Differential expression of Agouti-related Peptide in Avian Species and the Association of Appetite-related Neuropeptides with Nutrition Status

THESIS

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Chen Zhang, B.S.

Graduate Program in Animal Sciences

The Ohio State University

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Thesis Committee:

Dr. Kichoon Lee, Advisor
Dr. Pasha Lyvers-Peffer
Dr. David Latshaw
Abstract

In the poultry industry, the level of feed consumption plays an important role in determining the rate of growth and body composition achieved by animals. Especially for economic species, broiler and turkey, properly increasing feed intake and then elevating growth rate as well as body weight are among the critical goals for the benefit of producers. Therefore, the understanding of the regulatory mechanism of feed intake in the food animal species is of great importance, which is helpful in providing potential nutritional methods to overcome the negative environmental effects and to achieve the improvement of related animal production. The metabolism of food intake regulation is highly conserved along the evolution across different species, thus the knowledge gained from this study could also bring insights for the appetite control and the improvement of obesity in human.

Agouti-related peptide (AgRP), an orexigenic neuropeptide mainly expressing in the arcuate nucleus, is of great importance in the food intake regulation. It is elevated by states of negative energy balance and is considered as the major indicator of poor nutrition status. Besides AgRP, other hypothalamic neuropeptides including NPY, POMC and CART are sensitive to peripheral nutrition status and regulate feed intake behavior. The studies presented here investigate AgRP in the avian specie, especially focus on the differential expression of alternative transcripts and how nutrition status modulates the amount of appetite-associated neuropeptides in the hypothalamus.
Gamma amino-butyric acid (GABA) is a chief inhibitory neurotransmitter in the CNS, whose expression is restricted to the hypothalamus area. It plays a role in regulating neuronal excitability in the arcuate nucleus that is the hypothalamic area in charge of food intake regulation and energy homeostasis. In some feeding studies, GABA is considered as a nutritional supplement in order to overcome negative effects due to heat stress in poultry industry. The presented study aims to understand whether long-term dietary supplementation of GABA in different purity and dosages stimulates feed intake in chickens under normal ambient temperature.

This study reported two major transcript isoforms of AgRP in the avian species, also their differential expression patterns in various tissues. These results revealed AgRP-A form to be expressed in all the avian species, while AgRP-B form to be expressed in the chicken and turkey rather than in the quail. The expression patterns of AgRP A- and B form are different from each other in the chicken and turkey, and AgRP-A form is exclusively expressed in the hypothalamus in the quail. Importantly, the results of cloning genomic DNA and cDNA of AgRP in the chicken, turkey and quail as well as our bioinformatics analysis demonstrated that there is a deletion of 521bp in genomic DNA of AgRP-A form in the quail, which results in the absent expression of qAgRP-B form.

In order to investigate how the mRNA expressions of neuropeptides change with peripheral nutrition status, fast and re-feeding experiments were applied to 22-day old male quails. The results of real-time PCR on four appetite-related neuropeptides indicate that the amount of AgRP and NPY is increased (P<0.05) in
negative energy balance while levels of POMC and CART are decreased (P<0.05) in fast then are elevated (P<0.05) in re-feeding state.

Our GABA feeding experiments revealed that long-term dietary supplementations of both bacterial- and pure GABA in different doses fail to stimulate feed intake in chickens in normal ambient temperature. It suggests that GABA effects on promoting feed intake in the chickens in heat stress, which have been reported by other researchers, tend to release stress due to heat stress rather than to directly regulate appetites in chickens.

This study, to our knowledge, firstly reported the differential expression of alternative transcript isoforms of AgRP in the chicken, turkey and quail. The absent expression of AgRP-B form in the quail and varied expression pattern of two isoforms in various tissues in the avian species suggest the alternative promoter usages of AgRP A- and B form, which might result in the differed biological functions of them. The knowledge gained from present study is helpful in understanding feed intake regulation metabolism in the avian species, and in providing potential targets for appetite control in human.
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Wenner.
Vita

Sep. 2004- Jun. 2007 .................................... Changsha Railway No.1 High School

Sep. 2007- Jul. 2011 ............................................ B.S. Agriculture,

Hunan Agricultural University

Sep. 2011- present .......................................... Graduate Student,

Department of Animal Sciences,

The Ohio State University

Fields of Study

Major Field: Animal Sciences
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List of Abbreviations

GABA .................................................. Gamma Amino-Butyrate Acid

CNS .................................................. Central Nervous System

NPY .................................................. Neuropeptide Y

AgRP .................................................. Agouti-Related Peptide

POMC .................................................. Proopiomelanocortin

CART .............................................. Cocaine and Amphetamine Regulated Transcript

αMSH .................................................. alpha-Melanocyte Stimulating Hormone

CCK .................................................. Cholecystokinin

GLP-1 .................................................. Glucagon Like Peptide-1

PPY .................................................. Peptide YY

RER .................................................. Rough Endoplasmic Reticulum

MCR .................................................. melanocortin receptors

ICV .................................................. Intracerebroventricu
Chapter 1: Introduction

Feed intake regulation is of great interest in animal sciences, because the level of feed consumption is a basic and important factor that determines the rate of growth and body composition achieved by animals (Richards, 2003). Growth rate and meat production are under the control of feed intake and energy expenditure. The adaptive changes in these two factors over the long-term contribute to homeostatic regulation of body energy stores and the maintenance of a constant body weight. The coordinated regulation of feed intake and energy expenditure to achieve energy balance responds to both external environmental cues (feed availability, feed composition, temperature, stressors) and internal physiological signals, which are largely induced by external stimuli (Richards, 2003). Besides those peripheral signals, the brain, especially the hypothalamus, is mainly responsible for processing information provided by these input signals and generating appropriate responses.

The hypothalamus, as a central processor, has multiple negative feedback loops to form a distributed neural network for the regulation of feed intake and energy expenditure (Blevins et al., 2002; Berthoud. 2002). The genes encoding neuropeptides and their cognate receptors, extensively expressed in the hypothalamus, are fundamental to creating a sensing and signaling network in charge of food intake regulation and energy expenditure. Within the hypothalamus, specifically in the arcuate nucleus, there are two subpopulations of neurons involved in food intake regulation. The first of these neuron types is sensitive to negative energy balance and
secretes neuropeptide Y (NPY) as well as agouti-related protein (AgRP) when activated. A second neuronal cell type responds to positive energy balance and synthesizes and secretes melanocortin, which is the peptide cleaved from a precursor molecule called proopiomelanocortin (POMC) and another peptide called cocaine-amphetamine-related transcript (CART). This study mainly focused on mRNA expression patterns of appetite-related neuropeptides in avian species in order to determine the conservation of hypothalamic food intake regulation mechanism between birds and mammals (Philips-Singh et al., 2003) from a view of neuropeptides. Agouti-related peptide (AgRP) is one of the orexigenic neuropeptides, which is a paracrine-signaling molecule and is normally produced by AgRP neurons within the arcuate nucleus. It plays an important role in promoting increased feeding and decreased energy expenditure, and antagonizes melanocortin action. The hypothalamic AgRP was found to be elevated in obese and diabetic mice (Shutter et al., 1997), also overexpression of AgRP in transgenic mice or central administration resulted in hyperphagia and obesity (Graham et al., 1997). The antagonistic effects of AgRP to melanocortin are mainly exerted by binding to melanocortin receptors 3 and 4 (MC3R & MC4R), which are the primary targets for alpha Melanocyte Stimulating Hormone (α MSH) (Ollmann et al., 1997; Bultman et al., 1992). Also, AgRP neurons respond to information directly from leptin, an adipocyte-released hormone that circulates at levels proportional to fat mass. A previous study showed that increased AgRP mRNA levels were corresponded to diminished concentrations of circulating leptin (Schwartz et al., 2000).
Another important regulator of food intake regulation within the arcuate nucleus is gamma amino-butyric acid (GABA), which is considered a chief inhibitory neurotransmitter throughout the mammalian central nervous system (CNS). It is extensively involved in food intake regulation in the hypothalamus by modulating neuronal excitability in mammals. However, whether GABA’s positive association with food intake in mammals could be applied to chickens is still an open question.

Although there are extensive studies focusing on the involvement of AgRP and GABA in food intake regulation in mammals, the mechanism of these two important regulators involved in appetite control in avian species has remained unclear. Notably, there is a unique physiological structure in the avian digestive system called a proventriculus, which is the first chamber of birds’ stomach storing food before it progresses to the gizzard. It makes the feed digestion and thus feed intake regulation in avian species different from the mammals, which have simple-stomach, and also emphasizes the importance of this project. The current studies demonstrated that 1) there are alternative transcripts of AgRP existing in the chicken and turkey, but not in the quail. The absent expression of AgRP-B form in quail is due to a deletion of 521bp in genomic DNA of AgRP-A form. Also, mRNA expression patterns of these two isoforms of AgRP varied in tissue distribution, which suggests the alternative promoter usage of these two isoforms; 2) the amount of the appetite-related neuropeptides, such as AgRP/NPY and POMC/CART, corresponded to peripheral nutrition status in quail, but the changes in the level of AgRP mRNA were less responsive to replenishment of feed than other appetite-associated neuropeptides; 3)
long-term dietary GABA supplementation with different dosages failed to stimulate feed intake in chicken, no matter the bacterial- or pure GABA product was applied.
Specific Objectives

Objective 1): To determine the relationship between changes in the amounts of neuropeptides (POMC/CART and NPY/AgRP) and peripheral nutrition status. To measure hypothalamic gene expression of these four neuropeptides from quail following fasting and re-feeding.

Objective 2): To determine existence of alternative transcript isoforms of AgRP in avian species and to detect the different expression Identify the mRNA expression of AgRP A- and B forms in various tissues in the chicken, turkey and quail.

Objective 3): To demonstrate the effects of dietary GABA supplementation with different purity and varied doses on stimulating feed intake regulation in chickens. Compare the feed consumption and body weight among the groups with or without dietary GABA supplementation.
Chapter 2: Review of the Literature

2.1. Significance

Consumption of poultry products has been steadily increasing in the United States and worldwide. According to the ERS’s (Economic Research Service, 2012) food availability data, U. S. chicken availability per person has more than doubled since 1970, arriving at 58 pounds per person. Chicken began its upward climb in the 1940s, overtaking pork in 1996 as the second most consumed meat, and surpassing beef for the first time in 2010 (Bentley and Buzby, 2012). The elevating demand reflects the great economic importance of poultry production to the United States. The poultry industry must find ways to meet increasing demand for poultry products in the United States and worldwide, while reducing production costs.

Growth rate and meat production are economically important production traits of great concern in the poultry industry. To maximize growth rate and feed efficiency, changes in feed intake and energy expenditure must be coordinated and tightly regulated. Generally, the majority of energy intake is primarily metabolized to maintain basic metabolic rate, thermogenesis, and energy expenditure (in the form of muscle action), with excess energy stored as fat for future use. Increased body size in commercial chickens has been accompanied by unintended changes in correlated traits such as increases in voluntary feed intake and energy storage (Richards et al., 2007). Thus, it is meaningful to understand feed intake regulation in birds to develop and better manage commercial lines of poultry and to make them produce more meat.
2.2. Feed intake regulation

Regulation of food intake and energy expenditure is composed of highly complicated and extensively distributed networks. The major basis for food intake regulation metabolism is the interaction between the peripheral and the central components. The hypothalamus and the caudal brainstem are the major areas in the brain involved in food intake- and energy expenditure regulation. They receive the signals indicating the availability of ingested and stored nutrients, and in turn, generate appropriate behavioral- and endocrine output signals to maintain energy homeostasis.

Extensive investigations on appetite metabolism in mammalian species have demonstrated that the regulation of feed intake has two key components: one that involves the short-term control of feeding and the other that involves long-term regulation of energy balance by the central nervous system (Woods et al., 1998; McMinn et al., 2000; Jensen, 2001; Berthoud, 2002; Blevins et al., 2002). The short-term system is responsible for transmitting the meal-related signals (e.g., the presence of feed or specific nutrients) from the gastrointestinal tract to satiety centers located in the brainstem (Jenson, 2001). Then, the satiety signals are relayed to the hypothalamus to induce second-order responses and regulate feed intake activities. In addition, the long-term system enables the hypothalamus to interpret signals representing levels of energy stores (e.g., adipose tissue mass). To achieve energy homeostasis and maintain stable body weight, the meal-to-meal feed intake along with the long-term regulation of energy storage should be cumulatively regulated. Generally, feed intake is adjusted to ensure that energy and nutrients are stored in anticipation of periods of high demand or periods of shortage (Richards, 2003).
The hypothalamus, as a central processor, plays a pivotal role in integrating information received from afferent periphery pathways and generating appropriate responses to regulate feed intake. These components, including short-term- and long-term system along with the hypothalamus, build a controllable and highly regulated feed intake regulation network. Within this system, hormones, nutrients themselves as well as neuropeptides and neurotransmitters activated in the hypothalamus are critical regulators to transmit “information” between the CNS and peripheral components.

2.2.1. Short-term regulation of food intake

The short-term regulation of feed intake ensures that immediate available energy and nutritional requirements are met from meals in animals. It is based on the “depletion-repletion” models, in which some parameters (e.g. gut hormones, glucose and insulin levels etc.) representing immediately available energy are appropriately controlled. The “depletion-repletion” models can account for both meal onset and meal termination. In other words, a meal is initiated as the available energy falls to a threshold value and is terminated as substrate levels are sufficiently replenished (Woods et al., 1998). The regulators in short-term regulation of feed intake are mainly hormonal and neural signals that originate primarily in the gut but also in the pancreas and liver. These signals, which accurately reflect the status of food consumption, on the one hand, could be locally metabolized in the peripheral tissues or organs to generate immediate physical or hormonal responses. These first-order products could be transported to the brain and be involved in food intake regulation by influencing neurotransmitter synthesis or neuronal excitability in the hypothalamus. However, the
effects resulting from gut-derived peptides are relatively short-lived, and cannot efficiently affect feed intake by relaying message far away from the periphery into the central components. Thus, gut peptide signals are not thought to play the major role in mediating long-term changes in food intake and energy expenditure regulation (Richards, 2007).

**Cholecystokinin (CCK)**

Cholecystokinin (CCK) is a hormone released from the upper small intestine, particularly in the duodenum and jejunum, responding to food ingestion. CCK acts rapidly to reduce meal size and duration in animals, including humans (Gibbs et al., 1973; Kissileff et al., 1981) by stimulating gastric emptying and intestinal motility, as well as the release of pancreatic enzyme (Liddle et al. 1985; Moran et al., 1994).

The food intake suppressive effects of CCK act in a paracrine fashion on CCK\(_A\) receptors located on vagal sensory nerve terminals in the mucosal lamina propria (Raybould et al., 2006; Geary, 2004; Smith et al., 1985). Besides, CCK\(_A\) receptors are expressed widely in the CNS and the pancreas, this type of receptors has greater affinity to sulfated CCK than other types of CCK receptors. In addition, peripheral CCK may act directly on the CNS by crossing the blood brain barrier (Reidelberger et al., 2004). However, CCK has a half-life of only 1-2 minutes, thus the effects are short-lived. Although there are reports that demonstrate a long-term effect of CCK on balance of food intake and energy expenditure in mammalian species, it may be the result of interactions with other signals of adiposity such as leptin, for
instance, leptin can synergistically potentiate the satiating effect of CCK (Matson et al., 2000).

Alternatively, CCK is expressed within the CNS, acting as a neurotransmitter regulating reward behavior, anxiety, memory, and satiety (Crawley et al., 1994). Within the mammalian brain, CCK-8, one of the multiple CCK receptors expressed in the CNS, is served as the primary ligand in the CCK system (Beinfeld, 2001). Several reports indicated that endogenous CCK might be involved in the long-term effects of neuroleptics on midbrain dopaminergic neurons by application of a CCK antagonist (Chiodo et al., 1987; Crawley, 1991). In the nucleus accumbens, CCK increased the firing rate of glutamate-activated neurons (DeFrance et al., 1984; White et al., 1984) and antagonized the inhibitory actions of dopamine agonist in anesthetized rats (Liang et al., 1991; White et al., 1984; Yim et al., 1991). Furthermore, CCK levels in the brain have been reported to change after feeding, food deprivation and peripheral CCK administration, suggesting the effects of endogenous CCK in the CNS are under the control of nutritional status and peripheral level of CCK.

**Glucagon-like-peptide-1 (GLP-1)**

Glucagon-like-peptide-1 (GLP-1) is an incretin hormone, mainly released from the gut into the circulation system in response to nutrient ingestion, and circulating GLP-1 levels are inversely correlated with body mass, suggesting its role in inhibiting food intake (Holst et al., 1983; Naslund et al., 1999; Ranganath et al., 1996; Verdich et al., 2001). Peripheral injection of GLP-1 reduced food intake and activated c-fos in the brain stem (Tang-Christensen et al., 2001), and its inhibition of food intake was dose-
dependent in humans (Verdich et al., 2001). In addition, pre-prandial subcutaneous GLP-1 injection reduced calorie intake by 15% and resulted in 0.5 kg weight loss over 5 days in obese males (Naslund et al., 2003). The reduction in food intake and the slowed gastric emptying are accompanied by decreased orexigenic feelings and a prolonged period of postprandial satiety.

**Peptide YY (PYY)**

Peptide YY (PYY) is a 36 amino acid “hind gut” peptide released from the L cells of the gastrointestinal tract, with increasing concentration of cells found in the ileum, colon, and rectum (Adrian et al., 1985; Ekblad et al., 2002). The release of PYY is closely associated with calorie intake, it increases to a plateau 1-2 h after a meal and remains elevated for 6 h (Adrian et al., 1985). Although PYY shares 70% amino acid sequence identity with the orexigenic neuropeptide NPY, rather than stimulated appetite as NPY does, PYY reduced food intake and inhibited weight gain in rodents (Batterham et al., 2002). Batterham et al. showed that PYY injection in both lean and obese individuals caused a 30% reduction in calorie intake post infusion and decreased the 24h energy intake by 23% in lean and by 16% in the obese (Batterham et al., 2003).

There are two major forms of PYY that exist in the circulation: PYY\textsubscript{1-36} and PYY\textsubscript{3-36} (Grandt et al., 1994). PYY\textsubscript{3-36} is the cleaved product from PYY\textsubscript{1-36} by dipeptidyl pepridase IV (DPP-IV) (Eberlein et al., 1989), and it is PYY\textsubscript{3-36} that is the peripherally active anorectic signal that binds with greatest affinity at the cognate receptor. Although the ratio of these two forms of PYY was thought to change with
different nutritional status, the accurate proportion of \( \text{PYY}_{1-36} \) and \( \text{PYY}_{3-36} \) as well as how it changes with fasting or well-fed condition is yet to be clarified.

**Ghrelin**

Ghrelin is a 28-amino acid peptide that is primarily produced and released in the gut, it is also found in the brain, especially within the arcuate nucleus. Ghrelin is a potent orexigenic factor, whose postprandial reduction in circulation is regulated by calorie intake, peripheral nutritional signals (Sakata et al., 2002; Tschop et al., 2000), as well as energy stores. Studies showed that obese subjects did not demonstrate rapid postprandial fall in circulating ghrelin, and this in turn could contribute to continued food intake and obesity (English et al., 2002).

Ghrelin’s actions on food intake regulation also involve neuron’ activities in the arcuate nucleus. Central administration of ghrelin activates orexigenic neurons (Lawrence et al., 2002; Toshinai et al., 2003), and the stimulation of food intake by ghrelin administration is attenuated by adding anti-orexin antibody and in orexin null mice (Toshinai et al., 2003). However, ghrelin’s stimulation on food intake in animals and human seems short-lived. In addition, the long-term ghrelin blockade may not alter body weight, since ghrelin null mice does not demonstrate significantly increased appetite and altered body composition on a standard diet (Sun Y et al., 2003 & 2004; Chen et al., 2004).
2.2.2. Long-term regulation of energy balance

Energy homeostasis is under the control of the integration of the short-term and long-term food intake regulation, which serves a role in minimizing the impact of fluctuations in energy intake (Woods et al., 1998). Unlike the interaction between gut-derived peptides with the CNS in short-term regulation system, the long-term regulation is considered as a neuro-humoral system. In long-term food intake regulation, hormones, such as leptin that are secreted in proportion to body adiposity, are transported into the brain and directly bind to their candidate CNS targets on neurons. Getting the information about body energy status from the circulation enables these targets to stimulate specific second-order neurons to further generate orexigenic or anorexigenic signals as the unidirectional feedback. Within this system, the primary region involved in interpreting hormonal signals is the arcuate nucleus located at the hypothalamus. In this region there are two subpopulations of neurons involved in food intake regulation. The first of these neuron types is sensitive to negative energy balance and secretes neuropeptide Y (NPY) and agouti-related protein (AgRP) when activated. A second neuronal cell type synthesizes and secretes melanocortin, which is a peptide cleaved from a precursor molecule called proopiomelanocortin (POMC) and another peptide called cocaine-amphetamine-related transcript (CART).
2.2.2.1. Hormones

Leptin

The search for leptin was initiated by Coleman, back to 1978, he suggested the existence of a hormone that could inversely regulate food intake in proportion to body adiposity. The hypothesis was that the obese \(ob/ob\) mice lacked of this hormone, and that \(db/db\) mice were insensitive to the action of this hormone. But this statement was not confirmed until Zhang et al. (1994) found the \(ob\) mutation resides in the gene encoding leptin, a hormone secreted from adipocytes, and the \(db\) mutation is located in the leptin receptor gene (Chua et al., 1994; Lee et al., 1996).

The findings that intracerebroventricularly (ICV) administration of leptin lowered mice’s food intake, and that the leptin receptor involved in food intake regulation resided in the hypothalamus confirmed the anorexic role of leptin in appetite regulation (Mercer et al., 1996; Richards, 2003).
Leptin appears to be transported into the central nervous system (CNS) by a saturable receptor-mediated process (Banks et al., 1996), in which the efficiency is controlled by the ratio of plasma- and circulating leptin concentrations (Caro et al., 1996). In the CNS, the binding of leptin to its receptor generates the response of POMC-expressing neurons that produce $\alpha$-MSH to perform catabolic activities (Fig. 2.2.).

Expression, actions, and functional importance of leptin in mammalian species have been confirmed by intensive studies, and its critical role in food intake regulation and energy homeostasis was suggested to be conserved across species (Richard, 2007). However, significant species variation in leptin expression does exist between mammal and avian species (Ohkubo et al., 2000). Taouis et al. (1998) and Ashwell et al. (1999) reported the cloning and sequencing of a chicken leptin gene, however, their
findings raised other researchers’ doubts on it and stimulated a considerable number of studies on obtaining the full length of the leptin gene sequence and consistent leptin gene expression measurements in different tissues in birds. Unlike the controversy surrounding the chicken leptin gene, the leptin receptor gene has been clearly identified for both chickens and turkeys (Horev et al., 2000; Ohkubo et al., 2000; Richards et al., 2001)

**Insulin**

Insulin, which consists of A- and B- polypeptide chains that are linked together by disulfide bonds, is secreted from the β-cell of the islets of Langerhans. Its secretion is stimulated by several stimuli reflecting peripheral nutritional status, including ingested protein and plasma glucose concentrations. Insulin, as well as leptin, is the major regulator on carbohydrate and fat metabolism in the body. Basically, insulin lowers glucose level in the blood by stimulating cells in the liver, skeletal muscles, and fat tissue to absorb glucose and store as glycogen in the liver and skeletal muscles, and as triglyceride in the adipocyte.

Insulin is produced from its precursor proinsulin (Kahn et al., 2005), which is cleaved from preproinsulin. Preproinsulin is a 24-residue signal peptide that directs the nascent polypeptide chain to the rough endoplasmic reticulum (RER) and then the cleaved peptide is trans-located into the lumen of RER, forming proinsulin (reviewed based on Kahn et al., 2005). Similar with leptin, insulin enters the CNS by a receptor-mediated, saturable transport process across brain capillary endothelial cells (Baura et
al., 1993), and its receptor is co-located with leptin receptor within the hypothalamus (Baskin et al., 1988).

The regulation on energy balance and body weight maintenance of insulin and leptin is highly correlated (Fig. 2.3). Effects on reducing food intake and body weight of both leptin and insulin perform a dose dependent manner when they are centrally administrated (Chavez et al., 1995). Without functional leptin receptors, the genetically obese Zucker rats (fa/fa) did not reduce their food intake and body weight when given ICV insulin administration (Ikeda at al., 1986), suggesting central activities of insulin partially rely on leptin’s involvement. The significant difference between leptin and insulin in food intake regulation is that only the insulin secretion is stimulated acutely by immediate meals.

![Diagram](image)

**Figure 2.3** The regulations of leptin and insulin on food intake and body weight (Woods et al., 1998).
2.2.2.2. Central regulators of food intake

Arcuate Nucleus (ARC)

The hypothalamus is essential for appetite modulation and maintenance on energy balance to avoid wide variation in food intake and energy expenditure. Models of lateral hypothalamic feeding centers and ventromedial hypothalamic satiety centers were firstly proposed by Hetherington and Ranson (1940) and Anand and Brobeck (1951). The critical role of the arcuate nucleus (ARC) in appetite regulation was proposed when destruction of the ARC with systemic monosodium glutamate produced obesity and hyperphagia (Olney et al., 1969). Other hypothalamic regions, such as the paraventricular nucleus (PVN), dorsomedial nucleus (DMH) and ventromedial nucleus (VMH), are also involved in the regulation of food intake and energy expenditure (Figure 2.4).

Figure 2. 4 Morphologically defined regions of the hypothalamus (Stanley et al., 2005). Morphologically defined regions of the hypothalamus (Stanley et al., 2005). For references, ARC, arcuate nucleus; PVN, paraventricular nucleus; DMH, dorsomedial nucleus; VMH, ventromedial nucleus; LHA, lateral hypothalamic area; PFA, perifornical area.
The ARC lies in close proximity to the third ventricle, which lacks a complete blood-brain-barrier (BBB) (Broadwell and Brightman, 1976). This characteristic makes it directly contact with circulating hormonal signals (Fig. 2.4). In this region there are two subpopulations of neurons involved in food intake regulation (Cone et al., 2001). The first of these neuron types is sensitive to negative energy balance and secretes neuropeptide Y (NPY) and agouti-related protein (AgRP) when activated (Broberger et al., 1998). A second neuronal cell type synthesizes and secretes $\alpha$-melanocyte stimulating hormone ($\alpha$-MSH)( Kristensen et al., 1998; Elias et al., 1998), which are peptides cleaved from a precursor molecule called prooiomelanocortin (POMC) and another peptide called cocaine-amphetamine-related transcript (CART). Moreover, peripheral hormones, such as insulin and leptin, are transported to ARC and interact with these neurons to regulate food intake and energy homeostasis (Schwartz et al., 2000).

NPY

NPY, as one of the most abundant neurotransmitters in the CNS (Allen et al., 1983), is highly expressed in the ARC (Morris, 1989). NPY is also the most potent orexigenic neuropeptide known so far; repeated injections of NPY into third ventricle or PVN causes significant hyperphagia and obesity (Stanley et al., 1986; Zarjevski et al., 1993). Hypothalamic levels of NPY could be an indicator of peripheral nutritional status, with the NPY mRNA and NPY secretion increasing by fasting and decreasing after re-feeding (Kalra et al., 1991; Sanacora et al., 1990; Swart et al., 2002). Effects of NPY are not only exclusive to food intake regulation but also to energy homeostasis.
For example, central administration of NPY also inhibits brown fat thermogenesis (Billington et al., 1991) and the thyroid axis (Fekete et al., 2002) to reduce energy expenditure. Notably, NPY also promotes basal plasma insulin (Moltz and McDonald, 1985), effects which are independent of increased food intake.

POMC

The cleaved POMC products, their receptors and the endogenous melanocortin antagonists AgRP, as well as agouti, are the major factors in the melanocortin system. In contrary to NPY effects, hypothalamic POMC mRNA expression is lowered in fasting, but restored after at least 6h re-feeding or exogenous leptin administration (Swart et al., 2002; Schwartz et al., 1997). Human POMC gene mutations or abnormal POMC peptide processing gave rise to early-onset obesity (Krude et al., 1998), and haploinsufficiency of POMC gene triggered diet-induced obesity in mice (Challis et al., 2004).

There are five melanocortin receptors (MCR) identified, but only MCR3 and MCR4 are found to play a role in energy homeostasis (Stanley et al., 2004). They are widely expressed in the hypothalamus, but have higher expressions in the ARC, VMH, and PVN than other regions (Harrold et al., 1999; Mountjoy et al., 1994). MCR4 null mice demonstrate hyperphagia and result in diet-induced obesity (Fan et al., 1997; Huszur et al., 1997). Also, 1-6% of severe early-onset human obesity partially results from abnormalities of MCR4 (Farooqi et al., 2000). Central administration of MCR4 agonists suppresses food intake, while injection of the antagonists triggers hyperphagia (Benoit et al., 2000). In addition, mice lacking MCR3 have increased adiposity rather
than higher body weight, and preferentially metabolize carbohydrate than fat (Butler et al., 2000).

AgRP

Agouti-related peptide (AgRP) is a paracrine-signaling molecule, which is normally produced by AgRP neurons within the arcuate nucleus. It plays an important role in promoting increased feeding and decreased energy expenditure, and antagonizes with melanocortin action. AgRP mRNA expression is increased by fasting, and unlike NPY mRNA levels, which are reduced 6h after re-feeding, AgRP levels remain elevated (Swart et al., 2002). Also hypothalamic AgRP was found to be elevated in obese and diabetic mice (Shutter et al., 1997) while overexpression of AgRP gene in transgenic mice or central administration of AgRP in normal mice resulted in hyperphagia and obesity (Graham et al., 1997).

The antagonistic effects of AgRP with melanocortin are mainly exerted by binding to melanocortin receptors 3 and 4 (MC3R & MC4R), which are the primary targets for alpha Melanocyte Stimulating Hormone (α MSH) (Ollmann et al., 1997; Bultman et al., 1992). However, an in vivo study demonstrated that hypothalamic action of AgRP on energy homeostasis could be independent of α MSH and MC3R & MC4R, indicating the variety and complexity of AgRP action mode (Tolle et al., 2007).
CART

CART is the third most abundant transcripts within the hypothalamus and co-expressed with POMC in the ARC (Elias et al., 1998). The amount of mRNA expression of CART in the ARC is decreased by food deprivation, while it is stimulated by peripheral leptin replacement to ob/ob mice (Kristensen et al., 1998). Interestingly, there may be several populations of CART-expressing neurons in the ARC with different roles in feeding. Injection of CART-(1-102) and CART-(82-103) into the third ventricle suppresses both normal and NPY-stimulated feeding in rats (Kristensen et al., 1998), but administration of CART-(55-102) into the ARC could increase food intake (Abbott et al., 2001).

Even though extensive studies have been done on appetite control in mammals, there are only several studies focuses on feed intake regulation in birds. Notably, there are significant species variations in appetite control between birds and mammals. The stomach of bird has a unique chamber named “Proventriculus”, which can store the feed for a longer time than mammals do. Thus, the digestion of feed in birds and the gut-brain interactions could be varied. In addition, leptin’s contributions to feed intake regulation in birds have significant differences with those in mammals. Until now, researchers have not found endogenous leptin produced in the chickens even though sequencing and cloning of leptin receptor gene had been done long time ago. The lack of leptin regulations in appetite control contributes to the species variations in not only appetite regulation but also energy homeostasis in mammals and birds. The limited knowledge gained from feed intake metabolism, especially focuses on appetite-related
neuropeptides, as well as the species variations between birds and mammals underscore the importance of the presented study.
Chapter 3: Differential expression of multiple transcripts of Agouti-related peptide (AgRP) in avian species and its association with nutritional status

Chen Zhang*, Young M Choi*, Yeunsu Suh*, and Kichoon Lee*†

*Department of Animal Sciences and †The Ohio State Interdisciplinary Human Nutrition Programs, The Ohio State University, Columbus, OH, 43210

3.1. Abstract

Agouti-related peptide (AgRP) mainly serves as an endogenous antagonist of melancortin action. It is secreted by AgRP neuron in the arcuate nucleus, which is the major area within the brain to regulate food intake and energy homeostasis. The chicken AgRP gene was found to encode protein of three different sizes, with 233, 165 or 154 amino acid, respectively, depending on their alternative promoter usage and alternative splicing. Our bioinformatics analysis demonstrated that there were two, rather than three shown in the NCBI, isoforms of AgRP gene-- A- and B- forms -- in the chicken and turkey, but not both of them expressed in the quail. To confirm the existence of multiple transcripts of AgRP in the avian species, we cloned the AgRP gene and localized the expression of the AgRP gene in various tissues in the chicken, turkey and quail. RT-PCR analysis detected the AgRP mRNA in all tissues examined:
the hypothalamus, heart, liver, kidney, skeletal muscle and adipose tissues. We detected that in quail the AgRP A-isoform showed the highest expression in the hypothalamus, with undetectable expression in the other tissues examined. However, in the quail the AgRP B form was not expressed in all the tissues examined. Based on our RT-PCR analysis and the sequencing results of quail AgRP-A form genomic DNA, we found that there was a deletion of 521bp on the genomic DNA of quail AgRP A-form gene, resulting in the absence of the B isoform expression. In addition, the fast and re-feeding experiment was applied to determine how feed intake regulates the mRNA expression level of neuropeptides in the quail. Interestingly, the level of AgRP mRNA expression in the hypothalamus was less responsive to replenishment of feeding after 48h-fast than other orexigenic neuropeptides examined. These results implied that the different promoter structures of AgRP A- and B form in the 5’ UTR were related with the varied biological functions of both isoforms.

**Key words: Agouti-related peptide, Feed intake regulation, Avian specie.**
3.2. Introduction

Agouti-related peptide (AgRP) is a paracrine-signaling molecule, which is normally produced by AgRP neurons within the arcuate nucleus. It plays an important role in promoting increased feeding and decreased energy expenditure, and antagonizes melanocortin action. Hypothalamic AgRP was found to be elevated in obese and diabetic mice (Shutter et al., 1997) while overexpression of AgRP in transgenic mice or central administration resulted in hyperphagia and obesity (Graham et al., 1997). The antagonistic effects of AgRP with melanocortin are mainly exerted by binding to melanocortin receptors 3 and 4 (MC3R & MC4R), which are the primary targets for alpha melanocyte stimulating hormone (α MSH) (Ollmann et al., 1997; Bultman et al., 1992). However, a recent in vivo study demonstrated that hypothalamic action of AgRP on energy homeostasis could be independent of α MSH and MC3R & MC4R, indicating the variety and complexity of AgRP action mode (Tolle et al., 2007).

The human AgRP gene (hAgRP) consists of 4 exons, with one in the 5’ non-coding region and the other three in the coding area. It encodes a protein of a 132 amino acids in length, in which the 9-cysteine residues in the C-terminal region forming disulfide-bridges are critical for its biological function (Shutter et al., 1997). There are two alternatively expressed transcripts of hAgRP found in the arcuate nucleus and peripheral tissues (mostly the adrenal gland, but also the testis and the lung) (Shutter et al., 1997; Ollmann et al., 1997). The alternative isoforms of hAgRP differ in the 5’ un-translated exon that is truncated in the peripheral tissues (Shutter et al., 1997). Also, Brown et al. (2001) reported that the upstream non-coding exon had
significant promoter activity exclusively in a periphery-derived cell line, and suggested that this may be the minimal promoter driving expression of the short transcript in the periphery. Thus, great progress has been made over the years in the understanding of the expression patterns and the biological functions of AgRP in mammals.

However, biological activities of AgRP, specifically those related to feed intake regulations are poorly understood in birds. Even though some studies reported the gene structures of multiple transcripts of AgRP gene in chickens (Hiramatsu and Takeuchi, unpublished), whether other avian species, such as turkey and quail, also have different isoforms of AgRP gene expressed is yet an open question. Moreover, in avian species expression patterns of those isoforms of AgRP, which suggest that varied biological functions await clarification. To provide knowledge for further discovering different biological functions of multiple transcript isoforms of AgRP, and ultimately for elucidation of how these isoforms are involved in feed intake regulation in avian species, it is necessary to explore the existence of different transcripts of AgRP gene and their expression patterns in various tissues in the chicken, turkey and quail. Accordingly, the present study was designed to compare the gene structures and expression patterns of alternative transcripts of AgRP among the chicken, turkey, and quail. We describe here for the first time the absence of the AgRP-B isoform expression in the quail, and tissue distribution of expression of alternative transcripts of AgRP in three avian species.
3.3. Materials and Methods

3.3.1. Animal study and tissue harvest

The Institutional Animal Care and Use Committee at The Ohio State University approved all procedures involving animal handling. Fasting was performed for 48h, the fast & re-feeding group followed with 6h feeding before slaughtering. Twelve quails were equally grouped into 3 pens, which were considered as the control-, fasting- and fast & re-feeding- group. The brain, liver, adipose tissue, muscle, heart, lung, intestine, spleen, and kidney were collected from 22-day-old birds. Samples were immediately frozen in dry ices and stored in -80°C freezer for further analysis. Before the RNA isolation from the hypothalamus in quails, the frozen sample was trimmed into a small cube (3mm$^3$), which was cut off from the middle of third ventricle.

3.3.2. RT and SYBR green real-time PCR

Total RNA from collected brain was isolated using Trizol (Invitrogen) following the manufacturer’s instructions. The quantity and quality of RNA were evaluated by NanoDrop 100 (Thermo Scientific, Wilmington, DE) and, respectively by agarose gel. One ug of RNA was used in each reverse transcription reaction, which was performed using M-MLV reverse transcriptase (In-vitrogen); the conditions for reverse transcription were 65°C for 5 min, 37°C for 50 min, and 70°C for 15 min (Li et al., 2007). The primers for RT, SYBR Green real-time PCR assays and AgRP A- and B isoform tissue distribution are shown in the Table 3.1.
3.3.3. Cloning and Sequencing of Chicken, Turkey and Quail AgRP

For sequencing of the AgRP gene in the chicken, turkey and quail, the cDNA generated from RT-PCR was used as a template to amplify the entire coding sequence and 5’ genomic DNA (i.e. exon 1, exon 2 and the intervening intron) of chicken, turkey and quail AgRP. Two sets of forward (AgRP-A F: 5’-GGACATCGAGGCAGAGTGACT-3’; AgRP-B F: 5’-CTGTCACACGCAGACCTGAA-3’) and reverse (AgRP R: 5’TGCCACAGGGCTGCC-3’) primers were designed based on reported sequence information available in The National Center for Biotechnology Information (NCBI) database to cover the complete coding area of the AgRP A-and B form, respectively. The PCR was performed by using AmpliTaq Gold polymerase (Applied Biosystems, Carlsbad, CA) with the following conditions: 95°C for 10 min, 42 cycles of 94°C for 30s, 56°C for 1 min, and 72°C for 1 min, and an additional extension step at 72°C for 10 min. The PCR products were separated using an agarose gel (1.5%), and the bands observed were excised for DNA extraction using Qiagen Gel Extraction Kit (Qiagen Inc. Valencia, CA). The DNA fragments were then sent for sequencing at The Ohio State University Sequencing Core Facility using an Applied Biosystems 3730 DNA analyzer (Applied Biosystems). In the study for detecting the expression of AgRP A-and B form in various tissues, PCR was performed with the forward (5’-GGACATCGAGGCAGAGTGACT-3’) and reverse (5’-TCACCTCGGACCTAGCCA-3’) primers to cover the first two exons and the intervening intron of AgRP A form, and with the forward (5’-CTGTCACACGCAGACCTGAA-3’) as well as the same reverse (5’-
TACTGCAGGAAGATCAGCACCA-3′) primers to cover the last two exons of AgRP B form. The cDNA samples generated from total RNA isolation of adipose, muscle, heart, liver, lung, kidney and hypothalamus tissues were used as templates.

3.3.4. Quantitative Real Time PCR (qPCR)

Quantitative real time PCR was performed to evaluate relative gene expression of appetite-related neuropeptides in the quail hypothalamus. Primers (Table 3.1) were designed in different exons spanning genomic introns to avoid the amplification of any contaminating genomic DNA. Approximately equal amounts of cDNA of each sample were used in the qPCR as a template with AmpliTaq Gold polymerase (Applied Biosystems) and GeneAmp 10× PCR Buffer II containing 100-mM Tris-HCL, pH 8.3, 500-mM KCl. SYBR green was used to detect the amplification of the products. Reactions were performed in duplicate 25 µl volumes on an ABI 7300 Real-Time PCR Instrument (Applied Biosystems). Conditions for qPCR were 95°C for 10 min, and then 40 cycles of 94°C for 15 s, 60°C for 40s, and 72°C for 30s, an additional extension at 82°C for 33s. The observation of dissociation curve was based in the qPCR software.

Reference gene, ribosomal protein S13 (RPS13), was used for qPCR normalization and for determination of a suitable internal control in qPCR. The cycle threshold (CT) values for the target genes and the reference gene (RPS13) were determined by the ABI software, and were used to calculate gene expression of target. All target genes were normalized to RPS13 and displayed as a relative fold-change. For the tissue distributions of AgRP in the chicken, turkey and quail, ribosomal protein
S13 (RPS13), the stability of which has been described previously (de Jonge et al., 2007), was selected as the more suitable reference gene between tissues.

3.3.5. Bioinformatics and Statistical Analysis

For the gene homology analysis on AgRP full-length sequences, the basic local alignment search tool (BLAST) of the NCBI was used. Information for the cDNA sequence of human (NM_001138.1) and mouse (NM_001271806.1) AgRP was gained from NCBI database. The chicken, turkey and quail sequences that were used for homology analysis were from the current sequencing results. Alignment and comparison of cDNA sequences from human-, mouse-, chicken-, turkey- and quail AgRP were performed by BioEdit Sequence Alignment Editor (Version 7.0.9.0; http://www.mbio.ncsu.edu/bioedit/bioedit.html).

Data are presented as means ± SD. Comparisons among groups were analyzed by analysis of variance using the General Linear Model (GLM) procedure contained in the SAS statistical package. When the effect was significant (P<0.05), the means were separated using the probability option (PDIFF, a pairwise t-test).

3.4. Results

3.4.1. Different gene structures of AgRP A- and B- form in the avian species

The cDNA sequences of AgRP of chicken, turkey and quail showed that there were three exons in the coding sequence of avian AgRP. The AgRP A- and B form share the second and the third exons while AgRP-B form has a longer first exon than that of the A-form (Fig. 3.1 A). According to the protein subcellular localization prediction program WoLF PSORT (http://wolfsort.org), the predicted amino acid
sequence of AgRP-B form includes a nucleus localization signal “PWRRRPR” (Fig. 3.1 C), which endows the AgRP-B form with the ability to be transferred into the nucleus. But to confirm it, further study is needed. In addition, the cysteine-rich C’ terminal region named “Agouti superfamily”, which can form a disulfide bridge and is critical for its biological function, is highly conserved across the mammalian and avian species (Fig. 3.1.B).

Further, the gene structures of 5’ AgRP A- and B-forms demonstrated obvious differences. In the 5’ UTR, the AgRP-A form has a unique exon that is about 680 base pair away from the second exon in the coding sequence (Fig. 3.2). Importantly, compared to the sequences of AgRP-A form in chicken and turkey, there is a deletion of 521 base pair in the 5’ genomic sequence of AgRP-A form in the quail, resulting in the absence of quail AgRP-B form in all tissues examined (Fig. 3.3).

### 3.4.2. Differential expressions of AgRP A- and B-form in the avian species

The mRNA expressions of AgRP A- and B-form in the hypothalamus among three avian species (chicken, turkey and quail) were detected by amplification of RT-PCR products using forward primer AgRP-A F and reverse primer AgRP R1 for AgRP A-form, with forward primer AgRP-B F and reverse primer AgRP R for the B-form. In terms of expression of AgRP-A form mRNA in the hypothalamus, there was a band in length of about 150bp observed from the agarose gel separation in the chicken, turkey and quail, respectively (Figure 3.4 B) (apart from any non-specific band based on sequencing results). However, the mRNA of AgRP-B form did not express in the
quail hypothalamus while it had expressions in both the chicken and turkey, represented by a band, about 520bp, from the agarose gel separation (Figure 3.4 C).

The tissue distributions of AgRP A- and B-form mRNA expression were revealed by agarose gel. Thirty-nine cycles were applied in the amplification from RT-PCR products for each sample in order to detect their different expression levels in various tissues. However, these results were only qualitative rather than quantitative, in terms of indication to reveal the expression of AgRP A- and B-form transcripts.

As shown in Figure 3.5 A, chicken AgRP (cAgRP) A-form expressed in the majority of tissues examined, with the greatest expression in the hypothalamus and nearly undetectable expression in adipose tissue. Compared to the expression of cAgRP-A form, the expression of cAgRP-B form was more peripherally expressed. In the turkey the expression pattern of AgRP-A form was similar to the B-form (Figure 3.5 B). Notably, tissue distributions of mRNA expression of qAgRP-B form further confirmed the absence of B-form expression in the quail; the qAgRP-A form showed the greatest expression in the hypothalamus but undetectable expression in other tissues (Figure 3.5 C).

### 3.4.3 Nutritional status regulates the amount of orexigenic- and anorexigenic neuropeptides in quail hypothalamus

The changes in mRNA expression levels of appetite-related neuropeptide genes, POMC/CART and NPY/AgRP, in response to different nutritional status were detected by qRT-PCR. The total RNAs were isolated from the hypothalamus from the control, fasting, fasting and re-feeding groups in the quail, then were reversed
transcribed into cDNA for quantitative real-time PCR. As shown in Figure 3.5 C, in quail the NPY mRNA expression level significantly increased (p<0.01) during the period of 48h of fasting, and declined to the baseline after 6h of re-feeding following the fast. In contrast, POMC and CART were significantly decreased (p<0.01) after application of 48h of fasting and returned to the baseline after the 6h re-feeding. The hypothalamic expression level of qAgRP largely increased after the 48h fasting period but failed to decrease in the followed 6h re-feeding state as NPY did.

3.5. Discussion

The human AgRP (hAgRP) has two alternatively expressed transcripts in the arcuate nucleus and peripheral tissues (mostly the adrenal gland but also the testis and the lung) (Shutter et al., 1997; Ollmann et al., 1997). Their gene structures differ in the 5’ UTR, and the truncated form of hAgRP was mostly found in the peripheral tissues (Shutter et al., 1997). Also, Brown et al. (2001) suggested that there might be the minimal promoter driving expression of the short transcript in the peripheral tissues, which determined that the upstream non-coding exon had significant promoter activity exclusively in a peripherally-derived cell line. In this study, we determined the existence of only two major transcript isoforms of AgRP, A- and B-form, in avian species instead of three reported on NCBI by Hiramatsu and Takeuchi (unpublished). They claimed that there were three transcript isoforms of cAgRP, with the length of 1035bp in B-form, of 618bp in A-1 form and 701bp in A-2 form. However, we only detected the cAgRP A-1 form using the forward primer AgRP-A F and reverse primer AgRP R1 (Table 3.1). Our hypothesis for this discrepancy is that the cAgRP A-1 form
is dominantly expressed in the chicken over the A-2 form, but further study is required for confirmation.

To date, the expression profiles and functions of these transcript isoforms in birds have not been extensively elucidated. In this study, we identified differential mRNA expressions of AgRP A- and B-forms in various tissues in the chicken, turkey and quail. However, mRNA expression pattern of AgRP in various tissues differed among the avian species. In the chicken, AgRP-A form with a unique exon on 5’ UTR showed the highest expression in both the hypothalamus and heart muscle, while B-form had an undetectable expression in the hypothalamus but expressed more in heart and lung. According to the amino acid sequences of AgRP A- and B-form, there was no obvious cleavage site that produces the shorter form of AgRP. Thus the differential expression, in the CNS or in the periphery, of these alternative transcript isoforms did not result from the cytoplasmic secretion of cleavage-induced truncated product, which is the mechanism of multiple expression pattern of hAgRP mRNA.

Based on careful comparison of genomic DNA sequences of cAgRP A- and B-form, we found that there was a “TATA” box on the first exon of cAgRP-A form. We hypothesized that this “TATA” box could have the ability to regulate the promoter strength of the shorter B-form via binding with the TATA binding protein-related factors (TRFs). However, it is difficult to find an appropriate candidate sequence to act as a “TATA” box on upstream area of cAgRP B-form. In other word, cAgRP B-form is possibly a TATA-less gene, whose transcriptional activity is regulated by bonding with TRFs to the sequence located elsewhere rather than the “TATAA” sequence. Combined with the results from sequence alignments of cAgRP A- and B-form and
their varied mRNA expression pattern in tissue distribution, we hypothesized that alternative promoter usage, as a versatile mechanism to create diversity and flexibility in the regulation of gene expression (Ayoubi and Van De Ven, 1996), could be involved in multiple transcript expression of AgRP A- and B-form. Interestingly, Kealin et al. (2007) reported that a region upstream of cAgRP promoter containing both a STAT (signal transducers and activators of transcription) site and an IRE-like (inverted repeat elements) site, which serve as recognition sites for specific transcriptional factors. But the STAT and IRE sites that they pointed out in cAgRP sequences were about 2kb away from the first exon of cAgRP-A form, which means that the transcriptional factors binding to these sites could regulate the promoter activities of both cAgRP A- and B-form. Thus regulations on STAT and IRE sites could not be considered as the factor resulting in the differential expression activities of cAgRP A- and B-form in various tissues. Therefore, further study focusing on high-fidelity promoter profiling of cAgRP is required to elucidate the reasons for differential expression patterns of these two transcript isoforms.

In the turkey, the mRNA expression profiles of AgRP A- and B-form in various tissues were similar to each other. Since RT-PCR analysis is qualitative rather than quantitative, it is uncertain to claim that turkey AgRP (tAgRP)-A form had higher expression in the CNS than B form was (Fig. 3.4 B). But tAgRP-A form was more exclusively expressed in the hypothalamus than the B form did, according to the tissue distributions of AgRP A-form mRNA expression in the turkey. In comparison, the quail AgRP (qAgRP) only had the AgRP-A form expressed in the hypothalamus rather than expressed multiple transcript isoforms as cAgRP and tAgRP did. In order to
confirm the absence of qAgRP B-form mRNA expression, we detected the expression pattern of qAgRP B-form in various tissues, including the adipose tissue, skeletal muscle, heart, lung, liver, kidney and the arcuate nucleus (Fig. 3.4 C), but the results showed that there was no qAgRP-B form mRNA expression existed in any of the tissues examined.

In order to investigate whether the absence of qAgRP-B form mRNA expression in the quail is resulted from different genomic DNA structure, we amplified the cDNA and genomic DNA of AgRP A- and B form in the hypothalamus in three avian species, and separated the PCR products on 1.2% agarose gel. Bands in length of about 520bp represented the genomic DNA and the cDNA of AgRP-B form, which expressed in both chickens and turkeys but not in quail (Figure 3.3 B). In addition, bands representing the cDNA of AgRP-A form in three species had similar size in length, but band demonstrating the genomic DNA of qAgRP-A form is about 350bp rather than 850bp in length, as found in the chicken and turkey (Fig. 3.3 B). Thus we identified that there was a deletion of 521bp in the genomic DNA sequence of qAgRP A-form by sequence alignments of AgRP A-form in avian species and the results from PCR on cDNA and genomic DNA in the birds. It is the deletion that resulted in no expression of qAgRP-B form in the quail.

AgRP is an orexigenic peptide, its expression is largely restricted to a discrete population of neurons that sense the levels of peripheral energy stores. In coordination with other appetite-associated neuropeptides including NPY, POMC and CART, AgRP is part of a well-regulated network to integrate information indicating peripheral energy status and relay the signals to the next orders of neurons to control food intake.
The fasting and re-feeding experiments applied in the quail detected that the orexigenic peptides--AgRP and NPY--increased during the state of negative energy balance and returned to the baseline after re-feeding for several hours (Figure 3.5). Anorexigenic neuropeptides including POMC and CART, responded to the nutritional status in an opposite way to that of AgRP and NPY (Figure 3.5). In addition, changes of AgRP mRNA expression over the transition from fast to the following 6h re-feeding status were less responsive than other appetite-related peptides were. In other words, the mRNA expression of AgRP failed to diminish to the baseline after re-feeding for 6h while NPY, also as an orexigenic peptide, reduced to the similar level with the control group during re-feeding state (Figure 3.5).

In summary, this study reported the expression patterns of alternative transcripts of AgRP in avian species, and the comparisons of full-length gene sequences between those isoforms. Differential expression profiles of AgRP A- and B-form in the chicken, turkey and quail suggest the alternative promoter usage on these two isoforms, which might contribute to their varied biological function. Importantly, a deeper understanding in feed intake regulation, especially in economical important food animals, is helpful for us to enhance the meat production and animal products by properly stimulating animals feed intake.
<table>
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<th>Target gene</th>
<th>GenBank accession</th>
<th>Primer sequences</th>
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<td>NM_001031098.1</td>
<td>F: 5'-CCATGCTGGGAGAACAGCA-3'</td>
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<td></td>
<td></td>
<td>R: 5'-CCAGCGGAAATGCTCCAT-3'</td>
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<td>F: 5'-GCCCAGAGACACTGATCTCAGAC-3'</td>
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<td></td>
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<td>R: 5'-CTGCATGCACTGGGAATGA-3'</td>
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Table 3. 1. Nucleotide sequences of specific primers. POMC (proopiomelanocortin), CART (cocaine- and amphetamine-regulated transcript), NPY (neuropeptide Y), AgRP (agouti-related peptide).
**Figure 3.1.** (A) Scheme of the alternative transcripts of AgRP in avian species. For reference, the coding sequence is shown in black blocks, the non-coding sequence is in gray, and introns are shown in line connecting exons. The arrow with small letters illustrates the position of a specific primer. a: AgRP-A forward primer; b: AgRP-B forward primer; c: AgRP reverse primer; d: AgRP reverse primer-1. (B) Scheme of protein domain of AgRP A- and B form. (C) Alignment of provisional amino acid sequences of AgRP A- (GenBank: BAH28705.1) and B form (GenBank: BAH28706.1). The nucleus localization signal was highlighted.
Figure 3.2. (A) Sequence alignment of the full length of AgRP gene A-form among the avian species. (B) Comparison of full length of AgRP gene B form in the chicken and turkey. (C) Amino acid sequence alignment of AgRP gene among mammals and avian species. The conserved region named “Agouti superfamily” is highlighted by red blocks.
Figure 3.2 continued
**Figure 3.2 continued**

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Agouti superfamily
Figure 3.3. (A) Illustration of the deletion of 500bp in quail AgRP-A form. The deletion on qAgRP is between the first and second exons of AgRP-A form, which overlaps with the coding sequence of AgRP-B form. (B) Expression of genomic- and cDNA of AgRP A- and B forms in the chicken, turkey and quail.
Figure 3.4. Tissue distributions of AgRP A- & B form mRNA in the chicken, turkey and quail. For references; AF: abdominal fat; PM: pectoralis muscle; TM: thigh muscle; H: heart; Lu: lung; Li: liver; K: kidney; Hyp: hypothalamus.
Figure 3.5. The mRNA expression of AgRP, NPY, POMC and CART in the hypothalamus in response to fasting and re-feeding challenge in the quail. AgRP (agouti-related peptide), NPY (neuropeptide Y), POMC (proopiomelanocortin), CART (cocaine- and amphetamine-regulated
3.7 Supplementary data

**Figure 3.6.** Tissue distributions of AgRP A- and B-form mRNA in the chicken, turkey and quail. For references; AF: abdominal fat; PM: pectoralis muscle; TM: thigh muscle; H: heart; Lu: lung; Li: liver; K: kidney; Hyp: hypothalamus.
Chapter 4: Effects of dietary supplementation of Gamma Aminobutyric Acid (GABA) on feed intake and body weight gain in chickens

Chen Zhang*, Young Min Choi* and Kichoon Lee*,†

*Department of Animal Sciences and †The Ohio State Interdisciplinary Human Nutrition Programs, The Ohio State University, Columbus, OH, 43210

4.1. Abstract

This experiment was conducted on one-week old broilers to determine the effects of dietary gamma-aminobutyric acid (GABA) on growth performance in chickens. A total of 75 7-day-old male chicks were randomly assigned to five treatment groups (3 replicates of 6 birds per cage). Both bacterial GABA and pure GABA were supplied with diet in different dosages (0-, 25- and 100 mg/kg). The experiment lasted from 8 to 20 day post-hatch. The body weight and feed consumption were measured in every three days during the experimental period. However, there were no significant differences in feed intake and body weight among 5 treatment groups till the end of 20 days experiment. These results demonstrated that GABA could not stimulate feed intake and body weight gain in chickens in the normal feeding temperature.
4.2. Introduction

Feed intake regulation is of great interests in poultry sciences, since the level of feed consumption is a basic and important factor that determines the rate of growth and body composition achieved by animals (M.P. Richards, 2003). The coordinate regulation of feed intake and energy expenditure to achieve energy balance responds to both external environment cues (feed availability, feed composition, temperature, stressors) and internal physiological signals, which are partially induced by external stimuli. To maintain constant body weight and energy homeostasis, the feed intake regulation system is fine-tuned by the nutrients in the blood, specifically glucose, neurotransmitters, peptides and hormones (Zheng et al., 2003; Abizaid et al., 2006; Field et al., 2010). The hypothalamus and its surrounding regions in the brain are in charge of the coordination of the system and all the factors toward the food intake (King, 2006; Delaere et al., 2010).

Gamma aminobutyric acid (GABA) is a major inhibitory neurotransmitter within the brain, and it has a stimulatory effect on food intake in both birds and mammals. There are two types of GABA receptors known: GABA_A in which the receptor is part of a ligand-gated ion channel complex, and GABA_B that are G protein-coupled receptors. In rats ICV injection of GABA_A or GABA_B receptor agonists promotes food intake (Dunn-Meynell et al., 2009; Olgiati et al., 1980); in avian species ICV injection of GABA_A receptor agonists increase feed intake and administration of the antagonists attenuate the boost of appetite (Abizaid et al., 2006; Yadav et al., 2009;
Beverly et al., 2001). However, studies shown that ICV injection of the GABA$_B$ receptor agonist baclofen decreased feed intake in neonatal broiler chicks (Abizaid et al., 2006) while it had no effects on older broilers (Beverly et al., 2001), implying the complexity of GABAergic regulation of feed intake metabolism.

In poultry production maintaining an appropriate ambient temperature is of great importance, since the reduced performance and economic returns could be resulted from the heat stress (Dai et al., 2011). Notably, several reports demonstrated that dietary GABA (500mg/kg and 10mg/kg) increased performance and feed intake in broilers at high ambient temperature (Chen et al., 2002; Hu et al., 2008), suggesting the positive effects of GABA on feed intake regulation in chickens in heat stress. However, whether GABA increases feed intake through releasing stress due to heat challenge or it boosts the appetite by other means is yet an open question. To understand this, we applied pure- and bacterial GABA with different dosages to detect whether GABA could influence feed intake and body weight in normal feeding temperature.

4.3. Materials and methods

4.3.1. Animals and diets

All experimental procedures involving animal handling were approved by the Institutional Animal Care and Use Committee at The Ohio State University. A total of 75 chicks were applied to GABA experiments after one-week adaptive period. On day 8, chicks were randomly assigned to five treatment groups (3 replicates of 6 birds per cage) according to their average body weight (BW). The chicks were fed at ad libitum
with free access to water from day 8 to 20 post-hatch. The chicks of the five treatment groups (0mg GABA/kg of diet; 20mg pure- or bacterial GABA/kg of diet; 100mg pure- or bacterial GABA/kg of diet) were housed in optimal temperature controlled room. The chicks were fed a starter diet until 7 days of age, followed by a grower diet with or without GABA from day 8 to day 20. Ingredients and chemical composition of the basal diet are shown in Table 4.1. Small amounts of the basal diets were first mixed with the respective amounts of GABA as a small batch, and then remaining basal diets were added to them and mixed homogeneously. The basal diets were formulated to meet NRC (1994) nutrient requirements.

Bacterial GABA is produced by the bacterium Lactobacillus brevis, which is located from kimchi. One strain of Lactobacillus brevis, L. brevis GC01, has been isolated for mass production of GABA and was grown aerobically in fed-batch fermenter under condition of 5% monosodium glutamate is going in. After 48hr of incubation, old cells were harvested by centrifugation and all biomass dried at 60°C in drying chamber. Dried GABA is then blended with rice bran in a ratio of 25:75 with a final moisture level of 7%. Meanwhile, the pure GABA of 99.9% purity was bought from NutraBio.
Table 4.1. Ingredient and composition (%) of diets. *a*

<table>
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<th>Grower diet (8-20 days)</th>
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<tr>
<td>Ingredients (%)</td>
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<tr>
<td>Corn</td>
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<tr>
<td>Soybean meal</td>
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<tr>
<td>Corn gluten meal</td>
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<tr>
<td>Vegetable oil</td>
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<tr>
<td>Di-calcium phosphate</td>
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<td>Ground limestone</td>
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<tr>
<td>Iodized salt</td>
</tr>
<tr>
<td>Vitamin &amp; Trace mineral mix</td>
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4.3.2. Growth performance measurements

The body weight and feed intake were measured in every two days during the experimental period. Both body weight and feed intake were measured as a group rather than individually, and then divided by 6 to get the average as a unit. There were 3 replicates of each group in this study.

4.3.3. Statistical analysis

Comparisons among groups were analyzed by analysis of variance using the General Linear Model (GLM) procedure contained in the SAS statistical package.
When the effect was significant (P<0.05), the means were separated using the probability option (PDIFF, a pairwise t-test).

4.4. Results & discussion

4.4.1. Final body weight & body weight gain

No differences (P>0.05) existed among the five treatment groups for initial weight (8-day-old) (p<0.05) (Figure.1). We measured the group body weight in every three days, and divide it by 6 to get the average body weight as one unit. Every treatment had three replicates, in order to reduce the variation between the same treatments. However, there were no significant differences at any time-point between 5 treatments (0 mg GABA/kg of diet, 25 mg bacterial- or pure GABA/kg of diet, 100 mg bacterial- or pure GABA/kg of diet) (Figure. 4.1) in the normal feeding temperature.

Dai et al. (2011) reported that the addition of 100 mg/kg GABA increased body weight, as well as other carcass characteristics of broiler in heat stress, but we did not detect such differences due to addition of both of bacterial- or pure GABA in the normal ambient temperature. These results demonstrated that dietary supplementation of GABA, with different dosages or purity, failed to stimulate performance in chickens in the normal temperature. In addition, its stimulation on body weight gain in heat stress might closely related to its release of stress from the
eat challenge, rather than directly regulating food intake.

**Figure 4.1.** Body weight measurement. For references, Cont.: control groups; P25: 25 mg/kg pure GABA supplementation; B25: 25 mg/kg bacterial GABA supplementation; P100: 100 mg/kg pure GABA supplementation; B100: 100 mg/kg bacterial GABA supplementation. Bars indicate standard errors. Different letters denote significant differences (P<0.05)

### 4.4.2. Effects of bacterial- and pure GABA on food intake

We measured the group feed consumption with each treatment group in every three days, and divided by 6 to get the average food intake per group. Notably, with addition of 100 mg bacterial GABA/ kg diet the feed intake per chick was double during the 14-day old than those at 11-day old (Figure 4.2) (P<0.05). However, there were no significant differences in feed intake between each treatment at all time points during the experiment.
Figure 4.2. Food consumption measurement. For references, Cont.: control groups; P25: 25 mg/kg pure GABA supplementation; B25: 25 mg/kg bacterial GABA supplementation; P100: 100 mg/kg pure GABA supplementation; B100: 100 mg/kg bacterial GABA supplementation. Bars indicate standard errors. Different letters denote significant differences (P<0.05).

Previous studies have demonstrated that GABA supplementation with high dosage (100 mg/kg) could result in improved body weight, weight gain and feed consumption by 7.4%, 11.7% and 9.0% in chickens that are exposed to heat stress (Dai et al., 2011). Also, they suggested that the effects of GABA supplementation on increasing body weight as well as feed intake were due to its primary inhibitory neurotransmitter in the CNS in heat-stressed broilers (Jonaidi et al., 2002), but not to the improvement of intestinal digestibility of nutrients. Our studies confirmed this statement since in normal ambient temperature dietary GABA supplementation did not
improve feed consumption as well as body weight gain with different dosages and purities.
4.5 Supplemental data

**Figure 4.3.** Daily gain on body weight. For references, Cont.: control groups; P25: 25 mg/kg pure GABA supplementation; B25: 25 mg/kg bacterial GABA supplementation; P100: 100 mg/kg pure GABA supplementation; B100: 100 mg/kg bacterial GABA supplementation. Bars indicate standard errors. Different letters denote significant differences (P<0.05)
Figure 4. Feed-to-gain ratio. For references, Cont.: control groups; P25: 25 mg/kg pure GABA supplementation; B25: 25 mg/kg bacterial GABA supplementation; P100: 100 mg/kg pure GABA supplementation; B100: 100 mg/kg bacterial GABA supplementation. Bars indicate standard errors. Different letters denote significant differences (P<0.05)


King BM. The rise, fall and resurrection of the ventromedial hypothalamus in the regulation of feeding behavior and body weight. Physiol Beha 87:221-244.


