0.22^{b}	1.95 ± 0.35^{ab}	ND	0.78 ± 0.15
	VMH	ND	0.002 ± 0.0008^{a}	0.80 ± 0.13^{a}	ND	ND
	P value	<0.0001	<0.0001	<0.0001		
	Interaction					
	P value	0.39	0.007	0.13		

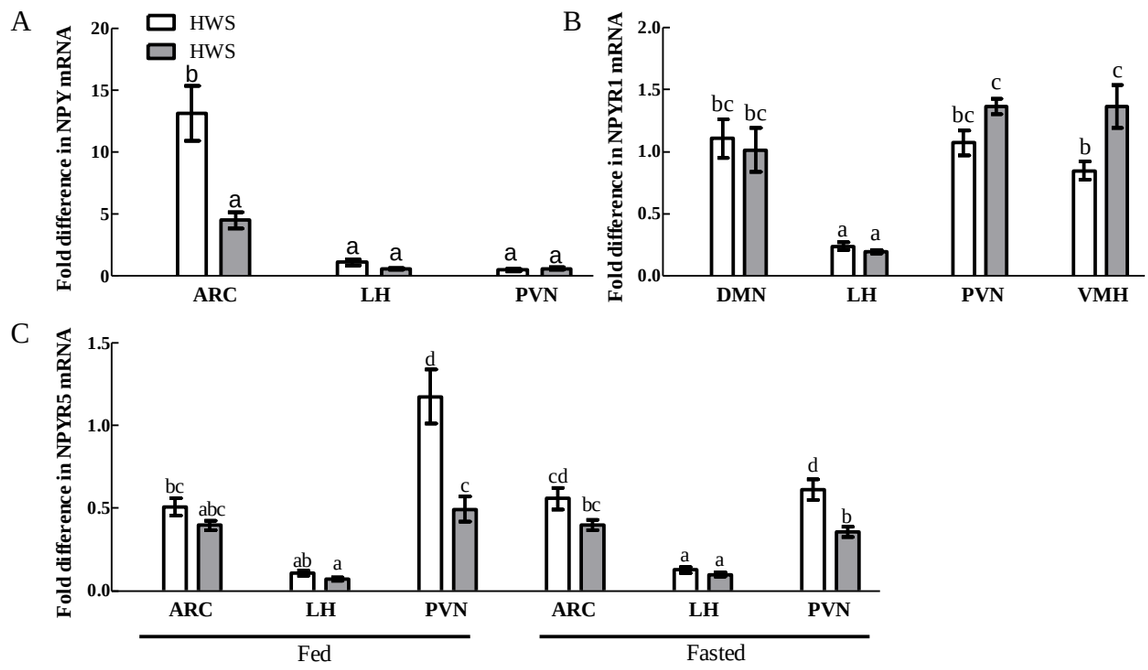

Figure 3-1. Significant interactions between genetic line and hypothalamic nuclei for the expression of of orexigenic factors. A. Significant interaction between genetic line and hypothalamic nuclei in fasted state (P = 0.01) for the expression of NPY. B. Significant interaction between genetic line and hypothalamic nuclei in fasted state (P = 0.007) for the expression of NPYR1. C. Significant interactions between genetic line and hypothalamic nuclei in both fed and fasted states (P = 0.0004 for fed and P = 0.02 for fasted) for the expression of NPYR5. Values represent the mean ± SE. The interactions were separated using Tukey's test.

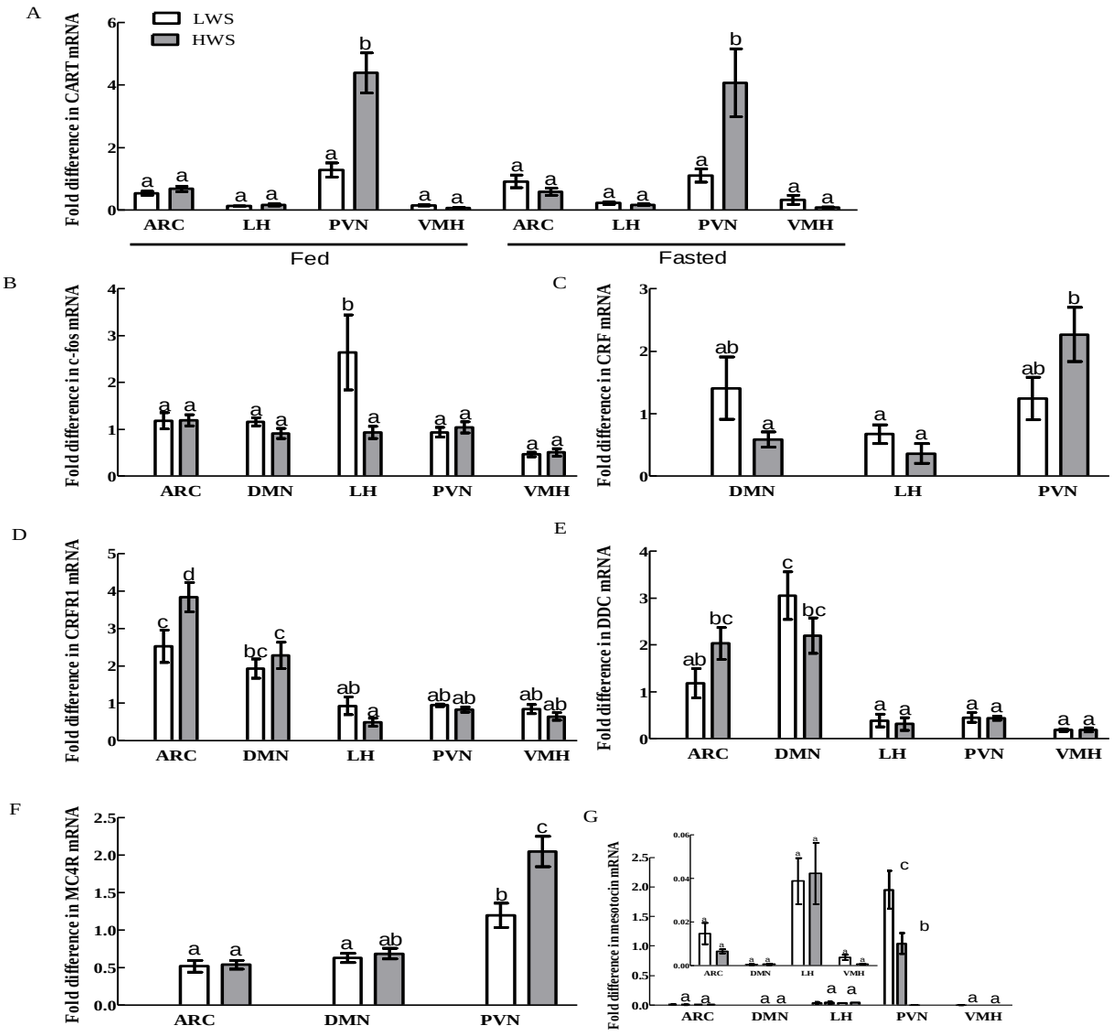

Figure 3-2. Significant interactions between genetic line and hypothalamic nuclei for the expression of anorexigenic factors. A. Significant interactions between genetic line and hypothalamic nuclei in both fed and fasted states ($P < 0.0001$ for both fed and fasted) for the expression of CART. B. Significant interaction between genetic line and hypothalamic nuclei in fed state ($P = 0.0002$) for the expression of c-fos. C. Significant interaction between genetic line and hypothalamic nuclei in fed state ($P = 0.01$) for the expression of CRF. D. Significant interaction between genetic line and hypothalamic nuclei in fasted state ($P = 0.02$) for the expression of CRFR1. E. Significant interaction between genetic line and hypothalamic nuclei in fed state ($P = 0.04$) for the expression of DDC. F. Significant interaction between genetic line and

Chapter 4

Anorexia is associated with stress-dependent orexigenic responses to exogenous neuropeptide Y

Abstract: Chicken lines that have been divergently selected for either low (LWS) or high (HWS) body weight at 56 days of age for more than 57 generations have different feeding behaviors in response to a range of intracerebroventricularly (ICV) injected neurotransmitters and the LWS have different severities of anorexia while HWS become obese. Previously, we demonstrated that LWS chicks did not respond while HWS chicks increased food intake after central injection of neuropeptide Y (NPY). The current study was designed to elucidate the molecular mechanisms underlying the loss of orexigenic function of NPY in LWS. Chicks were divided into 4 groups: stressed LWS and HWS on day of hatch, and control LWS and HWS. The stressor was a combination of food deprivation and acute cold exposure. On day 5 post-hatch, each chick received an ICV injection of vehicle or 0.2 nmol of NPY. Only the LWS stressed group did not increase food intake in response to ICV NPY. Hypothalamic mRNA abundance of appetite-associated factors was measured at 1 hour post-injection. Interactions of genetic line, stress, and NPY treatment were observed for the mRNA abundance of agouti-related peptide (AgRP) and synaptotagmin 1 (SYT1). ICV injection of NPY decreased and increased AgRP and SYT1 mRNA, respectively, in the stressed LWS while increasing AgRP mRNA in stressed HWS chicks. Stress was associated with increased NPY, orexin receptor 2, corticotropin-releasing factor receptor 1, melanocortin receptor 3 (MC3R), and growth hormone secretagogue receptor expression. In conclusion, the loss of responsiveness to exogenous NPY in stressed LWS chicks may be due to the decreased and increased hypothalamic expression of AgRP and MC3R, respectively. Such may induce an intensification of anorexigenic melanocortin signaling pathways in LWS chicks that block the orexigenic effect of exogenous NPY. These results provide insights onto the anorexic condition across species, and especially so for forms of inducible anorexia such as human anorexia nervosa.

Keywords: neuropeptide Y, chick, hypothalamus, anorexia, obesity

Introduction

Through more than 57 continuous generations of selection for low (LWS) or high (HWS) juvenile body weight, the Virginia lines of chickens have a 10-fold difference in body weight at selection age (56 days post-hatch) (1). The LWS line is comprised of lean individuals with

different severities of anorexia and the HWS are all hyperphagic and become obese (1, 2).

A portion of the LWS line chicks do not commence eating and die within the first week post-hatch (3), thus study at an early age is advantageous for understanding the most severe anorexic condition in this line. It has been proposed that differences in appetite regulation between the lines are of hypothalamic origin, as it was shown that the LWS became obese after lesioning of the ventromedial hypothalamus (VMH) (4). The hypothalamus, which is composed of several distinct nuclei that are related to appetite regulation, plays a crucial role in the regulation of food intake (5, 6).

For over a decade we have compared appetite-associated responses (including hypothalamic nuclei activation) of many centrally-injected neuropeptides and found differences between the lines. For example, the threshold in food intake response to anorexigenic factors, such as α- melanocyte-stimulating hormone (MSH) (7), corticotropin-releasing factor (CRF) (8), insulin (9), amylin (10), ghrelin (11), and neuropeptide AF (12) is lower in LWS than HWS. In contrast, HWS have a lower threshold response to neuropeptide S (13) and calcitonin and calcitonin gene-related peptide (14) than LWS while both ghrelin (11) and galanin (15) have similar thresholds of sensitivity in their effects on food intake in the lines. For two factors there is a complete loss of response in one line: the LWS does not respond to neuropeptide Y (NPY) with increased food intake (16) and the HWS does not decrease food intake in response to leptin (17).

The lack of an orexigenic effect of centrally-injected NPY in the LWS is intriguing because it is one of the most evolutionarily-conserved and potent hunger-stimulating factors in birds and mammals (18, 19). Moreover, both LWS and HWS had increased c-Fos immunoreactivity in the lateral hypothalamus and paraventricular nucleus (PVN) after intracerebroventricular (ICV) injection of NPY despite that the NPY did not affect food intake in the LWS (16). One interpretation of the non-responsiveness to NPY in LWS is based on known interactions between NPY and the melanocortin system. Although the anorexigenic tone of pro-opiomelanocortin (POMC) neurons in the arcuate nucleus (ARC) can be inhibited by NPY (20), when in excess it stimulates a negative feedback mechanism that increases anorexigenic tone through α-MSH release (21). In addition, that LWS chicks are more sensitive to α-MSH, which increases c-Fos immunoreactivity in the PVN in both lines (7), suggests that a hypersensitive anorexigenic mechanism exists which overrides NPY's orexigenic effect in LWS chicks, possibly

involving melanocortins.

It is therefore possible that the NPY induction of food intake in LWS is overridden by complex synergisms of anorexigenic neuropeptides acting in the same hypothalamic nuclei. In support of this idea, LWS chicks have greater hypothalamic mRNA abundance of satiety-associated neurotransmitters than HWS (22). Moreover, there are differences between the lines in hypothalamic mRNA abundance of NPY and receptor sub-types 1 and 5 (NPYR1 and 5, respectively) with greater expression of NPY in fasted LWS than HWS at 5 (23) and 90 days post-hatch (24), and greater expression of NPYR1 and 5 in LWS than HWS at 90 days post-hatch (24). Collectively these data suggest the induction of anorexigenic tone in the hypothalamus overrides the hunger-stimulating effects of NPY in LWS, rather than the lack of response to NPY in LWS being due to a non-functional NPY system.

This induction of anorexigenic tone could be mediated by various environmental factors as acute stressors have different physiological effects in the lines. For example, when stressed by handling restraint, plasma corticosterone (CORT) concentrations were greater in LWS than HWS, while similar in the non-stressed state (8). The threshold for exogenous CRF-mediated anorexia was lower in LWS than HWS and CRF receptor antagonism potently stimulated food intake in LWS but not HWS (8). In addition, Ka et al., (2009) reported that LWS chicks expressed higher levels of transcripts encoding avian leucosis virus subgroup-E (ALVE) (25), the higher expression of which may be associated with changes in physiology, disease resistance and egg production (26-28). These data suggest that the physiological response to stress may affect food intake regulation differently in LWS and HWS. Thus, while differences in hypothalamic gene expression between the lines have been explored under fed and fasted conditions, information is lacking on how NPY injection affects gene expression of appetite-associated factors and how early post-hatch exposure to stressors influences the feeding response to centrally-administered NPY. The experiment reported here was designed to measure food intake-related behavior and associated molecular mechanisms in the hypothalamus under stressed conditions in 5 day-old LWS and HWS chicks.

Materials and methods

Animals and experimental design

All animal protocols were approved by the Institutional Animal Care and Use Committee at Virginia Tech. The lines of chickens used in this experiment are from a long-term selection

experiment for low or high body weight at 56 days of age (29) with details of the selection program reported by (3, 30) . The founder population consisted of crosses of 7 inbred lines with the LWS and HWS lines maintained as a closed population. Eggs obtained from age contemporary parents from S_{56} generation parental stocks were incubated in the same machine. The diet was a mash (21.5% crude protein and 3,000 kcal ME/kg) and individual cages allowed visual and auditory contact with each other. Chicks were handled twice daily to adapt to handling. As the LWS is comprised of anorexic individuals, of which some do not survive the first week due to hypophagia (3), any chick which did not thrive or exhibited distress was euthanized prior to data collection in the present study. Details of handling and feeding after hatch are described in the experiments below.

Experiment 1 Food and water intake after stress and NPY injection

Chicks were removed from the hatcher on the morning of hatch and randomly assigned to either a stress or control group. The stress group chicks were placed inside United States Mail-approved cardboard chick shipping boxes (37 x 24 cm, n = 20 per box, without food or water) and immediately exposed to -20 ± 2 °C for 6 min followed by 22 ± 2 °C for 24 hours and then individually caged with visual and auditory contact in the same room with the control chicks. The control group, immediately following hatch, were group caged for 1 d with ad libitum access to both food and water and then transferred to individual cages in a room at 32 ± 1 °C and 50 ± 5% relative humidity.

Five days following hatch, chicks from both lines and stress groups were randomly assigned to receive either 0 (vehicle) or 0.2 nmol NPY by ICV injection. NPY was injected at 5:00 am on the day of experiment. The NPY dose and injection method was based on previous results (16). Body weights were recorded just prior to injection and were 21 ± 1.78, 42 ± 5.01, 23 ± 3.16, and 51 ± 4.74 for stressed LWS and HWS, and control LWS and HWS, respectively. The head of the chick was briefly inserted into a restraining device that left the cranium exposed to allow free-hand injection. Injection coordinates were 3 mm anterior to the coronal suture, 1 mm lateral from the sagittal suture, and 2 mm deep targeting the left ventricle. Anatomical landmarks were determined visually and by palpation. Injection depth was controlled by placing a plastic tubing sheath over the needle. The needle remained at injection depth in the un-anesthetized chick for 5 s post injection to reduce backflow. Chicken NPY (AnaSpec, San Jose, CA, USA) was dissolved in avian artificial cerebrospinal fluid for a total injection volume of 5 µl with

0.06% Evans Blue dye. After data collection or during sample collection, any chick without dye present in the lateral ventricle system was eliminated from the study. Because LWS and HWS chicks consume different amounts of food as a result of body weight differences, food and water intake data were adjusted for body weight at each time point. For example, when food intake is not converted to a body weight basis, LWS control chicks consumed 0.17 ± 0.03 g and HWS control chicks 1.17 ± 0.03 g of food by 180 min post injection.

Data for food and water intake were analyzed with the GLM procedure of SAS 9.3 (SAS Institute, Cary, NC, USA), with analyses at each time point within each line. The model included sex (male or female), stressor (stress or control), NPY dose (0 or 0.2 pmol), and their interactions. Effects involving sex were not significant, hence sex was removed from the statistical model. When interactions were significant, means were separated using Tukey's test. For all experiments, data are presented as means ± SE and differences considered as significant at $P < 0.05$.

Experiment 2 Hypothalamic mRNA abundance and blood glucose and corticosterone

The experimental design was similar to Experiment 1 except for the following modifications. After ICV injection of vehicle or NPY, chicks were returned to their cage without food. Sample collection was conducted between 8:00 and 12:00 h at 5 days post-hatch. At 1 hour post-injection, 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 seconds. 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 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 (31). This block was immediately submerged in RNAlater (Qiagen).

Trunk blood samples were collected immediately after decapitation in ethylene diamine tetraacetic acid (EDTA)-coated tubes, centrifuged for 15 min at 3000 ×g, and the supernatants stored at −80 °C until analysis. Whole blood glucose concentrations were measured on fresh whole blood samples with a glucometer (AgaMatrix, Inc). Plasma corticosterone (CORT)

concentrations were assayed with a commercial kit (Cayman Chemical, Ann Arbor, MI, USA).The intra-assay coefficient of variation was 13%.

The hypothalamus was homogenized using 5 mm stainless steel beads (Qiagen, Valencia, CA, USA), Isol-RNA 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) and validated for amplification efficiency before use (95-105%). Real time PCR reactions were performed in 10 µl reactions that contained 5 µl Fast SYBR Green Master Mix (Applied Biosystems), 0.5 µl each of forward and reverse primers (0.125 µM each), 1.5 µl nuclease free water, and 3 µl 10-fold diluted cDNA using a 7500 Fast 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 15s was performed at the end of each PCR reaction to ensure amplicon specificity.

Real-time PCR data were analyzed using the ΔΔCT method (32) with β-actin as the reference gene 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 genetic line, treatment, stressor, and their interactions. 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 for two-way interactions andslice analysis for three-way interactions, with significance assigned at $P < 0.05$.

Results

Food intake

Food intake in both lines as a function of stressor exposure and NPY injection is depicted

in Figure 4-1. For control chicks from both lines, NPY injection caused increased food intake (Fig. 4-1 left). The LWS line had a 60 min latency while the HWS responded at all observation times. For stressed chicks (Fig. 4-1 right), the LWS did not respond at any time whereas the HWS responded at all times to NPY injection. It should be noted that the food intake values for vehicle-only-injected chicks are similar between stress and control groups within a line, and that the HWS consumed twice the amount of food as did the LWS.

Figure 4-2 depicts water intake under the same conditions shown in Figure 4-1. Water intake was not affected by NPY injection or stress and followed a similar pattern in both lines.

Gene expression of appetite-associated factors and associated receptors

For brevity, only interactions on hypothalamic gene expression involving genetic line will be discussed. Higher order interactions involving genetic line are depicted as additional tables and figures as described below. Hypothalamic mRNA expression profiles of orexigenic neuropeptides and associated receptors are shown in Table 4-1. Only for AgRP was there a significant three-way interaction of line, stress, and NPY treatment. The expression of NPYR1 and visfatin did not differ between lines and their expression was not affected by stress or NPY treatment. There were several main effects of genetic line and stress. The LWS chicks had greater expression of NPY, NPYR5, and orexin receptor 2 (OREXR2) mRNA than HWS. The HWS expressed more NPYR2 and orexin mRNA than LWS. There was greater expression of NPY and decreased expression of OREXR2 mRNA in stressed than control chicks.

There were interactions between genetic line and stress on the expression of galanin and melanocyte concentrating hormone (MCH) (Table 4-4). Stress decreased the expression of galanin in HWS but not LWS, and expression was thus greater in HWS than LWS chicks under control conditions. Stress increased the expression of MCH in LWS but not in HWS chicks such that expression was greater in LWS than HWS under stressed conditions. There was an interaction of line and NPY treatment on NPYR2 mRNA (Table 4-5). While expression was similar in vehicle-injected chicks, NPYR2 mRNA after NPY injection was greater in HWS than LWS chicks (Table 4-5). There was a three-way interaction of genetic line, stress and NPY on the expression of AgRP (Figure 4-3). The injection of NPY increased the expression of AgRP in LWS but not HWS chicks under control conditions. After exposure to stress, however, injection of NPY decreased the expression of AgRP in LWS chicks while increasing the expression in HWS chicks relative to the vehicle-injected chicks.

The expression profiles of anorexigenic factors and associated receptors are depicted in Table 4-2. The expression of arginine vasotocin (AVT), calcitonin, CRF, CRF receptor 1 (CRFR1), dopamine decarboxylase (DDC), interleukin-1 beta receptor (IL1βR), leptin receptor (LEPR), melanocortin type 3 and 4 receptors (MC3R, MC4R), neuropeptide S (NPS), and oxytocin differed between LWS and HWS with AVT, calcitonin, CRF, CRFR1, DDC, LEPR, MC3R, NPS, and oxytocin more highly expressed in LWS than HWS, while IL1βR, and MC4R were greater in the HWS than LWS. There was an interaction between line and stress on the expression of pro-opiomelanocortin (POMC) (Table 4-4), interaction between genetic line and treatment on CRFR1 and DDC expression (Table 4-5), and three-way interaction of line, stress, and treatment on the expression of SYT1 (Figure 4-4). Stress decreased the expression of POMC in HWS chicks while having no effect on LWS chicks. The expression of POMC was greater in HWS than LWS chicks regardless of whether they had been stressed. However, stress decreased the magnitude of this difference between LWS and HWS chicks. The expression of CRFR1 was greater in LWS than HWS chicks under vehicle-injected conditions, while no difference was observed between LWS and HWS chicks injected with NPY. Injection of NPY had no effect on the expression of CRFR1 in both LWS and HWS chicks. The injection of NPY had no effect on the expression of DDC in both LWS and HWS chicks. The expression of DDC was greater in LWS than HWS chicks irrespective of NPY treatment. However, the injection of NPY decreased the magnitude of this difference between LWS and HWS chicks. The ICV injection of NPY increased SYT1 mRNA in stressed LWS chicks (Figure 4-4).

LWS chicks had greater concentrations of CORT than HWS chicks and stress increased plasma CORT (Table 4-3). There was a three-way interaction of genetic line, stress, and treatment on blood glucose (Figure 4-5). Under control conditions, NPY injection increased and decreased blood glucose in LWS and HWS chicks, respectively.

Discussion

In our previous report, the LWS failed to respond to ICV NPY at a wide range of doses (59 pmol to 5.2 nmol), whereas the HWS responded to all doses evaluated with increased food intake, although both lines have similar c-Fos hypothalamic expression patterns after ICV NPY (16). Thus, this effect became a major focus of attention and coincided with a move of the laboratory from one location (Radford University) to another (Virginia Tech), where the vivarium was then in proximity to the hatching location. Previously, the chicks were transported

via automobile in the winter from the hatchery to the vivarium, a 25 minute trip. At the new location the travel was shortened to about 3 min and exposure to cold thus minimized. Food intake data were collected in the new location after ICV NPY and both lines responded with increased food intake. We hypothesized that the transport was a stressor and thus responsible for the LWS being refractory to IVC NPY 5 days later. Hence the design of Experiment 1 with results supporting this thesis. The stress scheme was based on our former transport protocol and is similar to commercial poultry production as chicks may be transported for periods of up to 2 days through the postal service or commercial poultry transport without food or water, as a chick is nourished by yolk sac reserves for the first few days after hatch. During this time their thermoregulatory system is not developed and the environmental insults doing transfer from hatchery to rearing facilities. A potential caveat though is that the combined effects of thermal and nutritional stress makes it difficult to identify the contribution of the individual stressor to the bird's physiology. However, the protocol was designed to mimic conditions described in Newmyer et al. 2013 in order to replicate the findings and begin to elucidate the underlying mechanisms (16).

We interpret the result from Experiment 1 that the LWS can be induced to become refractory to NPY by a stressor for a duration of at least 4 days following removal of the stressor. The chick, although precocial, is hatched with a still-developing brain that is very plastic. It is well documented that stress and associated glucocorticoid concentrations affect plasticity (33, 34), especially thermal stressors (35). Additionally, previous studies in mammals demonstrate that stress from the environment very early in life can have a sustaining effect on food intake for the duration of the animal's life (36-38). Thus, that the stressor may have affected plasticity is supported by the result of Exp. 2 where hypothalamic synaptotagmin 1 (a neurotransmitter release regulator) (39) mRNA was affected by the stressor in LWS. This could be associated specifically with some orexigenic factors such as NPY in the LWS but not others, or perhaps strengthened other orexigenic factors because the LWS post-stress are driven to consume a similar amount of food as their non-stressed counterparts. Our unreported data with ICV injection of prolactin-releasing peptide (PrRP) in these lines supports this thesis as the LWS have a much lower threshold for the orexigenic effect of PrRP than the HWS.

The hypothalamic-pituitary-adrenal (HPA) axis is a major responder to stressor exposure (40, 41). While plasma corticosterone, a marker for HPA axis activity, was not different between

LWS and HWS, corticosterone was elevated in mildly stressed LWS but not HWS chicks (8). Results from central injection of CRF receptor antagonists, suggest that greater stimulation of anorexigenic signaling in LWS may contribute to their lower body weights via feeding suppression mediated by the stress response (8); lacking are studies to test the hypothesis that exposure to stress affects neuropeptide-induced feeding responses differently in LWS and HWS.

Yi et al., (2015) reported differences in hypothalamic gene expression of appetite-related factors between 5 day-old LWS and HWS chicks. The LWS chicks expressed more anorexigenic factors, such as calcitonin, CRFR1, leptin receptor, NPS, MC3R, and oxytocin than HWS, while no differences were observed between the lines for the mRNA abundance of NPY, CRF, and MC4R (22). However, here we found that LWS expressed more NPY and CRF, whereas HWS expressed more MC4R than LWS. These results may due to the differences in the experimental design. In the current study, we introduced two exogenous factors (NPY and stress) to understand why LWS chicks did not respond to ICV injection of NPY when stressed. Rice et al., (2014) reported that NPY mRNA was greater in fasted LWS than HWS chicks (23), consistent with the present study.

Because LWS chicks exhibit anorexia, the increased expression of NPY mRNA may be a compensatory mechanism. However, increased NPY mRNA in LWS may not necessarily reflect greater amounts of the biologically active peptide and it is possible that there is decreased translation or dysregulation in processing of the bioactive peptide, thus inducing dysfunction of NPY-related feeding circuits in anorectic LWS chicks. That NPY induced food intake in the LWS under non-stressed conditions suggests that the NPY-related neural circuit while intact is overridden by other factors in response to stress. Stress increased the mRNA abundance of NPY in the hypothalamus, which is consistent with Makino et al., (2000), where both acute and repeated immobilization stress increased NPY mRNA expression in the arcuate nucleus (42), although the increased NPY mRNA may not be directly related to appetite regulation. Not all of the NPY-expressing neurons in the ARC are involved in appetite regulation and only those NPY-containing neurons that co-express LEPR are associated with appetite regulation (43). Hence, the increased expression of NPY in the hypothalamus may be related to NPY's role in the control of stress (44). Kuo et al., (2005) reported that ICV injection of human recombinant leptin decreased food intake in LWS but not HWS chicks (17). Leptin also decreased food intake in broilers and leghorns (45), the former selected for meat production (46) and the latter for egg production (47),

respectively. In the current study, the mRNA abundance of leptin receptor in the hypothalamus was greater in the LWS than HWS chicks. More studies are needed to further demonstrate whether or not the increased expression of leptin receptor in LWS co-localizes with NPY-expressing neurons in the ARC.

In response to stress, there are several important physiological systems, such as the HPA axis, which is one of the most studied for its role in coping with stressful situations, the autonomic nervous system, especially the sympathetic response of the adrenal medulla and the sympathetic nerves, as well as several hormones or neurotransmitters like the so-called stress hormone glucocorticoids, including CRF, ACTH, epinephrine, norepinephrine and others. Stress increased plasma CORT, a major glucocorticoid secreted from cortex of the adrenal gland in birds and an indicator for the stress response (48). The secretion of CORT is stimulated by ACTH from the anterior pituitary, which in turn is released by the activation of CRF and arginine vasotocin (AVT) from the hypothalamus (40). However, here hypothalamic CRF mRNA did not differ between stressed and non-stressed chicks, which may due to the inhibiting effect of glucocorticoids on the production and release of CRF from the hypothalamus by a negative-feedback mechanism (49-51). Stressors also increase AVT, which may explain the increased CORT in the plasma.

NPY is one of the most potent orexigenic factors known to date in both mammals and chickens (18, 52), while CRF is very potent at inhibiting food intake (53, 54). The interactions between NPY and CRF in the regulation of food intake have been demonstrated. For instance, fasting increased and decreased the expression of NPY and CRF, respectively, in the hypothalamus in rats (55, 56). Heinrichs et al., (1993) reported that pre-treatment with a CRF antagonist attenuated NPY-induced food intake in the PVN of rats and hence concluded that CRF may have inhibitory control over NPY's orexigenic effect (57). Moreover, there is morphological evidence that NPY terminals are co-localized with CRF-containing neurons in the PVN (58) where the CRF receptors are also present (59). In the present study, both CRF and CRFR1 mRNA were greater in LWS than HWS chicks and stress increased the expression of CRFR1 in both lines. The CRFR1, which is highly expressed in the hypothalamus (60), is a G-protein coupled receptor and binds with high affinity to CRF (61), which functions as a potent anorexigenic neuropeptide in both mammals and chickens (53, 62). Even though ICV injection of CRF decreased food intake in both the LWS and HWS lines, the threshold was lower in the

LWS than HWS chicks (8). Hotta et al., (1999) reported that CRFR1 was involved in stress-induced inhibition of food intake in rats (63). Considering the already greater expression level of CRF and CRFR1 in the LWS, the increased expression of CRFR1 in LWS may further magnify the CRF signaling system and thereby dampen the orexigenic effect of NPY.

There was a significant three-way interaction of genetic line, stress, and injection of NPY on the mRNA abundance of AgRP. In mammals, AgRP is one of the most potent orexigenic neuropeptides (64, 65). AgRP exerts its orexigenic effects by inhibiting brain melanocortin signaling through antagonizing MC3R and MC4R in the hypothalamus (64). AgRP neurons positively regulate feeding behavior and the stimulation of only 800 AgRP neurons resulted in voracious feeding within min in mice (66). However, the effects of AgRP on food intake in chicks are controversial. Tachibana et al., (2001) reported that the central injection of AgRP increased food intake in layer-type chicks but had no effect on meat-type chicks (67). The ICV injection of AgRP also increased food intake in both LWS and HWS chicks (data not published), indicating that AgRP may have a role in appetite regulation in these genetic lines. Although injection of NPY increased AgRP mRNA in non-stressed LWS chicks but not in the non-stressed HWS, injection of NPY decreased the mRNA in stressed LWS chicks while increasing its expression in stressed HWS chicks relative to the vehicle-injected chicks. The result is intriguing in that the AgRP gene expression response to exogenous NPY was stress-dependent. One possible explanation for this is that if LWS chicks have a dysfunctional NPY system, they develop a more sensitive AgRP system as a compensatory mechanism to increase their food intake. This may be explained by the increased AgRP mRNA when they are injected with NPY under non-stressed conditions. Stress, however, may exacerbate the already disrupted NPY system and exogenous NPY may further cause the internalization of NPY-related receptors and down-regulate AgRP mRNA. Decreased expression of AgRP in LWS chicks would thus reduce its inhibitory effects on the melanocortin signaling system.

Avian leucosis viruses (ALVs) are a group of type C retroviruses, which include subgroups A, B, C, D, E, and J, associated with various neoplasms including lymphoid and myeloid leukosis (68, 69). Ka et al., (2009) reported that the greater expression of ALV subgroup E (ALVE) in LWS than chicks was associated with reduced growth in LWS chicks (25). The occurrence and frequency of ALVE integration in the genome of different chicken breeds are associated with changes in physiology, disease resistance and egg production (26-28). The higher

expression level of ALVE may serve as endogenous stress to hinder the growth of LWS chicks. In the current study, the loss of responsiveness of NPY's orexigenic effect may also due to the interaction between exogenous stress with endogenous stressors such as ALVE.

In the current study, a large number of measurements (29 genes) were analyzed by analysis of variance (ANOVA). Some of the significant differences in gene expression for the main effect may thus be false positives (although Tukey's test was used to separate the means, which would be very conservative in detecting differences).

In conclusion, results show that immediate post-hatch exposure to stress lead to elevated plasma CORT in both LWS and HWS chicks, and affected the food intake response to centrally-injected NPY in LWS but not HWS chicks at 5 days post-hatch. The LWS chicks that were stressed did not increase their food intake in response to exogenous NPY, and gene expression assays revealed differences in hypothalamic mRNA abundance of appetite-associated factors. These data suggest that the loss of responsiveness to NPY in stressed LWS chicks could be due to decreased and increased AgRP and MC3R mRNA, respectively, which may induce intensified anorexigenic melanocortin signaling pathways in LWS that block the orexigenic effect of exogenous NPY. Although beyond the scope of this study, measuring these factors at the protein level should clarify this hypothesis. The loss of responsiveness to hunger-inducing neurotransmitters after early-life exposure to a stressor may have relevance to understanding eating disorders in humans.

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Table 4-1. The effect of central neuropeptide Y (NPY) on hypothalamic orexigenic neuropeptide and receptor mRNA under stress.

Effect	**AgRP**	**galanin**	**MCH**	**NPY**	**NPYR1**	**NPYR2**
Line						
LWS	1.24 ± 0.11	1.11 ± 0.05	1.12 ± 0.06	1.14 ± 0.06	1.01 ± 0.03	0.97± 0.03
HWS	1.04 ± 0.10	1.27 ± 0.07	0.90 ± 0.03	0.91 ± 0.03	1.03 ± 0.03	1.09 ± 0.02
P-value	0.17	0.07	0.0003	0.0004	0.59	0.0004
Stress						
Control	0.95 ± 0.10	1.21 ± 0.07	0.96 ± 0.04	0.94 ± 0.04	0.99 ± 0.03	1.01 ± 0.03
Stressed	1.31 ± 0.11	1.16 ± 0.06	1.06 ± 0.05	1.11 ± 0.05	1.05 ± 0.03	1.05 ± 0.02
P-value	0.01	0.58	0.07	0.006	0.16	0.39
Treatment						
Vehicle	1.08 ± 0.10	1.16 ± 0.06	0.99 ± 0.04	1.06 ± 0.06	1.03 ± 0.03	1.04 ± 0.03
NPY	1.19 ± 0.11	1.21 ± 0.07	1.03 ± 0.05	1.00 ± 0.04	1.02 ± 0.03	1.02 ± 0.03
P-value	0.39	0.49	0.36	0.38	0.88	0.55
Interactions						
T × line	0.39	0.76	0.94	0.33	0.15	0.03
Line × stress	0.49	0.02	0.02	0.09	0.39	0.92
T × stress	0.33	0.44	0.13	0.90	0.60	0.19
T × line × stress	0.0001	0.26	0.09	0.55	0.96	0.63
Continued						
Effect	**NPYR5**	**orexin**	**OREXR2**	**PrRP**	**visfatin**	
Line						
LWS	1.04 ± 0.03	1.14 ± 0.05	0.99 ± 0.03	0.93 ± 0.06	0.99 ± 0.05	
HWS	0.79 ± 0.02	1.35 ± 0.05	0.86 ± 0.02	0.93 ± 0.05	1.02 ± 0.05	
P-value	<0.0001	0.004	0.0001	0.93	0.63	
Stress						
Control	0.89 ± 0.03	1.19 ± 0.05	0.89 ± 0.03	0.90 ± 0.05	1.03 ± 0.05	
Stressed	0.94 ± 0.03	1.30 ± 0.05	0.96 ± 0.03	0.97 ± 0.06	0.98 ± 0.05	
P-value	0.11	0.12	0.03	0.35	0.47	
Treatment						
Vehicle	0.92 ± 0.03	1.21 ± 0.05	0.92 ± 0.03	0.98 ± 0.05	1.00 ± 0.05	
NPY	0.91 ± 0.03	1.29 ± 0.06	0.93 ± 0.03	0.88 ± 0.06	1.01 ± 0.05	
P-value	0.75	0.24	0.89	0.20	0.92	
Interactions						
T × line	0.29	0.99	0.11	0.42	0.99	
Line × stress	0.44	0.10	0.28	0.73	0.96	
T × stress	0.70	0.37	0.04	0.02	0.20	
T × line × stress	0.88	0.54	0.27	0.43	0.84	

Relative quantity values were analyzed by ANOVA and the model included the main effects of genetic line (LWS: low body weight-selected; HWS: high body weight-selected), treatment (intracerebroventricular vehicle or neuropeptide Y injection), and stressor (control or stressed for 24 hours at hatch), and their interactions. Data in table are expressed as means ± standard error (n=9 to 10 per group) for main effect of genetic line, treatment, and stressor. Also shown are corresponding P-values for main effects, and two- and three-way interactions among genetic line, treatment, and stressor. Melanin concentrating hormone (MCH), NPY receptor sub-types 1, 2, and 5 (NPYR1, NPYR2, and NPYR5, respectively), prolactin releasing peptide (PrRP), agouti-related peptide (AgRP), orexin receptor 2 (OREXR2). T: treatment (vehicle or NPY).

Table 4-2. The effect of central neuropeptide Y (NPY) on hypothalamic anorexigenic neuropeptide and receptor mRNA under stress.

Effect	**amylin**	**AVT**	**calcitonin**	**CART**	**CRF**	**CRFR1**
Line						
LWS	1.01 ± 0.04	1.04 ± 0.08	1.02 ± 0.05	1.00 ± 0.08	1.11 ± 0.06	0.97 ± 0.03
HWS	0.94 ± 0.02	0.82 ± 0.05	0.73 ± 0.03	0.98 ± 0.07	0.90 ± 0.04	0.78 ± 0.02
P-value	0.11	0.01	<0.0001	0.79	0.004	<0.0001
Stress						
Control	0.93 ± 0.03	0.82 ± 0.04	0.85 ± 0.04	0.92 ± 0.06	0.94 ± 0.04	0.82 ± 0.03
Stressed	1.01 ± 0.03	1.05 ± 0.08	0.89 ± 0.05	1.06 ± 0.08	1.06 ± 0.07	0.93 ± 0.03
P-value	0.053	0.01	0.36	0.15	0.10	0.006
Treatment						
Vehicle	0.96 ± 0.03	0.96 ± 0.06	0.87 ± 0.04	1.00 ± 0.06	0.95 ± 0.05	0.88 ± 0.04
NPY	0.98 ± 0.03	0.91 ± 0.07	0.88 ± 0.05	0.98 ± 0.08	1.05 ± 0.06	0.87 ± 0.03
P-value	0.53	0.62	0.78	0.79	0.18	0.87
Interactions						
T × line	0.11	0.98	0.89	0.43	0.54	0.007
Line × stress	0.31	0.98	0.15	0.14	0.94	0.63
T × stress	0.19	0.72	0.02	0.26	0.04	0.02
T × line × stress	0.22	0.15	0.44	0.49	0.57	0.77
Effect	**CRFR2**	**DDC**	**ghrelin**	**GHSR**	**IL1βR**	**LEPR**
Line						
LWS	1.05 ± 0.05	0.89 ± 0.04	1.17 ± 0.07	1.00 ± 0.03	1.09 ± 0.06	0.95 ± 0.04
HWS	1.00 ± 0.05	0.69 ± 0.04	1.07 ± 0.05	0.95 ± 0.03	1.40 ± 0.07	0.82 ± 0.04
P-value	0.44	0.0004	0.25	0.21	0.002	0.02
Stress						
Control	1.11 ± 0.04	0.76 ± 0.04	1.07 ± 0.05	0.92 ± 0.03	1.23 ± 0.06	0.86 ± 0.03
Stressed	0.94 ± 0.05	0.82 ± 0.04	1.17 ± 0.07	1.03 ± 0.03	1.25 ± 0.07	0.90 ± 0.04
P-value	0.01	0.19	0.27	0.01	0.90	0.39
Treatment						
Vehicle	0.94 ± 0.05	0.83 ± 0.04	1.06 ± 0.07	0.99 ± 0.03	1.24 ± 0.07	0.89 ± 0.04
NPY	1.12 ± 0.05	0.75 ± 0.04	1.18 ± 0.05	0.96 ± 0.03	1.24 ± 0.07	0.88 ± 0.04
P-value	0.007	0.14	0.17	0.58	0.96	0.95
Interactions						
T × line	0.50	0.04	0.79	0.13	0.75	0.82
Line × stress	0.16	0.38	0.83	0.21	0.91	0.50
T × stress	0.24	0.04	0.31	0.06	0.98	0.04
T × line × stress	0.99	0.84	0.82	0.62	0.92	0.06

Table 4-2. Continued.

Effect	**MC3R**	**MC4R**	**NPS**	**oxytocin**	**POMC**	**SYT1**
Line						
LWS	0.96 ± 0.03	1.04 ± 0.04	0.97 ± 0.05	1.15 ± 0.06	1.00 ± 0.04	1.06 ± 0.04
HWS	0.78 ± 0.03	1.17 ± 0.03	0.45 ± 0.02	0.76 ± 0.04	1.41 ± 0.07	1.07 ± 0.04
P-value	<0.0001	0.007	<0.0001	<0.0001	<0.0001	0.99
Stress						
Control	0.82 ± 0.04	1.07 ± 0.04	0.71 ± 0.06	0.82 ± 0.05	1.27 ± 0.07	1.03 ± 0.04
Stressed	0.91 ± 0.03	1.14 ± 0.03	0.71 ± 0.06	1.10 ± 0.06	1.14 ± 0.05	1.10 ± 0.04
P-value	0.02	0.14	0.78	0.78	0.10	0.21
Treatment						
Vehicle	0.87 ± 0.04	1.10 ± 0.04	0.70 ± 0.06	0.97 ± 0.06	1.25 ± 0.07	1.03 ± 0.04
NPY	0.87 ± 0.03	1.12 ± 0.04	0.72 ± 0.06	0.94 ± 0.06	1.16 ± 0.06	1.10 ± 0.05
P-value	0.81	0.63	0.77	0.95	0.27	0.23
Interactions						
T × line	0.15	0.22	0.97	0.94	0.38	0.79
Line × stress	0.53	0.31	0.91	0.13	0.047	0.03
T × stress	0.07	0.30	0.11	0.20	0.88	0.18
T × line × stress	0.80	0.60	0.20	0.31	0.46	0.03

Relative quantity values were analyzed by ANOVA and the model included the main effects of genetic line (LWS: low body weight-selected; HWS: high body weight-selected), treatment (intracerebroventricular vehicle or neuropeptide Y injection), and stressor (control or stressed for 24 hours at hatch), and their interactions. Data in table are expressed as means ± standard error (n=9 to 10 per group) for main effect of genetic line, treatment, and stressor. Also shown are corresponding P-values for main effects, and two- and three-way interactions among genetic line, treatment, and stressor. Arginine vasotocin (AVT), cocaine and amphetamine regulated transcript (CART), corticotropin-releasing factor (CRF), corticotropin-releasing factor receptor sub-types 1 and 2 (CRFR1 and CRFR2, respectively), dopamine decarboxylase (DDC), growth hormone secretagogue receptor (GHSR), interleukin-1β receptor (IL-1βR), leptin receptor (LEPR), melanocortin receptor 3 and 4 (MC3R and MC4R, respectively), neuropeptide S (NPS), pro-opiomelanocortin (POMC), synaptotagmin 1 (SYT1). T, treatment (vehicle or NPY).

Table 4-3. The effect of central neuropeptide Y (NPY) on blood glucose and plasma CORT under stress.

Effect	CORT	glucose
Line		
LWS	3.50 ± 0.08	211.71 ± 3.97
HWS	3.07 ± 0.07	246.88 ± 5.84
P-value	0.0002	<0.0001
Stress		
Control	3.12 ± 0.07	229.39 ± 5.91
Stressed	3.46 ± 0.08	228.61 ± 5.73
P-value	0.005	0.91
Treatment		
Vehicle	3.31 ± 0.07	231.22 ± 6.23
NPY	3.25 ± 0.08	226.33 ± 5.08
P-value	0.50	0.56
Interactions		
T × line	0.32	0.02
Line × stress	0.56	0.96
T × stress	0.55	0.39
T × line × stress	0.63	0.04

Plasma corticosterone (CORT, ng/mL) and whole blood glucose concentrations (mg/mL) at 5 days post-hatch. The model included the main effects of genetic line (LWS: low body weight-selected; HWS: high body weight-selected), treatment (intracerebroventricular vehicle or neuropeptide Y injection), and stressor (control or stressed for 24 hours at hatch), and their interactions. Data are expressed as means ± standard error for main effect of genetic line, treatment, and stressor (n=7 to 9 per group). Also shown are corresponding P-values for main effects, and two- and three-way interactions among genetic line, treatment, and stressor. Data for CORT were transformed by log10(X) to reduce heterogeneity of variance.

Table 4-4. The interaction between genetic line and stress on the mRNA abundance of galanin, MCH, and POMC.

	Control	Stressed	*P*-value
galanin			
LWS	1.03 ± 0.08	1.19 ± 0.07	0.18
HWS	1.40 ± 0.09	1.14 ± 0.09	0.04
P-value	0.004	0.66	
MCH			
LWS	1.00 ± 0.07	1.25 ± 0.07	0.003
HWS	0.92 ± 0.04	0.88 ± 0.04	0.68
P-value	0.33	<0.0001	
POMC			
LWS	1.02 ± 0.07	1.02 ± 0.07	0.80
HWS	1.55 ± 0.10	1.26 ± 0.08	0.01
P-value	<0.0001	0.03	

Relative quantity of hypothalamic melanin concentrating hormone (MCH), galanin, and pro-opiomelanocortin (POMC) mRNA in vehicle or NPY-treated chicks from lines selected for low (LWS) or high (HWS) body weight under control or stressed conditions. There was an interaction between genetic line and treatment for all four genes ($P < 0.05$). Values represent means ± standard error (n=9 to 10 per group). The interactions were separated using Tukey's test.

Table 4-5. The interaction between genetic line and treatment on the mRNA abundance of NPYR2, CRFR1, DDC.

	Vehicle	NPY	*P*-value
NPYR2			
LWS	1.01 ± 0.04	0.92 ± 0.02	0.06
HWS	1.07 ± 0.03	1.12 ± 0.03	0.29
P value	0.26	0.0001	
CRFR1			
LWS	1.02 ± 0.05	0.91 ± 0.04	0.17
HWS	0.73 ± 0.03	0.83 ± 0.04	0.26
P-value	<0.0001	0.39	
DDC			
LWS	0.99 ± 0.06	0.79 ± 0.06	0.76
HWS	0.67 ± 0.04	0.71 ± 0.06	0.13
P-value	0.002	0.046	

Relative quantity of hypothalamic dopamine decarboxylase (DDC) and corticotropin-releasing factor receptor sub-type 1 (CRFR1) and neuropeptide Y receptor 2 mRNA in vehicle or NPY-treated chicks from lines divergently selected for low (LWS) or high (HWS) body weight. There was an interaction between genetic line and treatment (P = 0.007). Values represent means ± standard error (n=9-10 per group). The interactions were separated using Tukey's test.

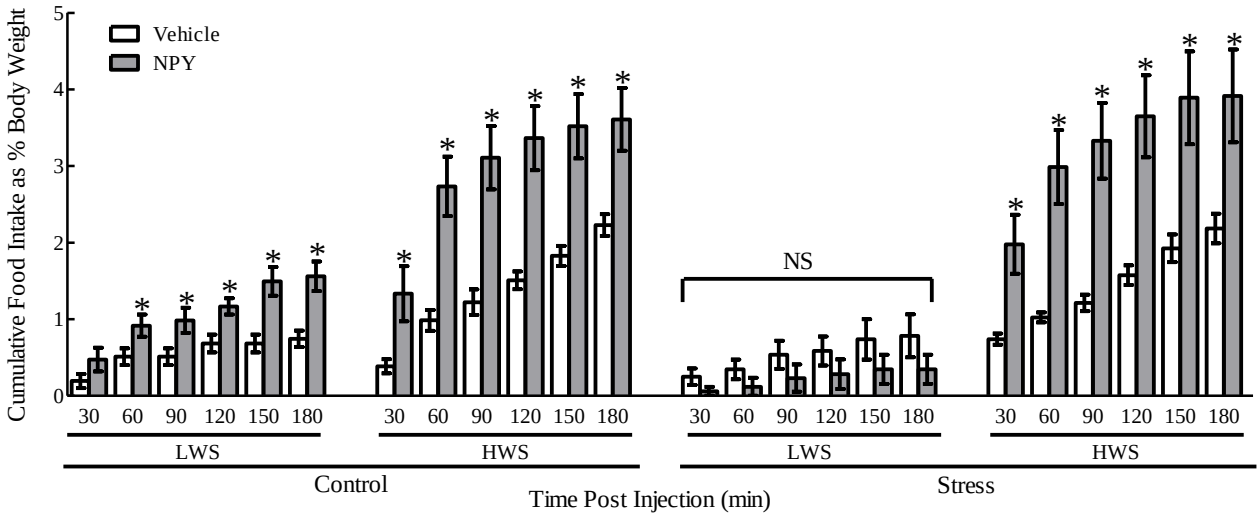

Figure 4-1. Means ± standard errors (n=7 to 10 per group) of cumulative food intake expressed as percentage of body weight following ICV injection of NPY in low (LWS) and high (HWS) body weight-selected lines of chicks with or without stress. *Different from vehicle-injected within a time point, line, and stress group ($P < 0.05$). Non-significance from 30-180 min post injection for stressed LWS is denoted as NS.

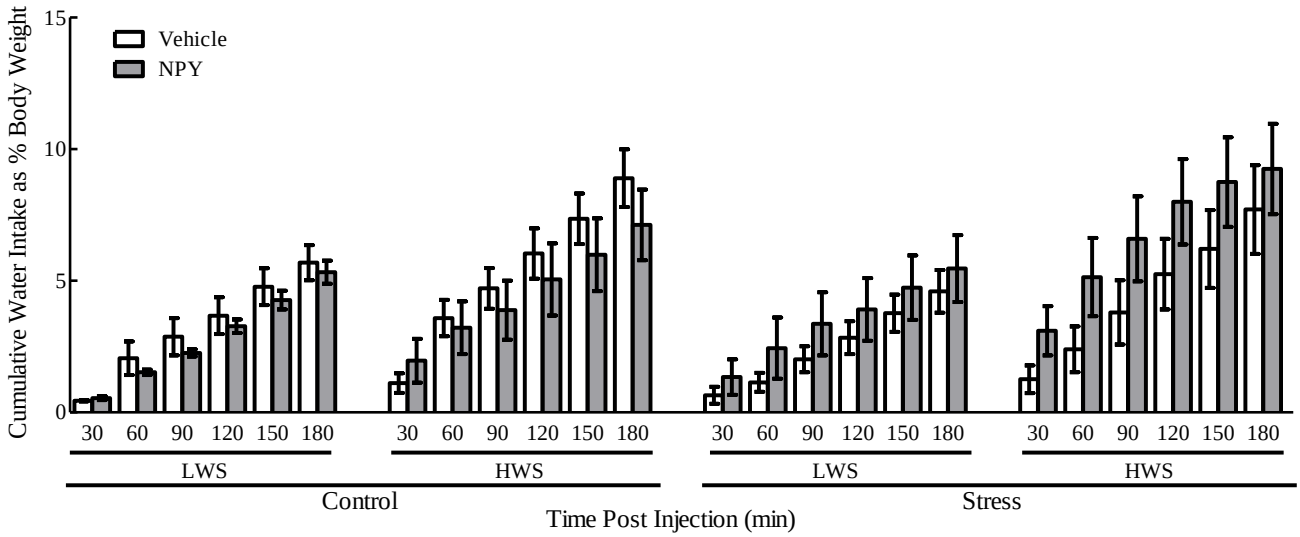

Figure 4-2. Means ± standard errors (n=7 to 10 per group) of cumulative water intake expressed as a percentage of body weight following ICV injection of NPY in low (LWS) or high (HWS) body weight-selected lines of chicks with or without stress. Water intake did not differ between vehicle and NPY-treated chicks.

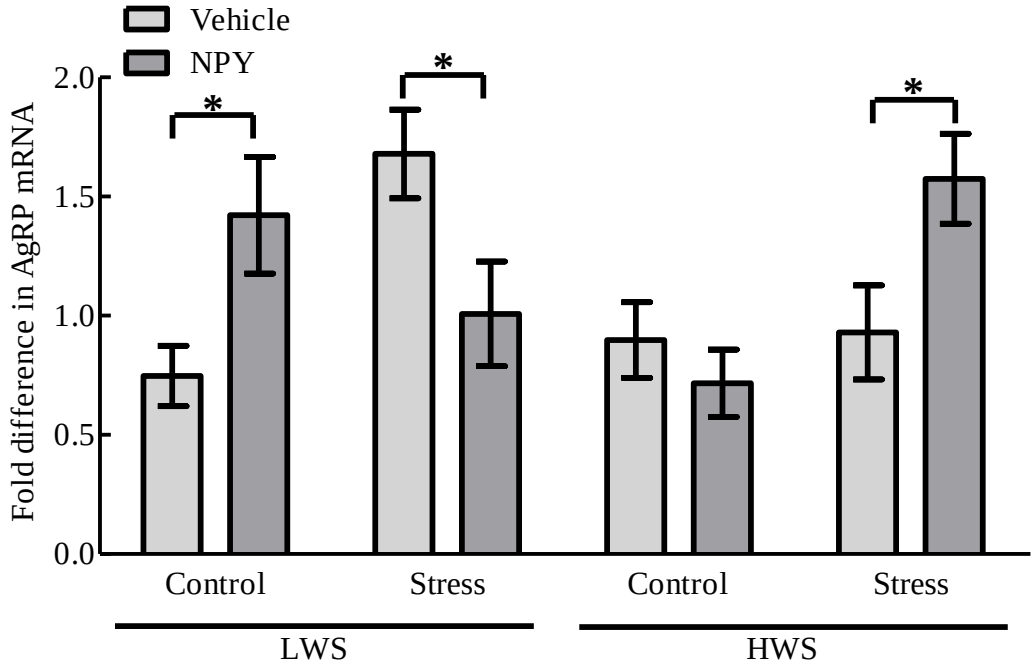

Figure 4-3. Relative quantity of hypothalamic agouti-related peptide (AgRP) mRNA in vehicle or NPY-treated chicks from lines selected for low (LWS) or high (HWS) body weight and exposed to stress. There was an interaction among genetic line, treatment, and stress (P = 0.0001). Values represent means ± standard error (n=9 to 10 per group). The three-way genetic line*treatment* stress interaction was sliced by genetic line and stress, considering only the treatment differences within one slice. *Different from vehicle-injected within a stress group and line ($P < 0.05$).

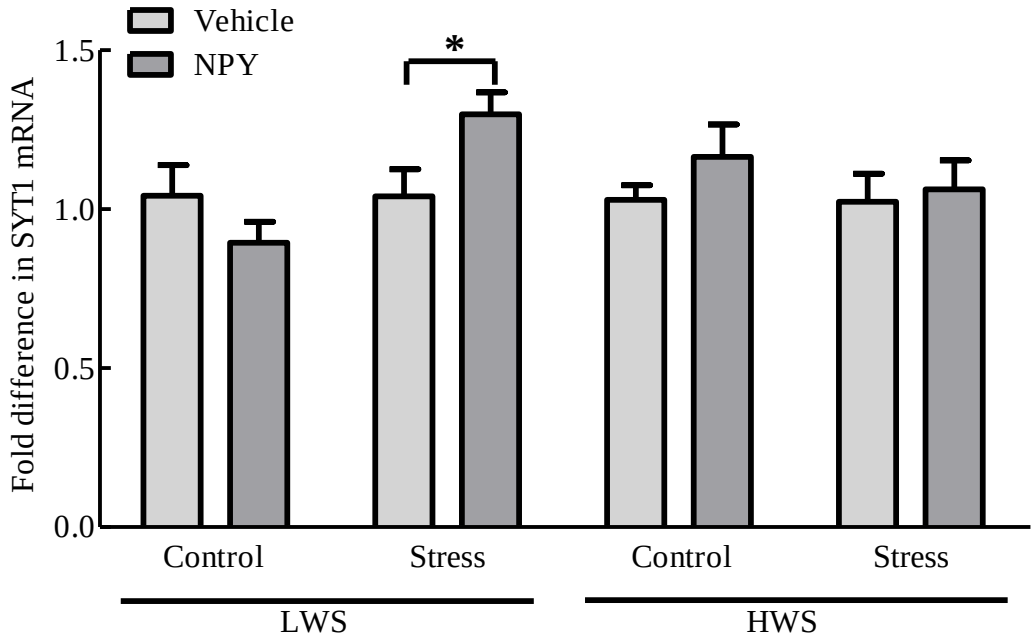

Figure 4-4. Relative quantity of hypothalamic synaptotagmin 1 (SYT1) mRNA in vehicle or NPY-treated chicks from lines selected for low (LWS) or high (HWS) body weight and exposed to stress. There was an interaction among genetic line, treatment, and stress ($P = 0.03$). Values represent means ± standard error (n=9 to 10 per group). The three-way genetic line*treatment* stress interaction was sliced by genetic line and stress, considering only the treatment differences within one slice. *Different from vehicle-injected within a stress group and line ($P < 0.05$).

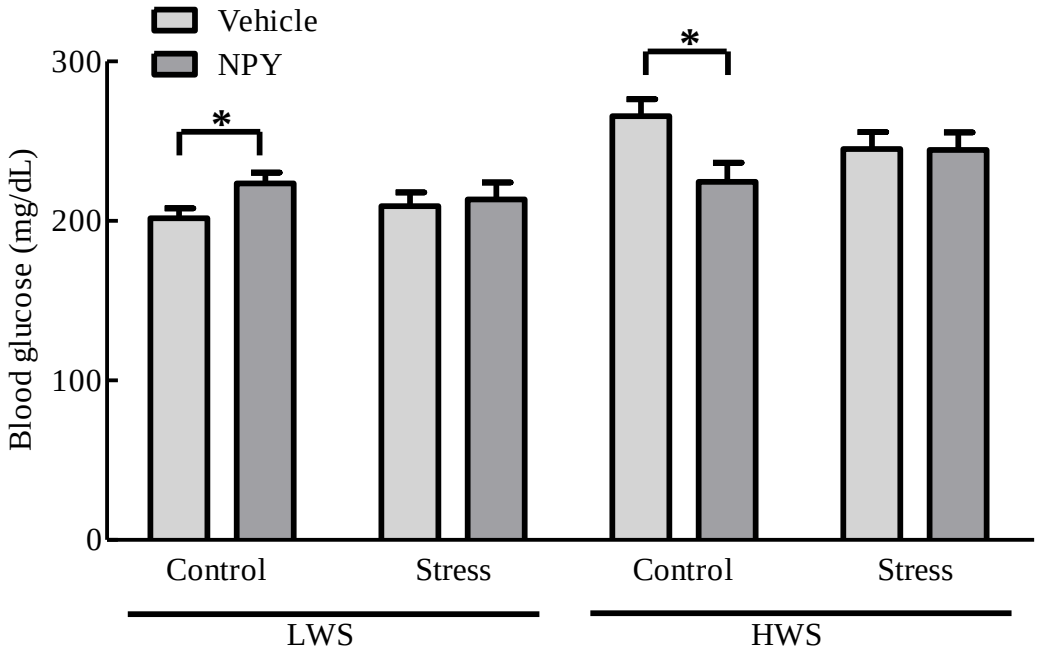

Figure 4-5. Blood glucose concentrations (mg/dL) in vehicle or NPY-treated chicks from lines divergently selected for low (LWS) or high (HWS) body weight and exposed to stress. There was an interaction among genetic line, treatment, and stress ($P = 0.02$). Values represent means ± standard error (n=9 to 10 per group). The three-way genetic line*treatment* stress interaction was sliced by genetic line and stress, considering only the treatment differences within one slice. *Different from vehicle-injected within a stress group and line ($P < 0.05$).

Chapter 5

Proteomic analysis of the hypothalamus in fed and fasted chicks from lines divergently selected for low or high body weight

Abstract: Divergent selection of chicken lines for either low (LWS) or high (HWS) body weight at 56 days of age for more than 57 generations resulted in different feeding behaviors. The LWS have varying severities of anorexia while all HWS become obese. Various studies have shown differences in hypothalamic mRNA abundance between LWS and HWS chicks. However, there are no reports on hypothalamic protein profiles in these genetic lines. Using label-free liquid chromatography tandem-mass spectrometry (LC-MS/MS), we characterized hypothalamic proteomes in LWS and HWS chicks. Hypothalamus was obtained from fed and 3 hour-fasted 5 day-old male LWS and HWS chicks (n=6 per group). A total of 741 proteins were identified. Four comparisons were performed: LWS fed vs LWS fasted (list 1), HWS fed vs HWS fasted (list 2), LWS fed vs HWS fed (list 3), and LWS fasted vs HWS fasted (list 4). KEGG pathway analysis was used to identify the metabolic processes and pathways associated with each list. In HWS, many of the enriched pathways were related to energy metabolism. Among the top differentially abundant proteins, neuronal growth regulator 1 was increased after fasting in the LWS. The protein abundance of aspartate aminotransferase, glutamate decarboxylase, and vesicular inhibitory amino acid transporter, which are involved in the synthesis and uptake of GABA, were greater in LWS than HWS. Long term selection for body weight led to correlated responses in appetite, which is associated with hypothalamic differences in proteins involved in metabolic processes, GABA synthesis, and protein ubiquitination. In addition, results suggest that HWS chicks are more sensitive in their hypothalamic response to short-term energy deprivation.

Keywords: chicken, anorexia, obesity, hypothalamus, proteome

Introduction

Through long term continuous selection (more than 57 generations) for low (LWS) or high (HWS) juvenile body weight, the Virginia lines of chickens have a 10-fold difference in body weight at selection age of 56 days post-hatch [1]. The LWS line is comprised of lean individuals with different severities of anorexia and the HWS are all hyperphagic and become obese as juveniles [1, 2].

It is advantageous to study the most severe anorexic condition in LWS chicks at an early age because a portion of chicks from this line do not start to eat and die within the first week post-hatch following yolk sac resorption [3]. It was proposed that differences in appetite regulation between the lines are of hypothalamic origin, as it was shown that the LWS became obese after lesioning of the ventromedial hypothalamus (VMH) [4]. We have compared feeding responses (including hypothalamic nuclei activation) of many appetite-associated neuropeptides and found differences between the lines. For example, the threshold in food intake response to anorexigenic factors, such as α- melanocyte-stimulating hormone (MSH) [5], corticotropin-releasing factor (CRF) [6], insulin [7], amylin [8], ghrelin [9], and neuropeptide AF [10] is lower in LWS than HWS. In contrast, HWS have a lower threshold response to neuropeptide S (NPS) [11] and calcitonin and calcitonin gene-related peptide [12] than LWS while galanin [13] has similar thresholds of sensitivity in their effects on food intake in the lines. For two factors there is a complete loss of response in one line: the LWS does not respond to exogenous neuropeptide Y (NPY) with increased food intake [14] and the HWS does not decrease food intake after central injection of leptin [15].

The hypothalamus, which consists of several distinct nuclei, plays a crucial role in the

regulation of appetite by integrating and coordinating multiple nutrient-related signals from both peripheral and central nervous systems [16, 17]. Ka et al., (2005, 2009, and 2011), Zhang et al., (2015), and Yi et al., (2015) reported differences in gene expression profiles in the hypothalamus between LWS and HWS chicks using techniques such as cDNA microarrays, real-time PCR, and RNA sequencing [18-22]. Yi et al., (2015) reported that 5 day-old LWS chicks expressed more mRNA for genes encoding anorexigenic factors, such as calcitonin, CRF receptor 1, leptin receptor, NPS, melanocortin receptor 3 (MC3R), and oxytocin than HWS, while no differences were observed between the lines for the mRNA abundance of neuropeptide Y (NPY), CRF, and melanocortin receptor 4 (MC4R) [22]. However, differences in hypothalamic protein profiles between LWS and HWS chicks are unreported. Here we present results generated by label-free liquid chromatography tandem-mass spectrometry (LC-MS/MS) to characterize the hypothalamic proteome in LWS and HWS chicks.

Materials and methods

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 [23] with details of the selection program reported elsewhere [24, 25]. The founder population consisted of crosses of 7 inbred lines with the LWS and HWS selected lines maintained as closed populations. Eggs obtained from age contemporary parents from S57 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 3,000 kcal metabolizable energy/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 = 6 for each group, male): LWS fed, LWS fasted, HWS fed and HWS fasted. The fasting duration was 180 min (to mimic our previous fasting duration) [6, 8, 12]. Chicks were provided access to drinking water during the fasting. 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 seconds. 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 per the following anatomy: perpendicular to the midline suture a cut was made at the septopallio-mesencephalic tract and at the third cranial nerves. Then 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 [26]. This block (comprised primarily of the hypothalamus) was immediately wrapped with sterile foil and stored at -80°C until further processing.

Protein extraction and LC-MS/MS analysis

Hypothalamus was homogenized using 5 mm stainless steel beads (Qiagen, MD, USA), lysis buffer (20 mM Tris-HCl, pH 6.8, 137 mM NaCl, 10% glycerol, and 2 mM EDTA) containing Halt Protease Inhibitor Cocktail (Thermo Scientific), and a Tissue Lyser II (Qiagen). After homogenization, samples were incubated in a rotating shaker at 4ºC for 2 hours. Samples were then centrifuged for 20 minutes at 12,000 x g at 4ºC and supernatant (1 ml) transferred to a fresh tube. Approximately 250 μg protein from each sample was precipitated by the addition of 1

ml LC/MS grade methanol (Spectrum Chemicals, New Brunswick, NJ) and incubation at -80ºC for 2 hours. Precipitated protein was collected at the bottom of each tube by centrifugation for 20 minutes at 12,000 x g. Each pellet was washed once with 1 ml LC/MS grade methanol. Protein pellets were resuspended in 250 µl freshly-prepared 8 M urea in 100 mM ammonium bicarbonate (AmBic). Proteins were denatured and disulfides reduced by incubation for 1 hour at 37ºC after the addition of 4.5 mM dithiothreitol (DTT) in 100 mM AmBic. Free sulfhydryls were then alkylated by incubation for 30 minutes at room temperature in the dark after the addition of freshly-prepared 10 mM iodoacetamide (IA) in 100 mM AmBic. Unreacted IA was then neutralized by the addition of 10 mM DTT in 100 mM AmBic and urea content diluted to 4 M by bringing the final volume to 500 µl by the addition of 100 mM AmBic. Endoproteinase LysC (Wako, Richmond, VA) was added at 1:100 (w/w) and digestions incubated with shaking at 37ºC overnight. The following day urea content was further diluted to 1.4 M by bringing the final volume to 1.43 ml by the addition of 100 mM AmBic and trypsin was added at 1:50 (w/w) and the digestions incubated for four hours with shaking at 37ºC.

Digestions were desalted using solid phase extraction (SPE) after acidification by the addition of 0.2% (v/v) trifluoroacetic acid (TFA) and 1% (v/v) formic acid. SPE utilized Sep-Pak® Vac 1cc/50 mg C18 cartridges (Waters Corporation, Milford, MA). Cartridges were conditioned and equilibrated using 1 ml LC/MS grade methanol followed by 1 ml solvent Y (98:2 LC/MS grade water: LC/MS grade acetonitrile containing 0.1% (v/v) TFA). The sample was then applied to the cartridge followed by 3 x 1 ml washes of solvent Y and desalted peptides were finally recovered using 1 ml solvent Z (50:50 LC/MS grade water: LC/MS grade acetonitrile containing 0.1% (v/v) TFA). Peptide samples were concentrated to dryness using a centrifugal vacuum concentrator, resolubilized in 250 µl solvent Y by sonication and stored at -

20°C.

Triplicate runs (3 µl injections) of each resolubilized peptide sample were separated through an Acquity I-class UPLC system (Waters Corporation, Milford, MA) utilizing a randomized sample queue. The mobile phases were solvent A (0.1% (v/v) formic acid (Sigma-Aldrich Corporation, St. Louis, MO) in LC/MS grade water (Spectrum Chemicals and Laboratory Products, New Brunswick, NJ)) and solvent B (0.1% (v/v) formic acid (Sigma-Aldrich Corporation, St. Louis, MO) in LC/MS grade acetonitrile (Spectrum Chemicals and Laboratory Products, New Brunswick, NJ)). The separation was performed using a CSH130 C18 1.7 µm, 1.0 x 150 mm column (Waters Corporation, Milford, MA) at 50 µL/min using a 110 minute gradient from 3-40% solvent B. The column temperature was maintained at 45˚C. A master mix, equal amount of all samples, was also processed in triplicate as described above.

Column effluent was analyzed using a Synapt G2-S mass spectrometer (Waters Corporation, Milford, MA) using an HDMSe (high-definition mass spectrometry with alternating scans utilizing low and elevated collision energies) acquisition method in continuum positive ion "resolution" MS mode. Source conditions were as follows: capillary voltage, 3.0 kV; source temperature, 120˚C; sampling cone, 60 V; desolvation temperature, 350˚C; cone gas flow, 50 L/hr; desolvation gas flow, 500 L/hr; nebulizer gas flow, 6 bar. Both low energy (4 V and 2 V in the trap and transfer region, respectively) and elevated energy (4 V in the trap and ramped from 20 to 50 V in the transfer region) scans were 1.2 seconds each for the m/z range of 50 to 1800. For ion mobility separation, the IMS and transfer wave velocities were 600 and 1200 m/sec, respectively. Wave height within the ion mobility cell was ramped from 10 to 40 V.

For lock-mass correction, a 1.2 second low energy scan was acquired every 30 seconds of a 100 fmol/µL [Glu1]-fibrinopeptide B (Waters Corporation, Milford, MA) solution (50:50

acetonitrile: water supplemented with 0.1 % formic acid) infused at 10 µL/min introduced into the mass spectrometer through a different source which was also maintained at a capillary voltage of 3.0 kV. The data for lock-mass correction were collected but not applied to sample data until data processing.

Protein identification and label-free quantification

Mass spectrometric data from 1-101 minutes of each chromatographic run were processed and analyzed utilizing ProteinLynx Global Server v. 3.0.2 (PLGS, Waters Corporation, Milford, MA). Average chromatographic and mass spectrometric peak width resolution were set to 0.3 min and 30000 FWHM, respectively. Mass values were lock-mass corrected based on the exact m/z value of the +2 charge state of [Glu1]-fibrinopeptide B (785.842). Peaks were defined based on the low energy, elevated energy and bin intensity thresholds of 250, 25 and 2000 counts, respectively. The final peak list for each sample was then searched against a protein database containing the complete *G. gallus* proteome including isoforms downloaded from Uniprot (www.uniprot.org) on 9/2/2014 and 3 randomized decoy entries for each real entry appended using PLGS. Workflow parameters for the protein identification searches were 2 possible missed cleavages utilizing Lys-C and trypsin as the protease combination, a fixed modification of carbamidomethylation of cysteine, possible modifications of Gln to pyroGlu when Gln is present at the N-terminus of a peptide and oxidation of Met. The software automatically determined peptide and peptide fragment mass tolerances. Protein identification searches using PLGS had a false discovery rate of no more than 3%.

Following analysis using PLGS, the results were imported into IsoQuant for further processing. Only those proteins identified by two or more peptides were included in the final data analysis. The protein false discovery rate was limited to less than 1%). Proteins were

quantitated utilizing the Top 3 peptide method by summing the precursor intensities of the three most intense peptides per protein. Peptides containing variable modifications and those with missed cleavages were used for protein quantitation in addition to those that were unmodified and trypsin-specific. The same 3 peptides were used for quantitation of each protein across all replicates.

Data analysis

Planned comparisons were used to compare the following groups: LWS fed vs LWS fasted (list 1), HWS fed vs HWS fasted (list 2), LWS fed vs HWS fed (list 3), and LWS fasted vs HWS fasted (list 4) (supplemental data) using Student's t-tests with a Bonferroni correction (< 0.0125) applied to the *P*-value. All statistical analyses were performed with JMP 11 Pro (SAS institute, Cary, NC, USA). The GO enrichment analysis on differentially expressed proteins was performed by using KEGG pathway mapping (http://www.genome.jp/kegg/).

Results

The present study aimed to reveal differences in the presence of hypothalamic proteins between LWS and HWS chicks in fed and fasted states at 5 days of age. Six male chicks per group were used for the proteomics analysis; LWS fed, LWS fasted, HWS fed, and HWS fasted. A total of 741 proteins were identified in the current experiment (supplemental data). Planned contrasts for data analysis were used to compare the following groups: LWS fed vs LWS fasted (list 1), HWS fed vs HWS fasted (list 2), LWS fed vs HWS fed (list 3), and LWS fasted vs HWS fasted (list 4) (supplemental data). Hence, the analysis resulted in four lists of differentially abundant (DA) proteins. Lists 1 to 4 depict significantly different proteins between the two groups in each comparison. Volcano plots were used to plot the 4 comparisons (Figure 5-1). Volcano plots are usually used in –omics studies to quickly identify genes or proteins that have

both large and highly significant changes [27]. In volcano plots, the x-axis shows the expression change for a given protein while y-axis represents the corresponding statistical *P* value.

In line with the neuroendocrine regulation of the hypothalamus, we identified some important proproteins (e. g. somatostatin and vasotocin-neurophysin) and proteins that are associated with vesicle trafficking (clathrin-, GTP-, Ras-related protein, and vesicle-trafficking protein), as well as calcium signaling (e. g. calcium/calmodulin-dependent kinase, calcium-transporting ATPase, and calcium binding proteins), neurotransmitter synthesis (glutamate dehydrogenase, peptidyl-prolyl cis-trans isomerase, prostaglandin E synthase, and aspartate aminotransferase), and synaptic vesicle function (e. g. synaptogyrin, syntaxins, synaptophysin, and synaptotagmins).

Protein differences between fed and fasted chickens

In LWS chicks, only 12 and 15 proteins (list 1) were downregulated and upregulated respectively in LWS chicks in response to fasting. By comparison, 113 proteins (list 2) changed after 3 hours of fasting, with 71 proteins downregulated and 42 proteins upregulated in HWS.

Protein differences between LWS and HWS chicks

A total of 154 and 198 proteins were different between LWS and HWS chicks in fed and fasted states, respectively. In the fed state, 79 proteins (list 3) were greater in HWS than LWS chicks and 75 proteins were greater in LWS chicks. In the fasted state, 111 proteins were greater in the LWS and 87 proteins were greater in the HWS.

KEGG pathway analysis

The KEGG pathway mapping was used in our gene ontology and pathway mapping analyses. Among the 327 DA proteins, 260 proteins could be mapped into various pathways. Only pathways that contained more than 3 proteins were considered to be significantly enriched.

In response to fasting, only one pathway was significant in LWS whereas there were 8 significant pathways in HWS. The number of significant pathways were 14 and 20 between LWS and HWS in the fed and fasted states respectively. Many of these significant pathways were related to energy metabolism, such as carbon metabolism, the citrate cycle, and oxidative phosphorylation.

Top differentially abundant proteins

We used a minimum ratio of 1.20 for defining the top downregulated DA proteins and 0.80 for the top upregulated DA proteins in each protein list comparison (supplemental data). The top differentially abundant proteins are also shown in Figure 5-1 as red (downregulated) and blue (upregulated) dots. Proteins of particular interest from each list are described below. In response to fasting, neuronal growth regulator 1 (NEGR1) was upregulated in LWS. Fasting decreased the protein abundance of vesicular inhibitory amino acid transporter (VIAAT) and aspartate aminotransferase in HWS. In the fed state, the abundance of ubiquitin-conjugating enzyme E2 D3 (UBE2D3) was increased in HWS. In the fasted state, protein abundance of aspartate aminotransferase, amino acid transporter, VIAAT, and glutamate decarboxylase 2 were greater in LWS whereas UBE2D3 was greater in HWS. The protein abundance of NEGR1 was greater in fasted LWS than HWS even though it was not one of the top DA proteins ($P = 0.006$, ratio = 1.168).

Discussion

The purpose of the current study was to demonstrate differences in the abundance hypothalamic proteins between LWS and HWS chicks in two feeding states using label-free LC-MS/MS. To our knowledge, few studies have been conducted to evaluate global protein profiles in chickens through the use of label-free LC-MS/MS, although the method has been successfully applied to study the proteome of serum, brain tissue, and human salivary and pituitary glands

[28-33]. Proteomic studies have been conducted to understand chicken post-hatch muscle and intestinal development, egg production, and embryonic development [34-38]. For example, Kuo et al., (2004) identified chicken hypothalamic proteins using two-dimensional gel electrophoresis (2-DE) coupled to LC-MS/MS, which demonstrated that six distinct proteins differed between high and low egg production strains of chickens [37]. In the current study, we identified 741 proteins in the hypothalamus of 5-day old chickens. Using similar methods, Stelzhammer et al., (2012) detected 622 unique proteins in rat hypothalamus [32].

The current study was a two-factor experimental design, whose purpose was to identify differences between LWS and HWS, with an emphasis on responses of the two lines to fasting assuming that the 3 hour fast would accentuate differences that are appetite-related. Although the hypothalamus is a major regulator of appetite, it is also the site for other physiological non-appetite-related processes. Thus, fasting was intended to reveal effects that were more related to feeding behavior. As a result, we used planned comparisons with Bonferroni corrections to control the family-wise error rate to be 0.05 or less. This produced 4 lists of proteins for each comparison.

The main observation was that LWS and HWS chicks responded differently to 3 hours of fasting as indicated by the change in a large number of proteins in HWS (113 proteins) but not LWS chicks (only 27). These results are intriguing in that HWS chicks are all hyperphagic and become obese whereas LWS chicks have different severities of anorexia, which may indicate that LWS chicks are more resistant to effects of short-term fasting on hypothalamic physiology. Pathway analysis on the 113 differentially abundant proteins in fasted HWS chicks revealed that many of these enriched pathways (contained more than 3 proteins) were involved in energy metabolism. Similar pathways were also present in differentially abundant proteins between

LWS and HWS chicks.

Using cDNA microarrays, Ka et al., (2009) observed that the expression of hypothalamic genes encoding factors that regulate neuroplasticity and growth were different between LWS and HWS chicks at day of hatch and 56 days post-hatch in a region of the brain containing the hypothalamus and brainstem [19]. Similarly, differences in hypothalamic neuroplasticity between 4 day-old LWS and HWS was also observed using oligonucleotide arrays [20]. Actin is one of the main components of the cytoskeleton and plays important roles in neuronal development, plasticity, and a variety of cellular processes including cell motility, cell division, cell morphogenesis, as well as intracellular protein trafficking [39]. The two studies mentioned above were focused at the gene level [19, 20]. At the protein level, we observed changes in proteins indicative of neuroplasticity between LWS and HWS chicks in both feeding states according to the enriched pathway of regulation of actin cytoskeleton in KEGG, which was supported by previous studies [19, 20].

Neuronal growth regulator 1 (NEGR1) is among the genes identified in human genome-wide association studies (GWAS) as common obesity loci [40]. It may be involved in the control of body weight and food intake. Lee et al. (2012) reported that NEGR1 mRNA was found in distinct hypothalamic nuclei and disruption of NEGR1 in mice resulted in reduction in body weight [41]. We found that LWS displayed increased protein abundance of NEGR1 after fasting, and NEGR1 was one of the top DA proteins. In rats, food restriction increased the mRNA expression of NEGR1 in the arcuate nucleus and VMH [42], which supported our finding that fasting increased NEGR1 abundance in LWS. However, NEGR1 abundance did not increase in HWS after fasting. NEGR1 is closely related to obesity. Considering that LWS are anorexic and HWS are obese, it is unclear why LWS had greater NEGR1 abundance than HWS and why

NEGR1 further increased after fasting in the LWS.

Hypothalamic gamma-aminobutyric acid (GABA), a dominant inhibitory amino acid neurotransmitter, plays an important role in the inhibitory circuits in the hypothalamus [43]. Glutamate decarboxylase catalyzes the decarboxylation of glutamate to GABA. Glutamate decarboxylase 2 was greater in LWS than HWS in the fasted state, which indicates that LWS may have higher levels of GABA in the hypothalamus. The fasted LWS also had more aspartate aminotransferase than HWS, which is an essential enzyme for the synthesis of glutamate [44]. In the central nervous system, vesicular storage and the subsequent exocytosis of GABA is the main pathway for inhibitory signal transmission [45]. The synaptic vesicle protein, VIAAT, is responsible for the vesicular storage of GABA and mediates the uptake of GABA [46]. The VIAAT protein was greater in LWS than HWS in the fasted state, which demonstrates that the uptake of GABA is different between LWS and HWS. Fasting decreased the abundance of aspartate aminotransferase and VIAAT in HWS but not LWS. Overall, the LWS had greater amounts of enzymes involved in the synthesis of glutamate and GABA as well as the vesicular transporter important for the uptake of GABA, which suggests that LWS may have strengthened GABA systems in the hypothalamus. George et al. (1977) reported that GABA plays a role in food intake in rats [47]. The differences in the hypothalamic GABA system between LWS and HWS may explain some of the differences in their feeding behavior.

Ubiquitination is the process of ubiquitin conjugation to other cellular proteins and plays important roles in a range of cell functions, such as protein degradation [48], transitions in the cell cycle [49], and the induction of the inflammatory response [50]. Ring finger proteins play critical roles in ubiquitination [51]. Rubin et al. (2010) reported that the mRNA expression of SH3 domain containing ring finger 2, which is one of the ring finger proteins, was detected in the

LWS but not HWS in the hypothalamus, and a deletion in the gene in HWS was identified as a candidate causal mutation to explain differences in body weight between the lines [52]. In the current study, UBE2D3, an enzyme that catalyzes the attachment of ubiquitin to the substrate protein, was greater in HWS than LWS in both feeding states. Due to the wide range of functions regulated by ubiquitination, differences in ubiquitin-related proteins between the lines indicate that ubiquitination-related functions may differ, which may be related to the differences in their feeding behavior.

One caveat of the current study was that the methods used to generate protein lysates were not specific for the capture of neuropeptides nor for the enrichment of membrane-associated proteins such as transporters and G protein-coupled receptors. Those neuropeptides and associated receptors include appetite regulatory factors and it is possible that the lack of enrichment for appetite or neuroplasticity-associated pathways was due to our method being more optimized for larger, cytosolic proteins.

In conclusion, the current study focused on identifying hypothalamic protein profiles in response to fasting in chicks from lines divergently selected long-term for low or high juvenile body weight. A large number of proteins were identified and many of these proteins were differentially expressed either between fed and fasted HWS or between fed LWS and HWS chicks. In response to fasting, HWS chicks were more sensitive than LWS chicks in their physiological response as demonstrated by the larger number of proteins changed in fasted HWS chicks. Functional analysis revealed that changes in metabolic processes and neuroplasticity differed between LWS and HWS. Compared to HWS chicks, LWS chicks had more proteins involved in the synthesis and uptake of GABA. In summary, long term selection for body weight has been associated with correlated responses in appetite and those differences in feeding

behavior may be associated with proteins involved in metabolic processes, synthesis of GABA, and protein ubiquitination.

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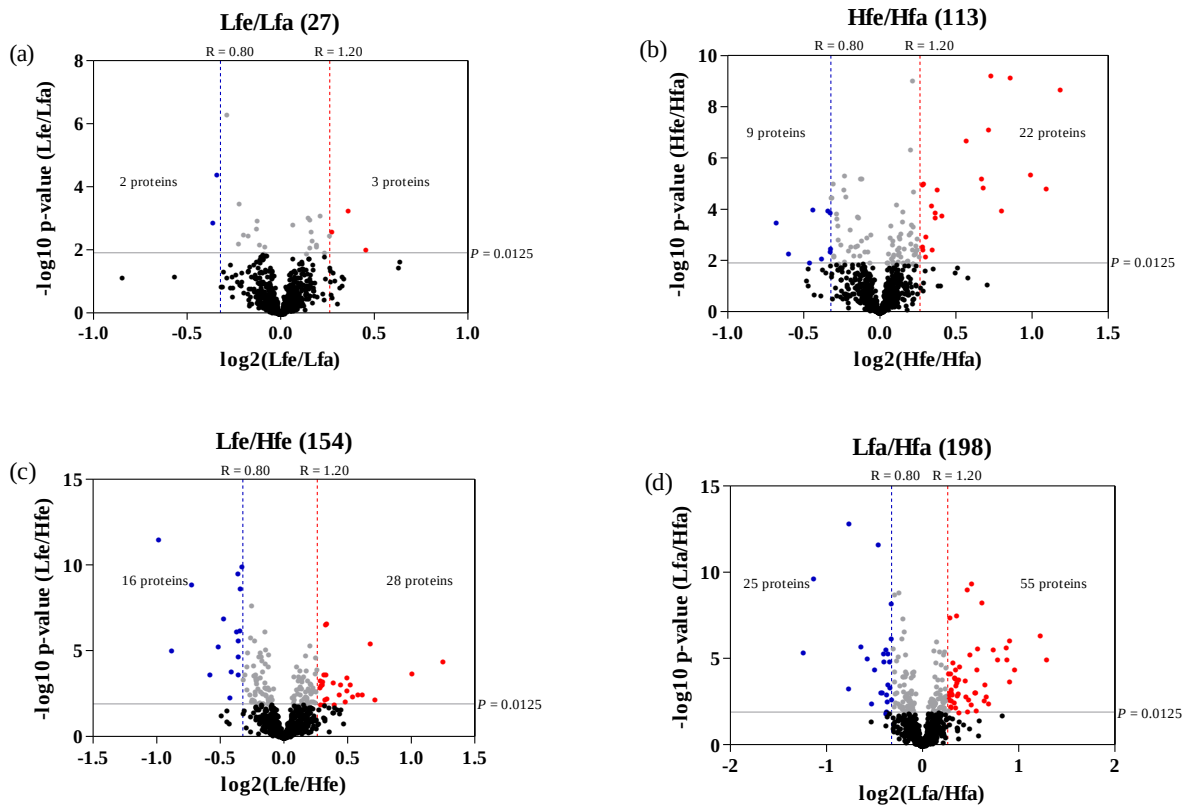

Figure 5-1. Volcano plots for the 4 comparisons. The log2 expression ratio is plotted versus the –log10 of the P value from t-test. (a) Plot comparing LWS fed and fasted (Lfe/Lfa). (b) Plot comparing HWS fed and fasted (Hfe/Hfa). (c) Plot comparing LWS and HWS in fed state (Lfe/Hfe). (d) Plot comparing LWS and HWS in fasted state (Lfa/Hfa). Proteins above the P value cutoff (0.0125, gray line) are condiered to have significantly changed. The total number of different proteins are given in parenthesis. Dots on the right side of the red-dashed line represent proteins with a ratio larger than 1.20 and dots on the left side of the blue-dashed line represent proteins with a ratio less than 0.80. The red and blue dots represent significantly changed proteins with a ratio larger than 1.20 (downregulated) and less than 0.80 (upregulated) respectively. The gray dots represent significantly changed proteins with a ratio between 0.80 and 1.20.

Chapter 6

Synthesis

This dissertation consists of a series of experiments designed to determine the expression and abundance of endogenous hypothalamic appetite-associated factors in chicken lines that have undergone long-term selection (over 57 generations) for either low (LWS) or high (HWS) body weight at 8 weeks of age. The LWS have different severities of anorexia while HWS become obese. Results from these studies can be used to explain molecular mechanisms of neurological pathways regulating energy balance in chickens, and may also be used to provide insights onto body weight dysfunctions in other species including humans.

In the first experiment, the expression of appetite-associated factors from the whole hypothalamus in the LWS and HWS lines was investigated in fed and fasted 5-d old chicks. The results showed that although mRNAs encoding orexigenic neuropeptides did not differ in expression between the lines, the expression of anorexigenic factors, including calcitonin, CRF receptor 1, leptin receptor, neuropeptide S, melanocortin receptor 3, and oxytocin were greater in LWS than HWS chicks. The greater expression of anorexigenic factors in the hypothalamus and their possible interactions may collectively contribute to the low appetite in LWS chicks. Due to the complexity of neuronal regulation of appetite and possible interactions of distinct hypothalamic nuclei in the control of feeding behavior, gene expression from whole hypothalamus can only help explain part of the differences in appetite regulation in the lines.

Hence we designed the second experiment to pinpoint the expression profiles of appetite-associated factors in distinct hypothalamic nuclei to further demonstrate the possible causes of different feeding behavior between the lines. We used the same experimental design as discussed above but collected samples from five appetite-related hypothalamic nuclei, which include the

ARC, DMN, LH, PVN and VMH. Gene expression results derived from these five hypothalamic nuclei demonstrated that different feeding behaviors between LWS and HWS may be due to differences in gene expression of appetite-associated factors in specific appetite-related nuclei and a complex interplay between them. The results showed that the mRNA expression of NPY in the ARC and mesotocin in the PVN were greater in fasted LWS than HWS chicks. The expression of NPYR5 in the PVN was greater in fed LWS than HWS chicks. The greater expression of mesotocin may play an important role in inducing anorexia in LWS chicks.

In experiment three, we demonstrated that exogenous NPY lost its orexigenic effect in 5-d old LWS but not HWS chicks after a combination of cold exposure and food deprivation after hatch and identified some molecular mechanisms. The ICV injection of NPY increased and decreased the expression of AgRP in non-stressed and stressed LWS chicks, respectively. Even though the effect of AgRP on food intake regulation is different in different breeds of chickens, the results demonstrated that endogenous AgRP may play a role in appetite regulation in LWS chicks.

Using label-free liquid chromatography tandem-mass spectrometry (LC-MS/MS), we characterized hypothalamic proteomes in LWS and HWS chicks in experiment four. Hypothalamus was obtained from fed and 3 hour-fasted 5 day-old male LWS and HWS chicks (n=6 per group). In response to fasting, more proteins were observed to be changed in HWS (113) than LWS (27) chicks, which clearly demonstrated that LWS chicks were less responsive to the change in energy balance imposed by the fasting. The KEGG pathway analyses of differentially abundant proteins demonstrated that many of these enriched pathways were related to energy metabolism. Among the top differentially abundant proteins, neuronal growth regulator 1 was increased after fasting in the LWS. The protein abundance of aspartate aminotransferase,

glutamate decarboxylase, and vesicular inhibitory amino acid transporter, which are involved in the synthesis and uptake of GABA, were greater in LWS than HWS. Long term selection for body weight led to correlated responses in appetite, which is associated with hypothalamic differences in proteins involved in metabolic processes, GABA synthesis, and protein ubiquitination.

Taken together, LWS chicks have greater hypothalamic expression of factors that drive anorexigenic tone than HWS chicks. Analysis of gene expression from hypothalamic nuclei further demonstrated the possible origin of this anorexigenic tone in the LWS chicks, which may be the mesotocin from the PVN that inhibits appetite in LWS chicks. Exposure of LWS to the stress dampened its NPY-related feeding circuity and this delineated that AgRP may function as an alternative to drive food intake in the LWS chicks. The proteomics data further demonstrated the quite different feeding behavior between the lines, namely that LWS chicks are less responsive than HWS chicks to fasting. Ultimately, long term selection for divergent body weight leads to quite different feeding behavior that may be due to differences in gene expression and protein abundance of appetite-associated factors in specific appetite-related nuclei and a complex interplay between them.