Involvement of AMP-activated protein kinase in differential regulation of appetite between lines of chickens selected for low or high juvenile body weight

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ABSTRACT

This study was to determine (1) if genetic selection for high (HWS) or low (LWS) body weight in chickens has altered the hypothalamic AMP-activated protein kinase (AMPK) system and (2) if this alteration contributes to the dissimilar feeding response to various appetite modulators between HWS and LWS lines. Compared to HWS, LWS chickens had higher levels of AMPK α and acetyl-CoA carboxylase (ACC) phosphorylation, which was caused by upregulation of the upstream factor calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK β). There was greater mRNA expression of carnitine palmitoyltransferase I (CPT1), leptin receptor (LEPR) and neuropeptide Y (NPY) and less mRNA expression of ACC α, fatty acid synthase (FAS), fat mass and obesity associated gene (FTO), pro-opiomelanocortin (POMC) and orexin in LWS than HWS chickens. At 5 days of age, intracerebroventricular (ICV) injection of AICAR, 5-amino-4-imidazolecarboxamide riboside, caused a quadratic dose-dependent decrease in food intake in LWS but not HWS chicks. Compound C, (6-(4-(2-piperidin-1-yl-ethoxy)-phenyl))-3-pyridin-4-yl-pyrazolo(1,5-a)-pyrimidine, caused a quadratic dose-dependent increase in food intake in HWS but not LWS chicks. The anorexigenic effect of AICAR in LWS chicks and orexigenic effect of Compound C in HWS chicks resulted from either activation or inhibition of other kinase pathways separate from AMPK. There is a lower threshold for the anorexigenic effect of ghrelin in LWS than HWS chicks, which was associated with differential hypothalamic AMPK signaling. ICV injection of ghrelin

inhibited corticotrophin-releasing hormone (CRH), 20-hydroxysteroid dehydrogenase (20HSD), glucocorticoid receptor (GR), CPT1 and FTO expression in LWS but not HWS chicks. Additionally, the hypothalamic mRNA level of ghrelin was significantly higher in LWS than HWS chicks, which may also contribute to the differential threshold response to ghrelin in these two lines. Obestatin caused a linear dose-dependent increase in food intake in HWS but not LWS chicks. The orexigenic effect of obestatin in HWS chicks was not associated with altered AMPK. Obestatin inhibited LEPR and FTO expression in HWS but not LWS chicks. Thus, selection for body weight may alter the hypothalamic response to ghrelin by the AMPK pathway, CRH pathway, CPT1 and FTO, and to obestatin by LEPR and FTO.

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INTRODUCTION AND LITERATURE REVIEW

1.1. AMP-ACTIVATED PROTEIN KINASE (AMPK)

1.1.1. AMPK History and Structure

AMP-activated protein kinase (AMPK) was first discovered for its ability to regulate the activity of acetyl-CoA carboxylase (ACC) (Carlson and Kim, 1973) and 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase) (Beg et al., 1973). In 1980, AMP was reported to stimulate the enzyme activity of ACC, suggesting that ACC might suppress fatty acid synthesis in response to falling energy charge (Yeh et al., 1980). Five years later, HMG-CoA reductase, the rate-controlling enzyme of the cholesterol synthesis pathway, was also found to be stimulated by AMP (Ferrer et al., 1985). Shortly after this, a single protein kinase, responsible for regulating both ACC and HMG-CoA reductase in response to AMP/ATP ratio, was identified and named AMP-activated protein kinase (AMPK) (Carling et al., 1989).

AMPK is known to exist as heterotrimeric complexes, consisting of one catalytic subunit, α, and two regulatory subunits, β and γ (Mitchelhill et al., 1994; Stapleton et al., 1994; Hardie and Carling, 1997), all of which are essential for full activity (Dyck et al., 1996). In mammals, each subunit is encoded by two or three genes resulting in two or three isoforms (α1, α2, β1, β2, γ1, γ2, γ3), and at least 12 potential complex configurations are possible (Stapleton et al., 1997; Thornton et al., 1998; Cheung et al., 2000). AMPK is a highly conserved heterotrimeric protein complex and homologue segments of the three

subunits have been identified in all eukaryotic species to date, from yeast to humans (Carling, 2004). In mammals, the α1 isoform is widely expressed and present in different body tissues, whereas the α2 isoform is the predominant isoform in heart, muscle and liver (Stapleton et al., 1996). Compared to β1, the β2 isoform is abundantly expressed in skeletal muscle (Thornton et al., 1998). Of the three γ-subunits identified, γ1 and γ2 are widely distributed in a variety of tissues, while γ3 is mainly expressed in skeletal muscle (Cheung et al., 2000).

The AMPK α subunit (63 kDa) consists of a N-terminal serine/threonine kinase catalytic domain and a C-terminal regulatory domain interacting with β and γ subunits, which contain an autoinhibitory region that inhibits the kinase in the absence of AMP (Crute et al., 1998). The β subunit contains 2 conserved regions: kinase interacting sequence (KIS) and association with SNF1 kinase complex (ASC), which bind to α and γ, respectively, to form a functional αβγ complex (Thornton et al., 1998; Hudson et al., 2003). Besides acting as a scaffold, the β subunit was also found to contain a glycogen-binding domain (GBD), indicating that the β subunit functions in energy sensing via glycogen binding (Hardie, 2003). The binding of glycogen to the GBD allosterically modifies the αβγ complex structure to inhibit AMPK activity and also suppresses the α subunit phosphorylation by altering the upstream kinase activity (Mangat et al., 2009; McBride et al., 2009). The γ subunits include particular cystathionine beta synthase (CBS) domains giving AMPK the ability to sensitively detect shifts in the AMP: ATP ratio (Adams et al., 2004). The cooperation of these different subunits can allosterically activate the enzyme by up to 5-fold (Carling et al., 1987).

1.1.2. AMPK Regulation

As the name implies, AMPK is allosterically regulated by the intracellular ratio of AMP to ATP (Hardie et al., 1999). AMP binds to the CBS domains on the γ subunit to activate AMPK by preventing association of the autoinhibitory segment with the kinase domain on the α subunit (Hardie and Hawley, 2001). The degree of activation depends on which isoforms of both the α and γ subunits are present in the complex. The complexes containing the α2 and γ2 isoforms can be activated by AMP up to 5-fold, while the complexes containing the γ3 can only be weakly activated less than 2-fold (Cheung et al., 2000).

Although AMP can allosterically activate the AMPK α subunit catalytic domain by approximately 5-fold, a majority of AMPK activity is dependent on the phosphorylation of an α subunit at threonine-172 which lies in the activation loop (Ojuka et al., 2002). By phosphorylation, AMPK is activated up to 1000-fold by upstream AMPK kinase (AMPKK) (Suter et al., 2006). Although the phosphorylation of AMPK regulation was discovered early the in 1980s, it took over 20 years to identify the upstream kinases in the AMPK cascade. In 2003, serine/threonine-protein kinase 11 (LKB1), a tumor suppressor protein kinase isolated from rat liver, was shown to regulate AMPK kinase activity (Woods et al., 2003). Like AMPK, LKB1 is also a heterotrimeric protein kinase and requires binding of two other regulatory subunits, STE20-related kinase adaptor (STRAD) and calcium-binding protein 39 (MO25), for activity (Hawley et al., 2003). LKB1 is allosterically activated by binding to the pseudokinase STRAD and the adaptor protein MO25. According to several *in vitro* studies (Hawley et al., 1995; 2003), the

LKB1/STRAD/MO25 complex was proposed to be the biologically active unit to phosphorylate and activate AMPK in response to an increase in the AMP/ATP ratio (Fig. 1.1). However, this concept was challenged by results of later studies, showing that various treatments in cells activated AMPK but did not alter LKB1 activity (Suter et al., 2006; Sanders et al., 2007). A possible mechanism to explain this inconsistency is that AMP could increase phosphorylation of AMPK via inhibition of the dephosphorylation step, which accounts for the observation that LKB1 is essential for AMPK regulation by AMP (Carling et al., 2008). Besides the LKB1/STRAD/MO25 complex, another possible upstream regulator responsible for phosphorylation of AMPK is calcium/calmodulin-dependent protein kinase kinase (CAMKK), especially the CAMKK β isoform (Hawley et al., 2005; Woods et al., 2005). CAMKK was first identified as a protein kinase that activates calmodulin-dependent protein kinase I (CAMK I) and calmodulin-dependent protein kinase IV (CAMK IV) (Tokumitsu et al., 1994). By using CAMKK purified from rat brain, it was suggested that AMP stimulates phosphorylation and activation of AMPK by CAMKK (Hawley et al., 1995). However, more recent studies indicate that CAMKK is activated by an increase in intracellular calcium instead of AMP (Woods et al., 2005; Stahmann et al., 2006), suggesting that CAMKK mediates the calcium-dependent AMPK activation pathway (Fig. 1.1).

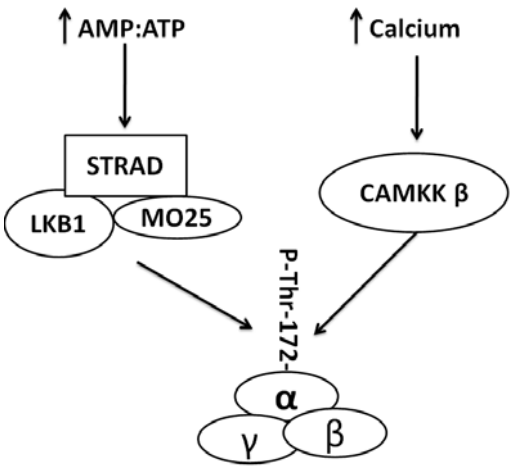

Fig. 1. 1. Upstream regulation of AMP-activated protein kinase (AMPK). Upstream AMPK kinases, including serine/threonine-protein kinase 11/STE20-related kinase adaptor/calcium-binding protein 39 (LKB1/STRAD/MO25) complex and calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK β), activate AMPK by phosphorylation of threonine 172 (Thr-172) on the catalytic α subunit. The LKB1/STRAD/MO25 complex is involved in activation of AMPK in response to an increase in the AMP:ATP ratio. CAMKK β is involved in activation of AMPK in response to an increase in the intracellular calcium concentration. (Adapted from Carling et al., 2008)

1.1.3. Targets of AMPK

During the past decade, there has been an explosive growth in our knowledge of the AMPK signaling cascade and numerous physiological roles of AMPK have been identified. AMPK signaling cascade exerts effects on glucose and lipid metabolism (Sambandam and Lopaschuk, 2003), and gene expression and protein synthesis to maintain intracellular energy balance (Chan and Dyck, 2005) (Fig. 1.2), all of which are

essential for metabolism balance in the liver, skeletal muscle, heart, adipose tissue, and pancreas.

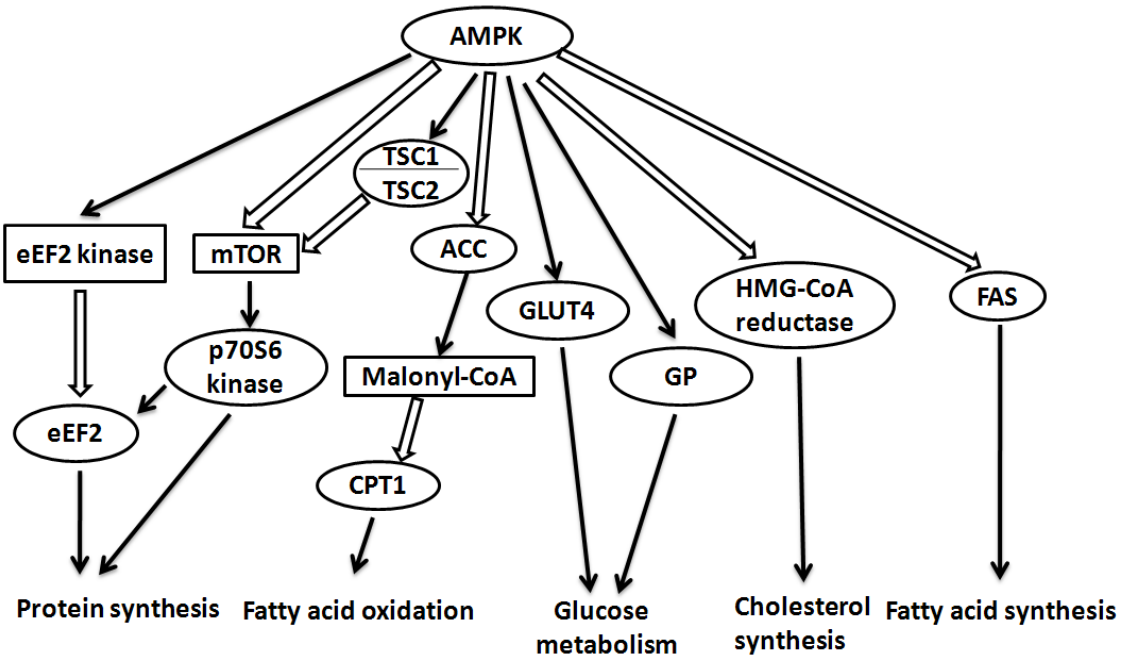

Fig. 1. 2. Targets of AMPK. AMPK regulates glucose and lipid metabolism, gene expression and protein synthesis to maintain intracellular energy balance. AMPK activates eukaryotic elongation factor-2 (eEF2) kinase, which phosphorylates and inactivates eEF2 to suppress protein synthesis. AMPK activation also inhibits mammalian target of rapamycin (mTOR) signaling and p70S6 kinase activity through the phosphorylation of TSC2-gene product/TSC1-gene product (TSC2/TSC1) to inhibit protein synthesis. The inhibition of acetyl CoA carboxylase (ACC) by AMPK decreases the synthesis of malonyl-CoA, which upregulates carnitine parmitoyltransferase 1 (CPT1) to stimulate fatty acid oxidation. On the other hand, AMPK inhibits fatty acid synthesis by suppressing the expression of fatty acid synthase (FAS). Additionally, AMPK also phosphorylates and inhibits the activity of 3-hydroxy-3-methylglutaryl CoA reductase

(HMG-CoA reductase) to inhibit cholesterol synthesis. AMPK stimulates glucose uptake by activating glucose transporter type 4 (GLUT4) as well as glycogenolysis by increasing glycogen phosphorylase (GP) activity. *Line arrows represent stimulation while block arrows represent inhibition*.

Accumulating evidence shows activation of AMPK increases glucose uptake and glycolysis (Abbud et al., 2000; Woods et al., 2000; Barnes et al., 2002; Yang et al., 2010). In rat liver epithelial cells, glucose uptake was acutely increased by oxidative phosphorylation and osmotic stress via a pathway involving AMPK (Barnes et al., 2002). *In vitro* administration of 5'-aminoimidazole-4-carboxamide ribonucleoside (AICAR), a pharmacological agonist of AMPK, stimulated glucose uptake and the translocation of glucose transporter type 4 (GLUT4) in skeletal muscle without engaging insulin-dependent signals (Russell et al., 1999). Chronic *in vivo* AICAR injections in rats increased the total amount of GLUT4 protein in skeletal muscle (Suwa et al., 2006), suggesting upregulated glucose transportation. Hypothalamic AMPK inhibition led to a significant suppression of glucose production, whereas hypothalamic AMPK activation antagonized the inhibition of hypothalamic nutrients on glucose production (Yang et al., 2010). AMPK activation also correlated well (+) with increased glycogen phosphorylase (GP) activity and an increased rate of glycogenolysis (Fraser et al., 1999), indicating that AMPK increases glycogenolysis by activating glycogen phosphorylase. Thus, AMPK plays an important role in glucose metabolism to maintain a critical level of energy stores.

One of the effects of AMPK activation in metabolic regulation is an increase in fatty acid metabolism, which provides more energy for the cell. ACC, a key regulatory enzyme of fatty acid oxidation, was reported early in 1973 to be inhibited by AMPK via phosphorylation (Carlson and Kim). This inhibition decreases the synthesis of malonyl-CoA, an inhibitor of carnitine palmitoyltransferase 1 (CPT1). CPT1 mediates the transportation of fatty acid into the mitochondria for oxidation (Winder, 2001). Therefore, inhibition of ACC by AMPK results in increased fatty acid transportation and subsequent oxidation. On the other hand, AMPK inhibits fatty acid synthesis by suppressing the expression of fatty acid synthase (FAS), a key enzyme for fatty acid synthesis (Woods et al., 2000). Additionally, AMPK also phosphorylates and inhibits the activity of HMG-CoA reductase (Beg et al., 1973), a key regulatory enzyme in cholesterol synthesis. The inhibition of HMG-CoA reductase suppresses the production of mevalonic acid, which is a key substrate for cholesterol synthesis. Thus, AMPK plays a role in fatty acid oxidation and synthesis as well as cholesterol synthesis.

AMPK, as an “energy sensor”, is also involved in the regulation of protein synthesis during times of energy or nutrient deprivation. Activation of AMPK by AICAR results in inhibition of eukaryotic elongation factor-2 (eEF2), a key component in protein translation machinery, suggesting a suppression of protein synthesis (McLeod and Proud, 2002). Later studies further demonstrated that eEF2 kinase is a direct substrate for AMPK (Browne et al., 2004), indicating that AMPK regulates eEF2 phosphorylation by control of eEF2 kinase activity. In addition to regulating eEF2 through eEF2 kinase, AMPK also controls the expression and activity of several other mediators of protein synthesis, including TSC2-gene product (TSC2), the mammalian target of rapamycin (mTOR), and

p70S6 kinase. AMPK activation inhibits mTOR signaling and p70S6 kinase activity through the phosphorylation of TSC2, which then inhibits cell size and growth (Bolster et al., 2002; Kimura et al., 2003). Thus, AMPK activation suppresses protein synthesis via a number of different pathways.

1.1.4. Regulation of Food Intake and Energy Balance by Hypothalamic AMPK

AMPK, known as a cellular "energy sensor", is not only involved in energy regulation within individual cells but also plays a similar role at the whole body level. In peripheral tissue, AMPK regulates body energy status in response to altered AMP/ATP ratio, nutrients, metabolites and hormones, whereas AMPK in the central nervous system (CNS), located in energy-sensing neurons and circuits for body energy homeostasis, acts as a "master regulator" of whole body energy balance (Ronnett et al., 2009). There is increasing evidence supporting the role of hypothalamic AMPK in food intake, energy expenditure, and body weight regulation in different species.

AMPK activity in hypothalamic neurons is altered by peripheral hormonal and nutrient signals and mediates the feeding response. Signals of energy deficit activate the hypothalamic AMPK pathway. Insulin-induced systemic hypoglycemia, which is a nutrient signal of energy deficit, increases hypothalamic AMPK phosphorylation and α2 activities in rats, suggesting hypothalamic AMPK is important for counter-regulatory hormonal responses (Han et al., 2005). Glucocorticoids, a stimulator of gluconeogenesis, upregulate neuropeptide Y (NPY) and agouti-related protein (AgRP) gene expression in the arcuate nucleus (ARC) through AMPK signaling to increase feeding (Shimizu et al., 2008). Ghrelin, an orexigenic neuropeptide secreted from the stomach, modulates

hypothalamic fatty acid metabolism specifically in the ventromedial hypothalamus (VMH) through AMPK signaling to control feeding (Lopez et al., 2008). Adiponectin, a peripheral energy signal secreted by adipose tissue, stimulates food intake and inhibits energy expenditure during fasting through its effect on the CNS AMPK system (Kubota et al., 2007). On the other hand, signals of energy surplus inhibit the hypothalamic AMPK pathway. AMPK activity is inhibited in ARC and paraventricular nucleus (PVN) by leptin, the anorexigenic hormone secreted from adipose tissue, and suppression of hypothalamic AMPK is necessary for the regulation effect of leptin on food intake and body weight (Minokoshi et al., 2004). Chronic insulin treatment prevents diabetes-induced changes in food intake as well as in hypothalamic AMPK activity, and intracerebroventricular (ICV) injection of insulin reduces hyperphagia and the enhanced hypothalamic AMPK activity in diabetic rats (Minokoshi et al., 2004; Namkoong et al., 2005). The adipocyte-derived hormone resistin profoundly suppresses hypothalamic fatty acid metabolism by increasing the phosphorylation level of AMPK and its downstream target ACC (Vazquez et al., 2008). Clearly, feeding-inhibiting factors appear to decrease hypothalamic AMPK activity and feeding-stimulating factors increase it. Various factors may regulate food intake by involving the AMPK signal pathway.

Pharmacological alteration of the activity of hypothalamic AMPK affects food intake and energy balance. ICV injection of AICAR, an activator of AMPK, into the PVN of the hypothalamus significantly increases food intake (Andersson et al., 2004). On the contrary, ICV (Kim et al., 2004) or intraparenchymal (Hayes et al., 2009) injection of a widely used AMPK antagonist (6-[4-(2-piperidin-1-yl-ethoxy)-phenyl)]- 3-pyridin-4-yl-pyrazolo[1,5-a] pyrimidine, which is also known as Compound C, suppressed food intake

and body weight gain by inhibiting AMPK. Compound C prevents AICAR-induced phosphorylation of AMPK and blocks the antilipolytic effect of AICAR on adipocytes in rats (Anthony et al., 2009). The phosphorylation effect of AICAR on ACC, a direct downstream target of AMPK, is blocked by Compound C (King et al., 2006). Additionally, ICV or intraperitoneal (IP) injection of C75 (3-carboxy-4-octyl-2-methylenebutyrolactone), a known FAS inhibitor with a potent anorexic action, reduces food intake and body weight by decreasing hypothalamic AMPK activity (Kim et al., 2004; Landree et al., 2004). AICAR reversed both the C75-induced anorexia and the decrease in phosphorylation levels of hypothalamic AMPK (Kim et al., 2004; Landree et al., 2004).

In mammals, various appetite-related neuropeptides, such as orexigenic neuropeptide NPY and AgRP as well as anorexigenic neuropeptide pro-opiomelanocortin (POMC), appear to be involved in the regulatory effect of AMPK on food intake and energy balance. *In vitro* administration of the AMPK activator AICAR to neurons isolated from the ARC increases intracellular calcium concentration of NPY-immunoreactive neurons (Kohno et al., 2008). C75 reduced NPY expression in the ARC of hypothalamus by decreasing AMPK α phosphorylation and phosphorylated cAMP response element-binding protein (pCREB), suggesting a possible mechanism by which C75 may regulate NPY expression via the AMPK pathway (Kim et al., 2004). In hypothalamic tissue cultures, *in vitro* administration of AICAR increases NPY and AgRP gene expression in the ARC of hypothalamus (Lee et al., 2005; Shimizu et al., 2008). By using genetic knockout mice, Claret et al. (2007) demonstrated that AMPK signaling in POMC and AgRP neurons plays an important role in long-term energy balance. Mice without AMPK

α2 in POMC neurons developed obesity due to reduced energy expenditure and dysregulated food intake but remained sensitive to leptin. In contrast, mice without AMPK α2 in AgRP neurons developed an age-dependent lean phenotype with increased sensitivity to a melanocortin agonist (Claret et al., 2007). All these results suggest that hypothalamic AMPK exerts its regulatory effect of food intake and energy balance by acting on various appetite-related neuropeptides.

1.1.5. AMPK in Chickens

A functional LKB1/AMPK pathway also exists in chickens with characteristics similar to the corresponding pathway in mammals (Proszkowiec-Weglarz et al., 2006). All isoforms of AMPK subunits, including α1, α2, β1, β2, γ1, γ2 and γ3 and AMPK upstream kinases LKB1/STRAD/MO25 complexes are expressed in different tissues of 3 week-old broiler chickens or chicken embryos (Proszkowiec-Weglarz et al., 2006; Proszkowiec-Weglarz and Richards, 2009). As in mammals, activated AMPK is also present in different hypothalamic nuclei, including the lateral hypothalamus (LH), periventricular nucleus (PVN), ventromedialis nucleus (VMN), infundibular nucleus (IN) and median eminence (ME), all of which are involved in regulation of food intake and energy balance, suggesting AMPK may have the same appetite and energy homeostasis regulatory effect in chickens (Proszkowiec-Weglarz et al., 2006).

AMPK activity is related to metabolic state and heat production in chickens. Alterations of energy level caused by fasting and refeeding did not have a major impact on the expression of AMPK α1 and α2 subunit genes in 3 week-old broiler chicken livers, which indicated that AMPK subunit gene transcription is not regulated during changes in

feeding state. However, the level of phosphorylated AMPK α subunits increased whereas ACC α, FAS, stearoyl-CoA desaturase-1 (SCD-1) and malic enzyme mRNA expression decreased after a 48 h fast (Proszkowiec-Weglarz et al., 2009), suggesting fasting may regulate the hepatic lipogenic process via the AMPK pathway in broiler chickens. In chicken embryos, chronic injection of AICAR significantly increased AMPK phosphorylation in skeletal muscle and in liver, which was paralleled by significant increases in heat production, glucose utilization, and liver and skeletal muscle mitochondrial capacity (Walter et al., 2010).

By using lines of chickens selected for high or low abdominal fatness, the role of AMPK in muscle glycogen metabolism was examined (Sibut et al., 2008). A 3-fold greater level of AMPK activation, as well as concomitant down- and upregulation for the gamma1 and gamma2 AMPK subunit isoforms, respectively, was found in the muscle of lean chickens. When compared to fat chickens, these differences were paralleled by greater gene expression for glycogen synthase, glycogen phosphorylase, and the gamma subunit of phosphorylase kinase in lean chicken muscles (Sibut et al., 2008). Together, these results indicate that selection for body fatness in chickens may regulate the muscle glycogen turnover and content by altering AMPK activity.

Clearly, a functional AMPK pathway with similar characteristics to mammals exits in chickens. However, the specific roles of the AMPK pathway in regulating food intake and energy balance in chickens and the kinase pathway or biochemical mechanisms involved still need to be clarified. A better understanding of how the AMPK pathway is

involved in food intake and body weight gain may contribute to devising strategies to either increase or decrease food intake in chickens.

1.2. GHRELIN AND OBESTATIN

1.2.1. Ghrelin History

The word ghrelin comes from "ghre," which means growth in Proto-Indo-European languages, for its ability to stimulate growth hormone (GH) release. Ghrelin was first discovered in the rat stomach and determined to be a natural ligand of the orphan growth hormone secretagogue receptor type 1 a (GHS-R1a) (Kojima et al., 1999). GHS-R1a is a G protein-coupled receptor expressed in the pituitary, hypothalamus and several peripheral tissues (Gnanapavan et al., 2002). Ghrelin can stimulate the release of growth hormone and modulate food intake by acting through this receptor (Kojima and Kangawa, 2005). Ghrelin not only plays an important role in the release of GH and the regulation of food intake, but also in other essential physiological effects, including gastric motility (Gourcerol et al., 2011), energy and glucose homeostasis (St-Pierre et al., 2003; Healy et al., 2011), cardiovascular (Dinca et al., 2010), pulmonary (Schwenke et al., 2010) and immune (Hirayama et al., 2010) function, cell proliferation (Rak-Mardyla and Gregoraszczuk; 2010) and differentiation and bone physiology (Koutroubakis et al., 2010).

The bioactive form of ghrelin in human and rodents is a 28-amino acid peptide with an octanoyl modification at the serine3 position (O-n-Octanoyl-Ser3). The acyl group modification is essential for ghrelin to bind and activate its classical receptor, GHS-R1a

(Kojima et al., 1999). However, in addition to ghrelin, various bioactive peptides are encoded by the ghrelin gene, in which des-acyl ghrelin and obestatin are the main forms (Hosoda et al., 2003). Unlike ghrelin, these ghrelin derivatives exert physiological function through receptors different from GHS-R1a. Obestatin was first reported to selectively bind to the orphan G protein-coupled receptor GPR39 (Zhang et al., 2005). However, study of the intracellular pathway indicated that obestatin was not the cognate ligand for GPR39 (Chartrel et al., 2007). Although the receptor for des-acyl ghrelin has not yet been defined, previous studies demonstrated that des-acyl ghrelin stimulates food intake by acting on the lateral hypothalamus (Toshinai et al., 2006) and stimulates peristaltic motion by acting on the spinal cord (Chen et al., 2005), suggesting that the undefined des-acyl ghrelin receptor may be present in the spinal cord and hypothalamus. It has been demonstrated that des-acyl ghrelin and obestatin have subtle but opposite physiological effects to ghrelin (Kojima and Kangawa, 2005; Zhang et al., 2005; Soares and Leite-Moreira, 2008). Research involving these three active products of the ghrelin gene suggest that there is a dynamic network with multiple effector elements which are dependent on each other.

1.2.2. Structure and Production of Ghrelin

The human ghrelin gene, located on chromosome 3p25-26, consists of five exons and four introns (Kojima and Kangawa, 2005). This ghrelin gene can generate a 117-amino acid precursor of ghrelin called preproghrelin, which is subsequently cleaved to yield ghrelin (Kojima et al., 1999). Ghrelin is a 28-amino acid peptide with an octanoyl modification at the serine3 position (C8:0), which is essentially for ghrelin's biological

activity and may also influence the transport of ghrelin across the blood-brain barrier (Banks et al., 2002). Natural molecular forms of ghrelin include des-Gln ghrelin and des-acyl ghrelin (Hosoda et al., 2003). Des-Gln ghrelin, a natural isoform that lacks glutamine at residue 14, is found in small amounts in the serum and has the same GH-releasing activity of full ghrelin (Hosoda et al., 2000a). Des-acyl ghrelin, the nonacylated form of ghrelin found mainly in both stomach and blood, lacks the ability to bind to GHS-R1a receptor and mediates no effects through this receptor (Hosoda et al., 2000b).

Additionally, a highly conserved region, encoding a 23-amino acid peptide called obestatin, was identified in the 76–98 segment of the proghrelin gene (Zhang et al., 2005). Obestatin appears to have opposite physiological effects to ghrelin on energy homeostasis, gastrointestinal function, body weight and food intake. The interrelationship of ghrelin and obestatin has added further complexity to ghrelin physiology.

1.2.3. Distribution of Ghrelin and Obestatin

There are two major forms of ghrelin in tissues and plasma: an endocrinologically inactive (des-acyl) and an active (octanoylated) form (Hosoda et al., 2000b). In human plasma, normal concentration of active ghrelin is 10-20 fmol/mL and total ghrelin, including both active and inactive forms, is 100-150 fmol/mL. Plasma ghrelin concentration is increased by fasting and is reduced by habitual feeding (Cummings et al., 2001). In all vertebrate species, the stomach is the main place where ghrelin is produced. Two-thirds of circulating ghrelin is produced by P/D1 cells lining the fundus of the human stomach and most of the remainder comes from epsilon cells of the pancreas (Ariyasu et al., 2001). Tissue distribution of ghrelin and des-acyl ghrelin appears to be

similar. The stomach has the highest level of ghrelin, with the next highest amounts found in the duodenum, jejunum, ileum, cecum and colon (Hosoda et al., 2000a). Besides the GI tract, ghrelin is also diversely expressed in other tissues including the hypothalamus, pituitary, adrenal gland, thyroid, breast, ovary, placenta, fallopian tube, testis, prostate, liver, gall bladder, pancreas, fat, lymphocytes, spleen, kidney, lung, skeletal muscle, myocardium, vein and skin (Gnanapavan et al., 2002; Ghelardoni et al., 2006), but at lower levels. Since expression of GHS-R1a receptor was also detected in many of these tissues, the local expression of ghrelin might be involved in physiological autocrine or paracrine effects.

The expression of obestatin was reported in the large and small intestines, stomach, spleen, cerebral cortex of rats (Zhang et al., 2005) and in the perinatal rat pancreas (Chanoine et al., 2006). Obestatin immunoreactivity was also detected in cells of the gastric mucosa, myenteric plexus and in the Leydig cells of the testis in mice (Dun et al., 2006). Additionally, double immunofluorescence demonstrated that obestatin binding cells were also immune-reactive for ghrelin (Gronberg et al., 2008), suggesting that obesatin and ghrelin are stored in the same secretory vesicles. Compared to ghrelin, obestatin had a lower ability to permeate the blood-brain-barrier (BBB) (Pan et al., 2006), which prevents peripheral obestatin from entering into the CNS and regulating feeding behavior (Kobelt et al., 2008).

1.2.4. The Receptors of Ghrelin and Obestatin

GHS-R1, the most investigated ghrelin receptor, has two variants, GHS-R1a and GHS-R1b, both of which originate from alternative processing of pre-mRNA of the same gene

(Gnanapavan et al., 2002). GHS-R1b is a widely expressed receptor like GHS-R1a, but lacks the ability to bind ghrelin or GH. The activation of GHS-R1a by ghrelin relies on three distinct systems of second messengers, including adenylate cyclase/protein kinase A (AC/PKA), phospholipase C/protein kinase C (PLC/PKC), and extracellular Ca^{2+} systems, which indicates that GHS-R1a is coupled to G_q and G_α protein subunits (Malagon et al., 2003). The participation of all three systems is required for ghrelin to activate the GHS-R1a receptor. Although GHS-R1b had no effect on ghrelin-mediated signaling, it did attenuate the constitutive activation of PLC by GHS-R1a, suggesting GHS-R1b may preferentially downregulate GHS-R1a signaling by attenuating GHS-R1a constitutive activation (Chu et al., 2007; Leung et al., 2007). The signal transduction pathway following ghrelin receptor activation was investigated in the rat pituitary cell line GH3. Ghrelin exerts a proliferative effect on GH3 both via a PKC/mitogen-activated protein kinase (PKC/MAPK)-dependent pathway and via a tyrosine kinase-dependent pathway (Nanzer et al., 2004). By using HepG2, a hepatoma cell line, ghrelin upregulated the tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), the association of the adaptormolecule growth factor receptor-bound protein 2 (Grb2) with IRS-1, and stimulated MAPK activity that is also potentiated by insulin (Murata et al., 2002). However, later studies demonstrated that HepG2 cells do not express GHS-R1a, which may suggest the existence of an alternative ghrelin receptor in these cells (Thielemans et al., 2007).

The highest level of GHS-R1a expression was detected in the hypothalamus and pituitary, which is consistent with its action of controlling appetite, food intake, and energy balance (Howard et al., 1996). Surprisingly, GHS-R1a is also expressed in other

parts of the brain, such as the hippocampus, pars compacta of the substantial nigra, ventral tegmental area, dorsal and pyriform cortex, which affects mood, cognition, short term memory and reward (Guan et al., 1997). Besides CNS, the expression of a ghrelin-receptor transcript product was also found in the vagal dorsal ganglion (Burdyga et al., 2006), suggesting that ghrelin may exert effects on multiple peripheral organs, such as the stomach, intestine, pancreas, thyroid, gonads, adrenals, kidney, heart and vasculature, bone, various tumors and cell lines expressing GHS-R1a, by activating the vagal dorsal ganglion. GHS-R1a is essential for the GH releasing effects of ghrelin as well as its stimulatory effects on food intake, as reported from experiments performed in GHS-R1a knockout mice (Sun et al., 2004). Nevertheless, ghrelin can bind to and activate cell lines devoid of GHS-R1a (Thielemans et al., 2007), suggesting GHS-R1a is not the sole receptor for ghrelin.

Since acylation at Ser3 is essential for GHS-1a activation, des-acyl ghrelin, the major circulating form of ghrelin (Hosoda et al., 2000b), is unable to bind GHS-R1a. Therefore, its effects should be mediated by other receptors. However, none of these have been characterized. *In vitro* studies conducted in cardiomyocytes (Lear et al., 2010), adipocytes (Rodriguez et al., 2009), prostatic (Cassoni et al., 2004) and skeletal muscle cells (Filigheddu et al., 2007) indicate that des-acyl ghrelin influences cell proliferation and metabolism and binds to cell membranes.

GPR39, a G-protein coupled receptor sharing homology with GHS-R1a, was initially suggested to be the receptor for obestatin (Zhang et al., 2005). *In vivo* studies conducted in the GPR39–knockout mouse indicated that the GPR39 receptor played an important

role in obestatin bioactivity regulating gastrointestinal and metabolic functions (Moechars et al., 2006). However, later studies found that I^{125}-obestatin does not bind to GPR39 and observed no effects of obestatin on GPR39-transfected cells in various functional assays using the same conditions as Zhang (Chartrel et al., 2007), suggesting that obestatin is not the cognate ligand for GPR39. Other groups also confirmed that it is the zinc ion, instead of obestatin, that could be a physiologically relevant agonist or modulator of GPR39 (Lauwers et al., 2006; Holst et al., 2007; Tremblay et al., 2007). Challenged by these feedback comments, Zhang et al. (2007) repeated their previous experiment and confirmed that the original findings on obestatin binding and activation of GPR39 receptors were not reproducible. Thus obestatin is unlikely to be the cognate ligand for GPR39 as initially claimed, and additional research is needed to reveal the exact native receptor for obestatin.

1.2.5. Effects of Ghrelin Gene-derived Products

1.2.5.1. GH Secretion

Ghrelin stimulates GH release both *in vitro* and *in vivo* in a dose-dependent manner by acting on the GHS-R1a (Takaya et al., 2000; Sun et al., 2004). Des-acyl ghrelin does not influence GH secretion, because it does not bind to GHS-R1a. Several studies demonstrated that neither intravenous (IV) nor ICV administration of obestatin affects the secretion of GH in rats (Zhang et al., 2005; Bresciani et al., 2006; Nogueiras et al., 2007; Samson et al., 2007; Yamamoto et al., 2007).

1.2.5.2. Food Intake and Energy Balance

In mammals, ICV, IC or subcutaneous (SC) administration of ghrelin can potently stimulate food intake (Kamegai et al., 2001; Nakazato et al., 2001; Shintani et al., 2001; Wren et al., 2001; Lawrence et al., 2002; Bomberg et al., 2007; Healy et al., 2011) and short-term fasting results in a pre-prandial rise in ghrelin levels. Additionally, hunger scores corresponded to ghrelin levels (Cummings et al., 2004; Guo et al., 2008; Healy et al., 2010). Among all identified orexigenic peptides, ghrelin has been found to be the most potent.

Ghrelin is an important peripheral signal produced in response to hunger and starvation to convince the central nervous system to stimulate feeding (Asakawa et al., 2005). There is a close relationship between ghrelin and ARC neurons peptides, including NPY and AgRP, as well as the anorexigenic peptides cocaine and amphetamine related transcript (CART) and POMC (Kamegai et al., 2001; Chen et al., 2004). Peripheral ghrelin signals reach the ARC of the hypothalamus by activating vagal afferents (Williams et al., 2003) or via the bloodstream (Hewson et al., 2000). Consequently, NPY and AgRP are activated, while CART and POMC are inhibited (Chen et al., 2004). Additionally, ghrelin secreted by hypothalamic neurons also plays an important role in its orexigenic effects (Couce et al., 2006).

Ghrelin also influences the activity of hunger-related neuropeptidergic systems outside the ARC. Orexin, an orexigenic neuropeptide expressed in the lateral hypothalamus area (LHA), appears to be involved in the feeding stimulatory effects of ghrelin (Toshinai et al., 2003). Ghrelin stimulates the activity of isolated orexin neurons, whereas glucose and leptin inhibit them (Yamanaka et al., 2003). Ghrelin-immunoreactive axonal terminals

make direct synaptic contacts with orexin-producing neurons in the LHA, and ICV injection of ghrelin induces Fos expression in these cells (Toshinai et al., 2003). These results indicate that the orexigenic effect of ghrelin is partly mediated via activating orexin secretion in LHA.

Despite the stimulatory effects on appetite, ghrelin also contributes to long-term energy balance and body weight gain. Plasma ghrelin levels are negatively correlated to body weight, decreasing with weight gain induced by overfeeding (Williams et al., 2006), pregnancy (Palik et al., 2007), or high-fat diet feeding (Otukonyong et al., 2005). In contrast, weight loss resulting from food restriction (Purnell et al., 2007), lung cancer (Shimizu et al., 2003), or long-term chronic exercise (Kraemer and Castracane, 2007) causes an elevation in plasma ghrelin levels.

Central ghrelin is physiologically related to cell metabolism in adipose tissue. Chronic ICV injection of ghrelin not only increases food intake, but also decreases energy expenditure, reduces utilization of fat and increases utilization of carbohydrates, which results in body weight gain and adiposity (Wortley et al., 2004). Chronic central ghrelin also results in stimulation of triglyceride (TG) uptake and lipogenesis and inhibition in lipid oxidation in white adipocytes (Theander-Carrillo et al., 2006). In a rat model of cancer cachexia, SC administration of ghrelin improved weight gain and lean body mass retention by limiting the loss of fat mass normally observed in tumor-implanted rats (DeBoer et al., 2007). Additionally, GHS-Ra1 downregulation in the PVN did not affect daily food intake but significantly reduced body weight and blood ghrelin levels,

suggesting that the central ghrelin system could regulate body weight without affecting food intake (Shrestha et al., 2009).

Both GHS-R-null and ghrelin-null mice studies suggested that ghrelin signaling is required for body energy balance. When fed a high-fat diet, GHSR-null mice had lower feed efficiencies and gross energetic efficiencies, incorporated less consumed food into body energy, and accumulated less body weight and adiposity as compared with wild-type controls (Zigman et al., 2005). Young ghrelin-null mice are protected from the rapid weight gain induced by chronic exposure to a high-fat diet. Compared to wild-type control, ghrelin-null mice had decreased adiposity and increased energy expenditure and locomotor activity (Wortley et al., 2005).

The regulatory effect of des-acyl ghrelin on food intake has been reported to be either negative (Asakawa et al., 2005; Chen et al., 2005; Matsuda et al., 2006) or positive (Toshinai et al., 2006). Nonetheless, most available studies suggest des-acyl ghrelin can inhibit food intake. The food-inhibiting effect seems to be specific for central administration and mediated by increased gene expression of CART and urocortin on the PVN and the ARC in the hypothalamus (Asakawa et al., 2005). In addition, desacyl ghrelin over-expressing mice exhibited a moderately decreased linear growth pattern with a decrease in body weight, food intake, gastric emptying and fat pad mass weight, indicating that des-acyl ghrelin generates a negative energy balance (Asakawa et al., 2005).

Reports about the effects of obestatin on food intake are inconsistent. Some studies reported that obestatin inhibited food intake and suppressed body weight (Zhang et al.,

2005; Bresciani et al., 2006; Green et al., 2007; Lagaud et al., 2007; Zizzari et al., 2007) whereas others reported no effect (Seoane et al., 2006; Sibilia et al., 2006; Gourcerol et al., 2007; Noguerias et al., 2007). The anorexia effect of obestatin was firstly reported by Zhang et al. (2005), intraperitoneal (IP) and ICV injection of human obestatin suppressed food intake, while treatment of non-amidated obestatin was less effective. However, many other groups failed to reproduce the results using different doses and source of obestatin injection (Seoane et al., 2006; Sibilia et al., 2006; Gourcerol et al., 2007; Lagaud et al., 2007; Noguerias et al., 2007). In one of these studies, IP administration of obestatin inhibited food intake and body weight gain in rodents with an unusual U-shaped dose-response relationship, which may explain the difficulties in reproducing the feeding regulation effect of obestatin reported by some groups (Lagaud et al., 2007). Although there is inconsistency in the anorexigenic action of obestatin, this peptide, when co-administered with ghrelin, inhibits orexigenic actions of ghrelin on food intake and demonstrated that it also blocks its action on GH secretion (Zizzari et al., 2007).

1.2.5.3. Gastrointestinal Functions

The IV or ICV injection of ghrelin dose-dependently increased gastric acid secretion and stimulated gastric motility (Masuda et al., 2000; Date et al., 2001; Dornonville et al., 2004). Central ghrelin increased gastric acid secretion in a dose-dependent manner by activating the nucleus of the solitary tract and the dorsomotor nucleus of the vagus nerve (Date et al., 2001). Although des-acyl ghrelin does not appear to influence gastric secretion (Dornonville et al., 2004), it inhibits gastric emptying without altering small intestinal transit (Asakawa et al., 2005; Chen et al., 2005). IV injection of obestatin

stimulates the secretion of pancreatic juice enzymes through a vagal pathway in anaesthetized rats (Kapica et al., 2007) and IP injection of obestatin led to a sustained suppression of gastric emptying activity (Zhang et al., 2005).

1.2.5.4. Cardiovascular Functions

Ghrelin improves left ventricular function in men and rats by inhibiting cardiomyocyte apoptosis and expression of proinflammatory cytokines (Dubinski et al., 2007). It also protects cardiac muscle from injury induced by isoproterenol in rats (Li et al., 2006). *In vitro*, ghrelin administration decreases inotropism (Bedendi et al., 2003), and inhibits apoptosis of cardiomyocytes (Kui et al., 2009). Research about the role of obestatin in the cardiovascular system is still limited. The IV injection of obestatin does not change blood pressure of spontaneously hypertensive rats (Li et al., 2009). *In vitro,* treatment of obestatin does not change cardiomyocyte viability and metabolism (Iglesias et al., 2007). Additional studies are needed to determine the effects of obestatin on cardiovascular functions.

1.2.6. Chicken Ghrelin

Chicken ghrelin is a 26 amino acid peptide (GSSFLSPTYKNIQQQKDTRKPTARLH) that shows a very high (54%) sequence identity to human ghrelin (Kaiya et al., 2002). The third serine residue is conserved in the chicken and mammalian species with an acylation by either n-octanoic or n-decanoic acid. Like mammalian species, chicken ghrelin mRNA is predominantly expressed in the stomach, but mainly observed in the proventricular region of the stomach and absent in the gizzard. However, low levels of

ghrelin mRNA expression were also detectable in brain, lung, and intestine (Kaiya et al., 2002). Chicken ghrelin has the same GH-releasing effect as rat or human ghrelin in rats and chicks (Ahmed et al., 2002; Kaiya et al., 2002; Baudet and Harvey, 2003).

The ICV, IV and SC injection of ghrelin stimulated feeding in mammals (Kamegai et al., 2001; Nakazato et al., 2001; Shintani et al., 2001; Wren et al., 2001; Lawrence et al., 2002; Bomberg et al., 2007; Healy et al., 2011), whereas ICV injection of ghrelin strongly suppressed feeding in chickens regardless of age, sex, and time of injection or dosages (Furuse et al., 2001; Saito et al., 2005; Chen et al., 2008). Like ghrelin, ICV injection of growth hormone-releasing peptide-2, which also stimulates release of GH, inhibits food intake in neonatal chicks (Furuse et al., 2001; Saito et al., 2002). These results indicate that although the GH releasing activity of ghrelin is evolutionarily conserved, the different ghrelin structures result in species-specific food intake regulation functions in the chicken (Kaiya et al., 2002).

As opposed to the orexigenic effect of ghrelin in mammals, which is mediated by ARC neuropeptides such as NPY and orexin (Kamegai et al., 2001; Chen et al., 2004), the anorexigenic effect of ghrelin in chickens is independent from the NPY pathway and may be mediated by corticotropin-regulating factor (CRF) (Saito et al., 2005). These results indicate that the mechanisms underlying the food intake regulation activity of ghrelin in chickens are different from those in mammals. The reason for this discrepancy between mammals and birds still needs to be clarified.

1.3. OBJECTIVE OF THE STUDY

The objectives of this study were to determine if genetic selection for high (HWS) or low (LWS) body weight in chickens has altered the hypothalamic AMP-activated protein kinase (AMPK) system and if this alteration contributes to the different feeding responses to various appetite modulators between HWS and LWS lines. The objectives were:

(1) to determine the relationship between high and low body weight phenotypes and the expression of genes encoding AMPK subunits and associated proteins.
(2) to investigate central feeding responses to various food intake modulators in HWS and LWS chickens.
(3) to determine if genetic selection for body weight has altered the central responses to the AMPK activator AICAR and AMPK inhibitor Compound C and if this alteration was due to an altered AMPK system.
(4) to determine if genetic selection for body weight has altered the central response to ghrelin and obestatin and if this alteration was due to an altered AMPK system.
(5) to determine if altered central responses to ghrelin and obestatin involve the alteration of the neuronal corticotrophin-releasing hormone (CRH) pathway or obesity and appetite related factors.

1.4. REFERENCES

Abbud, W., S. Habinowski, J. Z. Zhang, J. Kendrew, F. S. Elkairi, B. E. Kemp, L. A. Witters, and F. Ismail-Beigi. 2000. Stimulation of AMP-activated protein kinase (AMPK) is associated with enhancement of Glut1-mediated glucose transport. Arch. Biochem. Biophys. 380:347-352.

Adams, J., Z. P. Chen, B. J. Van Denderen, C. J. Morton, M. W. Parker, L. A. Witters, D. Stapleton, and B. E. Kemp. 2004. Intrasteric control of AMPK via the gamma1 subunit AMP allosteric regulatory site. Protein Sci. 13:155-165.

Ahmed, S., and S. Harvey. 2002. Ghrelin: a hypothalamic GH-releasing factor in domestic fowl (Gallus domesticus). J. Endocrinol. 172:117-125.

Andersson, U., K. Filipsson, C. R. Abbott, A. Woods, K. Smith, S. R. Bloom, D. Carling, and C. J. Small. 2004. AMP-activated protein kinase plays a role in the control of food intake. J. Biol. Chem. 279:12005-12008.

Anthony, N. M., M. P. Gaidhu, and R. B. Ceddia. 2009. Regulation of visceral and subcutaneous adipocyte lipolysis by acute AICAR-induced AMPK activation. Obesity (Silver Spring) 17:1312-1317.

Ariyasu, H., K. Takaya, T. Tagami, Y. Ogawa, K. Hosoda, T. Akamizu, M. Suda, T. Koh, K. Natsui, S. Toyooka, G. Shirakami, T. Usui, A. Shimatsu, K. Doi, H. Hosoda, M. Kojima, K. Kangawa, and K. Nakao. 2001. Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. J. Clin. Endocrinol. Metab. 86:4753-4758.

Asakawa, A., A. Inui, M. Fujimiya, R. Sakamaki, N. Shinfuku, Y. Ueta, M. M. Meguid, and M. Kasuga. 2005. Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. Gut 54:18-24.

Banks, W. A., M. Tschop, S. M. Robinson, and M. L. Heiman. 2002. Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure. J. Pharmacol. Exp. Ther. 302:822-827.

Barnes, K., J. C. Ingram, O. H. Porras, L. F. Barros, E. R. Hudson, L. G. Fryer, F. Foufelle, D. Carling, D. G. Hardie, and S. A. Baldwin. 2002. Activation of GLUT1 by metabolic and osmotic stress: potential involvement of AMP-activated protein kinase (AMPK). J. Cell Sci. 115:2433-2442.

Baudet, M. L., and S. Harvey. 2003. Ghrelin-induced GH secretion in domestic fowl in vivo and in vitro. J. Endocrinol. 179:97-105.

Bedendi, I., G. Alloatti, A. Marcantoni, D. Malan, F. Catapano, C. Ghe, R. Deghenghi, E. Ghigo, and G. Muccioli. 2003. Cardiac effects of ghrelin and its endogenous derivatives des-octanoyl ghrelin and des-Gln14-ghrelin. Eur. J. Pharmacol. 476:87-95.

Beg, Z. H., D. W. Allmann, and D. M. Gibson. 1973. Modulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity with cAMP and wth protein fractions of rat liver cytosol. Biochem. Biophys. Res. Commun. 54:1362-1369.

Bolster, D. R., S. J. Crozier, S. R. Kimball, and L. S. Jefferson. 2002. AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling. J. Biol. Chem. 277:23977-23980.

Bomberg, E. M., M. K. Grace, M. M. Wirth, A. S. Levine, and P. K. Olszewski. 2007. Central ghrelin induces feeding driven by energy needs not by reward. Neuroreport 18:591-595.

Bresciani, E., D. Rapetti, F. Dona, I. Bulgarelli, L. Tamiazzo, V. Locatelli, and A. Torsello. 2006. Obestatin inhibits feeding but does not modulate GH and corticosterone secretion in the rat. J. Endocrinol. Invest. 29:RC16-18.

Browne, G. J., S. G. Finn, and C. G. Proud. 2004. Stimulation of the AMP-activated protein kinase leads to activation of eukaryotic elongation factor 2 kinase and to its phosphorylation at a novel site, serine 398. J. Biol. Chem. 279:12220-12231.

Burdyga, G., A. Varro, R. Dimaline, D. G. Thompson, and G. J. Dockray. 2006. Ghrelin receptors in rat and human nodose ganglia: putative role in regulating CB-1 and MCH receptor abundance. Am. J. Physiol. Gastrointest Liver Physiol. 290:G1289-1297.

Carling, D. 2004. The AMP-activated protein kinase cascade--a unifying system for energy control. Trends Biochem. Sci. 29:18-24.

Carling, D., P. R. Clarke, V. A. Zammit, and D. G. Hardie. 1989. Purification and characterization of the AMP-activated protein kinase. Copurification of acetyl-CoA carboxylase kinase and 3-hydroxy-3-methylglutaryl-CoA reductase kinase activities. Eur. J. Biochem. 186:129-136.

Carling, D., M. J. Sanders, and A. Woods. 2008. The regulation of AMP-activated protein kinase by upstream kinases. Int. J. Obes. (Lond) 32 Suppl 4:S55-59.

Carling, D., V. A. Zammit, and D. G. Hardie. 1987. A common bicyclic protein kinase cascade inactivates the regulatory enzymes of fatty acid and cholesterol biosynthesis. FEBS Lett. 223:217-222.

Carlson, C. A., and K. H. Kim. 1973. Regulation of hepatic acetyl coenzyme A carboxylase by phosphorylation and dephosphorylation. J. Biol. Chem. 248:378-380.

Cassoni, P., C. Ghe, T. Marrocco, E. Tarabra, E. Allia, F. Catapano, R. Deghenghi, E. Ghigo, M. Papotti, and G. Muccioli. 2004. Expression of ghrelin and biological

activity of specific receptors for ghrelin and des-acyl ghrelin in human prostate neoplasms and related cell lines. Eur. J. Endocrinol. 150:173-184.

Chan, A. Y., and J. R. Dyck. 2005. Activation of AMP-activated protein kinase (AMPK) inhibits protein synthesis: a potential strategy to prevent the development of cardiac hypertrophy. Can. J. Physiol. Pharmacol. 83:24-28.

Chanoine, J. P., A. C. Wong, and V. Barrios. 2006. Obestatin, acylated and total ghrelin concentrations in the perinatal rat pancreas. Horm. Res. 66:81-88.

Chartrel, N., R. Alvear-Perez, J. Leprince, X. Iturrioz, A. Reaux-Le Goazigo, V. Audinot, P. Chomarat, F. Coge, O. Nosjean, M. Rodriguez, J. P. Galizzi, J. A. Boutin, H. Vaudry, and C. Llorens-Cortes. 2007. Comment on "Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake". Science 315:766; author reply 766.

Chen, C. Y., Y. Chao, F. Y. Chang, E. J. Chien, S. D. Lee, and M. L. Doong. 2005. Intracisternal des-acyl ghrelin inhibits food intake and non-nutrient gastric emptying in conscious rats. Int. J. Mol. Med. 16:695-699.

Chen, H. Y., M. E. Trumbauer, A. S. Chen, D. T. Weingarth, J. R. Adams, E. G. Frazier, Z. Shen, D. J. Marsh, S. D. Feighner, X. M. Guan, Z. Ye, R. P. Nargund, R. G. Smith, L. H. Van der Ploeg, A. D. Howard, D. J. MacNeil, and S. Qian. 2004. Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein. Endocrinology 145:2607-2612.

Chen, L. L., Q. Y. Jiang, H. Kaiya, X. T. Zhu, G. Shu, K. Kangawa, P. Gao, and Y. L. Zhang. 2008. Central administration of chicken ghrelin inhibits food intake in

adult chickens. Pages 75 in 9th International Symposium on Avian Endocrinology.

Cheung, P. C., I. P. Salt, S. P. Davies, D. G. Hardie, and D. Carling. 2000. Characterization of AMP-activated protein kinase gamma-subunit isoforms and their role in AMP binding. Biochem. J. 346 Pt 3:659-669.

Chu, K. M., K. B. Chow, P. K. Leung, P. N. Lau, C. B. Chan, C. H. Cheng, and H. Wise. 2007. Over-expression of the truncated ghrelin receptor polypeptide attenuates the constitutive activation of phosphatidylinositol-specific phospholipase C by ghrelin receptors but has no effect on ghrelin-stimulated extracellular signal-regulated kinase 1/2 activity. Int. J. Biochem. Cell Biol. 39:752-764.

Claret, M., M. A. Smith, R. L. Batterham, C. Selman, A. I. Choudhury, L. G. Fryer, M. Clements, H. Al-Qassab, H. Heffron, A. W. Xu, J. R. Speakman, G. S. Barsh, B. Viollet, S. Vaulont, M. L. Ashford, D. Carling, and D. J. Withers. 2007. AMPK is essential for energy homeostasis regulation and glucose sensing by POMC and AgRP neurons. J. Clin. Invest. 117:2325-2336.

Couce, M. E., D. Cottam, J. Esplen, R. Teijeiro, P. Schauer, and B. Burguera. 2006. Potential role of hypothalamic ghrelin in the pathogenesis of human obesity. J. Endocrinol. Invest. 29:599-605.

Crute, B. E., K. Seefeld, J. Gamble, B. E. Kemp, and L. A. Witters. 1998. Functional domains of the alpha1 catalytic subunit of the AMP-activated protein kinase. J. Biol. Chem. 273:35347-35354.

Cummings, D. E., R. S. Frayo, C. Marmonier, R. Aubert, and D. Chapelot. 2004. Plasma ghrelin levels and hunger scores in humans initiating meals voluntarily without time- and food-related cues. Am. J. Physiol. Endocrinol. Metab. 287:E297-304.

Cummings, D. E., J. Q. Purnell, R. S. Frayo, K. Schmidova, B. E. Wisse, and D. S. Weigle. 2001. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes 50:1714-1719.

Date, Y., M. Nakazato, N. Murakami, M. Kojima, K. Kangawa, and S. Matsukura. 2001. Ghrelin acts in the central nervous system to stimulate gastric acid secretion. Biochem. Biophys. Res. Commun. 280:904-907.

DeBoer, M. D., X. X. Zhu, P. Levasseur, M. M. Meguid, S. Suzuki, A. Inui, J. E. Taylor, H. A. Halem, J. Z. Dong, R. Datta, M. D. Culler, and D. L. Marks. 2007. Ghrelin treatment causes increased food intake and retention of lean body mass in a rat model of cancer cachexia. Endocrinology 148:3004-3012.

Dinca, M., I. L. Dumitriu, M. B. Gurzu, S. M. Slatineanu, L. Foia, L. Vata, E. Cojocaru, and G. Petrescu. 2010. Ghrelin and Ang 1-7 have vasodilatory cumulative effects on pulmonary vessels. Rev. Med. Chir. Soc. Med. Nat. Iasi. 114:803-807.

Dornonville de la Cour, C., E. Lindstrom, P. Norlen, and R. Hakanson. 2004. Ghrelin stimulates gastric emptying but is without effect on acid secretion and gastric endocrine cells. Regul. Pept. 120:23-32.

Dubinski, A., and Z. Zdrojewicz. 2007. [Ghrelin and its influence on cardiovascular system]. Wiad Lek 60:352-355.

Dun, S. L., G. C. Brailoiu, E. Brailoiu, J. Yang, J. K. Chang, and N. J. Dun. 2006. Distribution and biological activity of obestatin in the rat. J. Endocrinol. 191:481-489.

Dyck, J. R., G. Gao, J. Widmer, D. Stapleton, C. S. Fernandez, B. E. Kemp, and L. A. Witters. 1996. Regulation of 5'-AMP-activated protein kinase activity by the noncatalytic beta and gamma subunits. J. Biol. Chem. 271:17798-17803.

Ferrer, A., C. Caelles, N. Massot, and F. G. Hegardt. 1985. Activation of rat liver cytosolic 3-hydroxy-3-methylglutaryl coenzyme A reductase kinase by adenosine 5'-monophosphate. Biochem. Biophys. Res. Commun. 132:497-504.

Filigheddu, N., V. F. Gnocchi, M. Coscia, M. Cappelli, P. E. Porporato, R. Taulli, S. Traini, G. Baldanzi, F. Chianale, S. Cutrupi, E. Arnoletti, C. Ghe, A. Fubini, N. Surico, F. Sinigaglia, C. Ponzetto, G. Muccioli, T. Crepaldi, and A. Graziani. 2007. Ghrelin and des-acyl ghrelin promote differentiation and fusion of C2C12 skeletal muscle cells. Mol. Biol. Cell 18:986-994.

Fraser, H., G. D. Lopaschuk, and A. S. Clanachan. 1999. Alteration of glycogen and glucose metabolism in ischaemic and post-ischaemic working rat hearts by adenosine A1 receptor stimulation. Br. J. Pharmacol. 128:197-205.

Furuse, M., T. Tachibana, A. Ohgushi, R. Ando, T. Yoshimatsu, and D. M. Denbow. 2001. Intracerebroventricular injection of ghrelin and growth hormone releasing factor inhibits food intake in neonatal chicks. Neurosci. Lett. 301:123-126.

Ghelardoni, S., V. Carnicelli, S. Frascarelli, S. Ronca-Testoni, and R. Zucchi. 2006. Ghrelin tissue distribution: comparison between gene and protein expression. J. Endocrinol. Invest. 29:115-121.

Gnanapavan, S., B. Kola, S. A. Bustin, D. G. Morris, P. McGee, P. Fairclough, S. Bhattacharya, R. Carpenter, A. B. Grossman, and M. Korbonits. 2002. The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. J. Clin. Endocrinol. Metab. 87:2988.

Gourcerol, G., D. Adelson, M. Million, L. Wang, and Y. Tache. 2011. Modulation of gastric motility by brain-gut peptides using a novel non-invasive miniaturized pressure transducer method in anesthetized rodents. Peptides 32(4):737-46.

Gourcerol, G., D. H. St-Pierre, and Y. Tache. 2007. Lack of obestatin effects on food intake: should obestatin be renamed ghrelin-associated peptide (GAP)? Regul. Pept. 141:1-7.

Green, B. D., N. Irwin, and P. R. Flatt. 2007. Direct and indirect effects of obestatin peptides on food intake and the regulation of glucose homeostasis and insulin secretion in mice. Peptides 28:981-987.

Gronberg, M., A. V. Tsolakis, L. Magnusson, E. T. Janson, and J. Saras. 2008. Distribution of obestatin and ghrelin in human tissues: immunoreactive cells in the gastrointestinal tract, pancreas, and mammary glands. J. Histochem. Cytochem. 56:793-801.

Guan, X. M., H. Yu, O. C. Palyha, K. K. McKee, S. D. Feighner, D. J. Sirinathsinghji, R. G. Smith, L. H. Van der Ploeg, and A. D. Howard. 1997. Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. Brain. Res. Mol. Brain Res. 48:23-29.

Guo, Z. F., A. J. Ren, X. Zheng, Y. W. Qin, F. Cheng, J. Zhang, H. Wu, W. J. Yuan, and L. Zou. 2008. Different responses of circulating ghrelin, obestatin levels to

fasting, re-feeding and different food compositions, and their local expressions in rats. Peptides 29:1247-1254.

Han, S. M., C. Namkoong, P. G. Jang, I. S. Park, S. W. Hong, H. Katakami, S. Chun, S. W. Kim, J. Y. Park, K. U. Lee, and M. S. Kim. 2005. Hypothalamic AMP-activated protein kinase mediates counter-regulatory responses to hypoglycaemia in rats. Diabetologia. 48:2170-2178.

Hardie, D. G. 2003. Minireview: the AMP-activated protein kinase cascade: the key sensor of cellular energy status. Endocrinology 144:5179-5183.

Hardie, D. G., and D. Carling. 1997. The AMP-activated protein kinase--fuel gauge of the mammalian cell? Eur. J. Biochem. 246:259-273.

Hardie, D. G., and S. A. Hawley. 2001. AMP-activated protein kinase: the energy charge hypothesis revisited. Bioessays 23:1112-1119.

Hardie, D. G., I. P. Salt, S. A. Hawley, and S. P. Davies. 1999. AMP-activated protein kinase: an ultrasensitive system for monitoring cellular energy charge. Biochem. J. 338 (Pt 3):717-722.

Hawley, S. A., J. Boudeau, J. L. Reid, K. J. Mustard, L. Udd, T. P. Makela, D. R. Alessi, and D. G. Hardie. 2003. Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. J. Biol. 2:28.

Hawley, S. A., D. A. Pan, K. J. Mustard, L. Ross, J. Bain, A. M. Edelman, B. G. Frenguelli, and D. G. Hardie. 2005. Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. Cell Metab. 2:9-19.

Hawley, S. A., M. A. Selbert, E. G. Goldstein, A. M. Edelman, D. Carling, and D. G. Hardie. 1995. 5'-AMP activates the AMP-activated protein kinase cascade, and Ca2+/calmodulin activates the calmodulin-dependent protein kinase I cascade, via three independent mechanisms. J. Biol. Chem. 270:27186-27191.

Hayes, M. R., K. P. Skibicka, K. K. Bence, and H. J. Grill. 2009. Dorsal hindbrain 5'-adenosine monophosphate-activated protein kinase as an intracellular mediator of energy balance. Endocrinology 150:2175-2182.

Healy, J. E., J. L. Bateman, C. E. Ostrom, and G. L. Florant. 2011. Peripheral ghrelin stimulates feeding behavior and positive energy balance in a sciurid hibernator. Horm. Behav.

Healy, J. E., C. E. Ostrom, G. K. Wilkerson, and G. L. Florant. 2010. Plasma ghrelin concentrations change with physiological state in a sciurid hibernator (Spermophilus lateralis). Gen. Comp. Endocrinol. 166:372-378.

Hewson, A. K., and S. L. Dickson. 2000. Systemic administration of ghrelin induces Fos and Egr-1 proteins in the hypothalamic arcuate nucleus of fasted and fed rats. J. Neuroendocrinol. 12:1047-1049.

Hirayama, T., T. Kawabe, M. Matsushima, Y. Nishimura, Y. Kobe, Y. Ota, K. Baba, and K. Takagi. 2010. Ghrelin and obestatin promote the allergic action in rat peritoneal mast cells as basic secretagogues. Peptides 31:2109-2113.

Holst, B., K. L. Egerod, E. Schild, S. P. Vickers, S. Cheetham, L. O. Gerlach, L. Storjohann, C. E. Stidsen, R. Jones, A. G. Beck-Sickinger, and T. W. Schwartz. 2007. GPR39 signaling is stimulated by zinc ions but not by obestatin. Endocrinology 148:13-20.

Hosoda, H., M. Kojima, H. Matsuo, and K. Kangawa. 2000a. Purification and characterization of rat des-Gln14-Ghrelin, a second endogenous ligand for the growth hormone secretagogue receptor. J. Biol. Chem. 275:21995-22000.

Hosoda, H., M. Kojima, H. Matsuo, and K. Kangawa. 2000b. Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochem. Biophys. Res. Commun. 279:909-913.

Hosoda, H., M. Kojima, T. Mizushima, S. Shimizu, and K. Kangawa. 2003. Structural divergence of human ghrelin. Identification of multiple ghrelin-derived molecules produced by post-translational processing. J. Biol. Chem. 278:64-70.

Howard, A. D., S. D. Feighner, D. F. Cully, J. P. Arena, P. A. Liberator, C. I. Rosenblum, M. Hamelin, D. L. Hreniuk, O. C. Palyha, J. Anderson, P. S. Paress, C. Diaz, M. Chou, K. K. Liu, K. K. McKee, S. S. Pong, L. Y. Chaung, A. Elbrecht, M. Dashkevicz, R. Heavens, M. Rigby, D. J. Sirinathsinghji, D. C. Dean, D. G. Melillo, A. A. Patchett, R. Nargund, P. R. Griffin, J. A. DeMartino, S. K. Gupta, J. M. Schaeffer, R. G. Smith, and L. H. Van der Ploeg. 1996. A receptor in pituitary and hypothalamus that functions in growth hormone release. Science 273:974-977.

Hudson, E. R., D. A. Pan, J. James, J. M. Lucocq, S. A. Hawley, K. A. Green, O. Baba, T. Terashima, and D. G. Hardie. 2003. A novel domain in AMP-activated protein kinase causes glycogen storage bodies similar to those seen in hereditary cardiac arrhythmias. Curr. Biol. 13:861-866.

Iglesias, M. J., A. Salgado, R. Pineiro, B. K. Rodino, M. F. Otero, L. Grigorian, R. Gallego, C. Dieguez, O. Gualillo, J. R. Gonzalez-Juanatey, and F. Lago. 2007.

Lack of effect of the ghrelin gene-derived peptide obestatin on cardiomyocyte viability and metabolism. J. Endocrinol. Invest. 30:470-476.

Kaiya, H., S. Van Der Geyten, M. Kojima, H. Hosoda, Y. Kitajima, M. Matsumoto, S. Geelissen, V. M. Darras, and K. Kangawa. 2002. Chicken ghrelin: purification, cDNA cloning, and biological activity. Endocrinology 143:3454-3463.

Kamegai, J., H. Tamura, T. Shimizu, S. Ishii, H. Sugihara, and I. Wakabayashi. 2001. Chronic central infusion of ghrelin increases hypothalamic neuropeptide Y and Agouti-related protein mRNA levels and body weight in rats. Diabetes 50:2438-2443.

Kapica, M., M. Zabielska, I. Puzio, A. Jankowska, I. Kato, A. Kuwahara, and R. Zabielski. 2007. Obestatin stimulates the secretion of pancreatic juice enzymes through a vagal pathway in anaesthetized rats - preliminary results. J. Physiol. Pharmacol. 58 Suppl. 3:123-130.

Kim, E. K., I. Miller, S. Aja, L. E. Landree, M. Pinn, J. McFadden, F. P. Kuhajda, T. H. Moran, and G. V. Ronnett. 2004. C75, a fatty acid synthase inhibitor, reduces food intake via hypothalamic AMP-activated protein kinase. J. Biol. Chem. 279:19970-19976.

Kimura, N., C. Tokunaga, S. Dalal, C. Richardson, K. Yoshino, K. Hara, B. E. Kemp, L. A. Witters, O. Mimura, and K. Yonezawa. 2003. A possible linkage between AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signalling pathway. Genes Cells 8:65-79.

King, T. D., L. Song, and R. S. Jope. 2006. AMP-activated protein kinase (AMPK) activating agents cause dephosphorylation of Akt and glycogen synthase kinase-3. Biochem. Pharmacol. 71:1637-1647.

Kobelt, P., A. S. Wisser, A. Stengel, M. Goebel, N. Bannert, G. Gourcerol, T. Inhoff, S. Noetzel, B. Wiedenmann, B. F. Klapp, Y. Tache, and H. Monnikes. 2008. Peripheral obestatin has no effect on feeding behavior and brain Fos expression in rodents. Peptides 29:1018-1027.

Kohno, D., H. Sone, Y. Minokoshi, and T. Yada. 2008. Ghrelin raises [Ca2+]i via AMPK in hypothalamic arcuate nucleus NPY neurons. Biochem. Biophys. Res. Commun. 366:388-392.

Kojima, M., H. Hosoda, Y. Date, M. Nakazato, H. Matsuo, and K. Kangawa. 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402:656-660.

Kojima, M., and K. Kangawa. 2005. Ghrelin: structure and function. Physiol. Rev. 85:495-522.

Koutroubakis, I. E., C. Zavos, J. Damilakis, G. Papadakis, J. Neratzoulakis, N. Karkavitsas, and E. A. Kouroumalis. 2010. Role of Ghrelin and Insulin-like Growth Factor Binding Protein-3 in the Development of Osteoporosis in Inflammatory Bowel Disease. J. Clin. Gastroenterol.

Kraemer, R. R., and V. D. Castracane. 2007. Exercise and humoral mediators of peripheral energy balance: ghrelin and adiponectin. Exp. Biol. Med. (Maywood) 232:184-194.

Kubota, N., W. Yano, T. Kubota, T. Yamauchi, S. Itoh, H. Kumagai, H. Kozono, I. Takamoto, S. Okamoto, T. Shiuchi, R. Suzuki, H. Satoh, A. Tsuchida, M. Moroi, K. Sugi, T. Noda, H. Ebinuma, Y. Ueta, T. Kondo, E. Araki, O. Ezaki, R. Nagai, K. Tobe, Y. Terauchi, K. Ueki, Y. Minokoshi, and T. Kadowaki. 2007. Adiponectin stimulates AMP-activated protein kinase in the hypothalamus and increases food intake. Cell Metab. 6:55-68.

Kui, L., Z. Weiwei, L. Ling, H. Daikun, Z. Guoming, Z. Linuo, and H. Renming. 2009. Ghrelin inhibits apoptosis induced by high glucose and sodium palmitate in adult rat cardiomyocytes through the PI3K-Akt signaling pathway. Regul. Pept. 155:62-69.

Lagaud, G. J., A. Young, A. Acena, M. F. Morton, T. D. Barrett, and N. P. Shankley. 2007. Obestatin reduces food intake and suppresses body weight gain in rodents. Biochem. Biophys. Res. Commun. 357:264-269.

Landree, L. E., A. L. Hanlon, D. W. Strong, G. Rumbaugh, I. M. Miller, J. N. Thupari, E. C. Connolly, R. L. Huganir, C. Richardson, L. A. Witters, F. P. Kuhajda, and G. V. Ronnett. 2004. C75, a fatty acid synthase inhibitor, modulates AMP-activated protein kinase to alter neuronal energy metabolism. J. Biol. Chem. 279:3817-3827.

Lauwers, E., B. Landuyt, L. Arckens, L. Schoofs, and W. Luyten. 2006. Obestatin does not activate orphan G protein-coupled receptor GPR39. Biochem. Biophys. Res. Commun. 351:21-25.

Lawrence, C. B., A. C. Snape, F. M. Baudoin, and S. M. Luckman. 2002. Acute central ghrelin and GH secretagogues induce feeding and activate brain appetite centers. Endocrinology 143:155-162.

Lear, P. V., M. J. Iglesias, S. Feijoo-Bandin, D. Rodriguez-Penas, A. Mosquera-Leal, V. Garcia-Rua, O. Gualillo, C. Ghe, E. Arnoletti, G. Muccioli, C. Dieguez, J. R. Gonzalez-Juanatey, and F. Lago. 2010. Des-acyl ghrelin has specific binding sites and different metabolic effects from ghrelin in cardiomyocytes. Endocrinology 151:3286-3298.

Lee, K., B. Li, X. Xi, Y. Suh, and R. J. Martin. 2005. Role of neuronal energy status in the regulation of adenosine 5'-monophosphate-activated protein kinase, orexigenic neuropeptides expression, and feeding behavior. Endocrinology 146:3-10.

Leung, P. K., K. B. Chow, P. N. Lau, K. M. Chu, C. B. Chan, C. H. Cheng, and H. Wise. 2007. The truncated ghrelin receptor polypeptide (GHS-R1b) acts as a dominant-negative mutant of the ghrelin receptor. Cell Signal 19:1011-1022.

Li, L., L. K. Zhang, Y. Z. Pang, C. S. Pan, Y. F. Qi, L. Chen, X. Wang, C. S. Tang, and J. Zhang. 2006. Cardioprotective effects of ghrelin and des-octanoyl ghrelin on myocardial injury induced by isoproterenol in rats. Acta. Pharmacol. Sin. 27:527-535.

Li, Z. F., S. W. Song, Y. W. Qin, J. L. Zhang, X. X. Zhao, B. L. Zhang, A. J. Ren, Z. F. Guo, and X. Zheng. 2009. Bolus intravenous injection of obestatin does not change blood pressure level of spontaneously hypertensive rat. Peptides 30:1928-1930.

Lopez, M., R. Lage, A. K. Saha, D. Perez-Tilve, M. J. Vazquez, L. Varela, S. Sangiao-Alvarellos, S. Tovar, K. Raghay, S. Rodriguez-Cuenca, R. M. Deoliveira, T. Castaneda, R. Datta, J. Z. Dong, M. Culler, M. W. Sleeman, C. V. Alvarez, R. Gallego, C. J. Lelliott, D. Carling, M. H. Tschop, C. Dieguez, and A. Vidal-Puig. 2008. Hypothalamic fatty acid metabolism mediates the orexigenic action of ghrelin. Cell Metab. 7:389-399.

Malagon, M. M., R. M. Luque, E. Ruiz-Guerrero, F. Rodriguez-Pacheco, S. Garcia-Navarro, F. F. Casanueva, F. Gracia-Navarro, and J. P. Castano. 2003. Intracellular signaling mechanisms mediating ghrelin-stimulated growth hormone release in somatotropes. Endocrinology 144:5372-5380.

Mangat, S., D. Chandrashekarappa, R. R. McCartney, K. Elbing, and M. C. Schmidt. 2009. Differential roles of the glycogen-binding domains of beta subunits in regulation of the Snf1 kinase complex. Eukaryot. Cell 9:173-183.

Masuda, Y., T. Tanaka, N. Inomata, N. Ohnuma, S. Tanaka, Z. Itoh, H. Hosoda, M. Kojima, and K. Kangawa. 2000. Ghrelin stimulates gastric acid secretion and motility in rats. Biochem. Biophys. Res. Commun. 276:905-908.

Matsuda, K., T. Miura, H. Kaiya, K. Maruyama, S. Shimakura, M. Uchiyama, K. Kangawa, and S. Shioda. 2006. Regulation of food intake by acyl and des-acyl ghrelins in the goldfish. Peptides 27:2321-2325.

McBride, A., S. Ghilagaber, A. Nikolaev, and D. G. Hardie. 2009. The glycogen-binding domain on the AMPK beta subunit allows the kinase to act as a glycogen sensor. Cell Metab. 9:23-34.

McLeod, L. E., and C. G. Proud. 2002. ATP depletion increases phosphorylation of elongation factor eEF2 in adult cardiomyocytes independently of inhibition of mTOR signalling. FEBS Lett. 531:448-452.

Minokoshi, Y., T. Alquier, N. Furukawa, Y. B. Kim, A. Lee, B. Xue, J. Mu, F. Foufelle, P. Ferre, M. J. Birnbaum, B. J. Stuck, and B. B. Kahn. 2004. AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. Nature 428:569-574.

Mitchelhill, K. I., D. Stapleton, G. Gao, C. House, B. Michell, F. Katsis, L. A. Witters, and B. E. Kemp. 1994. Mammalian AMP-activated protein kinase shares structural and functional homology with the catalytic domain of yeast Snf1 protein kinase. J. Biol. Chem. 269:2361-2364.

Moechars, D., I. Depoortere, B. Moreaux, B. de Smet, I. Goris, L. Hoskens, G. Daneels, S. Kass, L. Ver Donck, T. Peeters, and B. Coulie. 2006. Altered gastrointestinal and metabolic function in the GPR39-obestatin receptor-knockout mouse. Gastroenterology 131:1131-1141.

Murata, M., Y. Okimura, K. Iida, M. Matsumoto, H. Sowa, H. Kaji, M. Kojima, K. Kangawa, and K. Chihara. 2002. Ghrelin modulates the downstream molecules of insulin signaling in hepatoma cells. J. Biol. Chem. 277:5667-5674.

Nakazato, M., N. Murakami, Y. Date, M. Kojima, H. Matsuo, K. Kangawa, and S. Matsukura. 2001. A role for ghrelin in the central regulation of feeding. Nature 409:194-198.

Namkoong, C., M. S. Kim, P. G. Jang, S. M. Han, H. S. Park, E. H. Koh, W. J. Lee, J. Y. Kim, I. S. Park, J. Y. Park, and K. U. Lee. 2005. Enhanced hypothalamic AMP-

activated protein kinase activity contributes to hyperphagia in diabetic rats. Diabetes 54:63-68.

Nanzer, A. M., S. Khalaf, A. M. Mozid, R. C. Fowkes, M. V. Patel, J. M. Burrin, A. B. Grossman, and M. Korbonits. 2004. Ghrelin exerts a proliferative effect on a rat pituitary somatotroph cell line via the mitogen-activated protein kinase pathway. Eur. J. Endocrinol. 151:233-240.

Nogueiras, R., P. Pfluger, S. Tovar, M. Arnold, S. Mitchell, A. Morris, D. Perez-Tilve, M. J. Vazquez, P. Wiedmer, T. R. Castaneda, R. DiMarchi, M. Tschop, A. Schurmann, H. G. Joost, L. M. Williams, W. Langhans, and C. Dieguez. 2007. Effects of obestatin on energy balance and growth hormone secretion in rodents. Endocrinology 148:21-26.

Ojuka, E. O., T. E. Jones, L. A. Nolte, M. Chen, B. R. Wamhoff, M. Sturek, and J. O. Holloszy. 2002. Regulation of GLUT4 biogenesis in muscle: evidence for involvement of AMPK and Ca(2+). Am. J. Physiol. Endocrinol. Metab. 282:E1008-1013.

Otukonyong, E. E., M. G. Dube, R. Torto, P. S. Kalra, and S. P. Kalra. 2005. High-fat diet-induced ultradian leptin and insulin hypersecretion are absent in obesity-resistant rats. Obes. Res. 13:991-999.

Palik, E., E. Baranyi, Z. Melczer, M. Audikovszky, A. Szocs, G. Winkler, and K. Cseh. 2007. Elevated serum acylated (biologically active) ghrelin and resistin levels associate with pregnancy-induced weight gain and insulin resistance. Diabetes Res. Clin. Pract. 76:351-357.

Pan, W., H. Tu, and A. J. Kastin. 2006. Differential BBB interactions of three ingestive peptides: obestatin, ghrelin, and adiponectin. Peptides 27:911-916.

Proszkowiec-Weglarz, M., and M. P. Richards. 2009. Expression and activity of the 5'-adenosine monophosphate-activated protein kinase pathway in selected tissues during chicken embryonic development. Poult. Sci. 88:159-178.

Proszkowiec-Weglarz, M., M. P. Richards, B. D. Humphrey, R. W. Rosebrough, and J. P. McMurtry. 2009. AMP-activated protein kinase and carbohydrate response element binding protein: a study of two potential regulatory factors in the hepatic lipogenic program of broiler chickens. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 154:68-79.

Proszkowiec-Weglarz, M., M. P. Richards, R. Ramachandran, and J. P. McMurtry. 2006. Characterization of the AMP-activated protein kinase pathway in chickens. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 143:92-106.

Purnell, J. Q., D. Cummings, and D. S. Weigle. 2007. Changes in 24-h area-under-the-curve ghrelin values following diet-induced weight loss are associated with loss of fat-free mass, but not with changes in fat mass, insulin levels or insulin sensitivity. Int. J. Obes. (Lond) 31:385-389.

Rak-Mardyla, A., and E. Gregoraszczuk. 2010. Effect of ghrelin on proliferation, apoptosis and secretion of progesterone and hCG in the placental JEG-3 cell line. Reprod. Biol. 10:159-165.

Rodriguez, A., J. Gomez-Ambrosi, V. Catalan, M. J. Gil, S. Becerril, N. Sainz, C. Silva, J. Salvador, I. Colina, and G. Fruhbeck. 2009. Acylated and desacyl ghrelin

stimulate lipid accumulation in human visceral adipocytes. Int. J. Obes. (Lond) 33:541-552.

Ronnett, G. V., S. Ramamurthy, A. M. Kleman, L. E. Landree, and S. Aja. 2009. AMPK in the brain: its roles in energy balance and neuroprotection. J. Neurochem. 109 Suppl. 1:17-23.

Russell, R. R., 3rd, R. Bergeron, G. I. Shulman, and L. H. Young. 1999. Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. Am. J. Physiol. 277:H643-649.

Saito, E. S., H. Kaiya, T. Tachibana, S. Tomonaga, D. M. Denbow, K. Kangawa, and M. Furuse. 2005. Inhibitory effect of ghrelin on food intake is mediated by the corticotropin-releasing factor system in neonatal chicks. Regul. Pept. 125:201-208.

Saito, E. S., H. Kaiya, T. Takagi, I. Yamasaki, D. M. Denbow, K. Kangawa, and M. Furuse. 2002. Chicken ghrelin and growth hormone-releasing peptide-2 inhibit food intake of neonatal chicks. Eur. J. Pharmacol. 453:75-79.

Sambandam, N., and G. D. Lopaschuk. 2003. AMP-activated protein kinase (AMPK) control of fatty acid and glucose metabolism in the ischemic heart. Prog. Lipid Res. 42:238-256.

Samson, W. K., M. M. White, C. Price, and A. V. Ferguson. 2007. Obestatin acts in brain to inhibit thirst. Am. J. Physiol. Regul. Integr. Comp. Physiol. 292:R637-643.

Sanders, M. J., P. O. Grondin, B. D. Hegarty, M. A. Snowden, and D. Carling. 2007. Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. Biochem. J. 403:139-148.

Schwenke, D. O., J. T. Pearson, K. Kangawa, P. A. Cragg, and M. Shirai. 2010. Exogenous ghrelin accentuates the acute hypoxic ventilatory response after two weeks of chronic hypoxia in conscious rats. Acta. Physiol. (Oxf) 200:279-287.

Seoane, L. M., O. Al-Massadi, Y. Pazos, U. Pagotto, and F. F. Casanueva. 2006. Central obestatin administration does not modify either spontaneous or ghrelin-induced food intake in rats. J. Endocrinol. Invest. 29:RC13-15.

Shimizu, H., H. Arima, M. Watanabe, M. Goto, R. Banno, I. Sato, N. Ozaki, H. Nagasaki, and Y. Oiso. 2008. Glucocorticoids increase neuropeptide Y and agouti-related peptide gene expression via adenosine monophosphate-activated protein kinase signaling in the arcuate nucleus of rats. Endocrinology 149:4544-4553.

Shimizu, Y., N. Nagaya, T. Isobe, M. Imazu, H. Okumura, H. Hosoda, M. Kojima, K. Kangawa, and N. Kohno. 2003. Increased plasma ghrelin level in lung cancer cachexia. Clin. Cancer Res. 9:774-778.

Shintani, M., Y. Ogawa, K. Ebihara, M. Aizawa-Abe, F. Miyanaga, K. Takaya, T. Hayashi, G. Inoue, K. Hosoda, M. Kojima, K. Kangawa, and K. Nakao. 2001. Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. Diabetes 50:227-232.

Shrestha, Y. B., K. Wickwire, and S. Giraudo. 2009. Effect of reducing hypothalamic ghrelin receptor gene expression on energy balance. Peptides 30:1336-1341.

Sibilia, V., E. Bresciani, N. Lattuada, D. Rapetti, V. Locatelli, V. De Luca, F. Dona, C. Netti, A. Torsello, and F. Guidobono. 2006. Intracerebroventricular acute and

chronic administration of obestatin minimally affect food intake but not weight gain in the rat. J. Endocrinol. Invest. 29:RC31-34.

Sibut, V., E. Le Bihan-Duval, S. Tesseraud, E. Godet, T. Bordeau, E. Cailleau-Audouin, P. Chartrin, M. J. Duclos, and C. Berri. 2008. Adenosine monophosphate-activated protein kinase involved in variations of muscle glycogen and breast meat quality between lean and fat chickens. J. Anim. Sci. 86:2888-2896.

Soares, J. B., and A. F. Leite-Moreira. 2008. Ghrelin, des-acyl ghrelin and obestatin: three pieces of the same puzzle. Peptides 29:1255-1270.

St-Pierre, D. H., L. Wang, and Y. Tache. 2003. Ghrelin: a novel player in the gut-brain regulation of growth hormone and energy balance. News Physiol. Sci. 18:242-246.

Stahmann, N., A. Woods, D. Carling, and R. Heller. 2006. Thrombin activates AMP-activated protein kinase in endothelial cells via a pathway involving Ca2+/calmodulin-dependent protein kinase kinase beta. Mol. Cell Biol. 26:5933-5945.

Stapleton, D., G. Gao, B. J. Michell, J. Widmer, K. Mitchelhill, T. Teh, C. M. House, L. A. Witters, and B. E. Kemp. 1994. Mammalian 5'-AMP-activated protein kinase non-catalytic subunits are homologs of proteins that interact with yeast Snf1 protein kinase. J. Biol. Chem. 269:29343-29346.

Stapleton, D., K. I. Mitchelhill, G. Gao, J. Widmer, B. J. Michell, T. Teh, C. M. House, C. S. Fernandez, T. Cox, L. A. Witters, and B. E. Kemp. 1996. Mammalian AMP-activated protein kinase subfamily. J. Biol. Chem. 271:611-614.

Stapleton, D., E. Woollatt, K. I. Mitchelhill, J. K. Nicholl, C. S. Fernandez, B. J. Michell, L. A. Witters, D. A. Power, G. R. Sutherland, and B. E. Kemp. 1997. AMP-activated protein kinase isoenzyme family: subunit structure and chromosomal location. FEBS Lett. 409:452-456.

Sun, Y., P. Wang, H. Zheng, and R. G. Smith. 2004. Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. Proc. Natl. Acad. Sci. U S A 101:4679-4684.

Suter, M., U. Riek, R. Tuerk, U. Schlattner, T. Wallimann, and D. Neumann. 2006. Dissecting the role of 5'-AMP for allosteric stimulation, activation, and deactivation of AMP-activated protein kinase. J. Biol. Chem. 281:32207-32216.

Suwa, M., T. Egashira, H. Nakano, H. Sasaki, and S. Kumagai. 2006. Metformin increases the PGC-1alpha protein and oxidative enzyme activities possibly via AMPK phosphorylation in skeletal muscle in vivo. J. Appl. Physiol. 101:1685-1692.

Takaya, K., H. Ariyasu, N. Kanamoto, H. Iwakura, A. Yoshimoto, M. Harada, K. Mori, Y. Komatsu, T. Usui, A. Shimatsu, Y. Ogawa, K. Hosoda, T. Akamizu, M. Kojima, K. Kangawa, and K. Nakao. 2000. Ghrelin strongly stimulates growth hormone release in humans. J. Clin. Endocrinol. Metab. 85:4908-4911.

Theander-Carrillo, C., P. Wiedmer, P. Cettour-Rose, R. Nogueiras, D. Perez-Tilve, P. Pfluger, T. R. Castaneda, P. Muzzin, A. Schurmann, I. Szanto, M. H. Tschop, and F. Rohner-Jeanrenaud. 2006. Ghrelin action in the brain controls adipocyte metabolism. J. Clin. Invest. 116:1983-1993.

Thielemans, L., P. J. Peeters, H. Jonckheere, W. Luyten, R. de Hoogt, B. Coulie, and J. Aerssens. 2007. The hepatocarcinoma cell line HepG2 does not express a GHS-R1a-type ghrelin receptor. J. Recept. Signal Transduct Res. 27:309-322.

Thornton, C., M. A. Snowden, and D. Carling. 1998. Identification of a novel AMP-activated protein kinase beta subunit isoform that is highly expressed in skeletal muscle. J. Biol. Chem. 273:12443-12450.

Tokumitsu, H., D. A. Brickey, J. Glod, H. Hidaka, J. Sikela, and T. R. Soderling. 1994. Activation mechanisms for Ca2+/calmodulin-dependent protein kinase IV. Identification of a brain CaM-kinase IV kinase. J. Biol. Chem. 269:28640-28647.

Toshinai, K., Y. Date, N. Murakami, M. Shimada, M. S. Mondal, T. Shimbara, J. L. Guan, Q. P. Wang, H. Funahashi, T. Sakurai, S. Shioda, S. Matsukura, K. Kangawa, and M. Nakazato. 2003. Ghrelin-induced food intake is mediated via the orexin pathway. Endocrinology 144:1506-1512.

Toshinai, K., H. Yamaguchi, Y. Sun, R. G. Smith, A. Yamanaka, T. Sakurai, Y. Date, M. S. Mondal, T. Shimbara, T. Kawagoe, N. Murakami, M. Miyazato, K. Kangawa, and M. Nakazato. 2006. Des-acyl ghrelin induces food intake by a mechanism independent of the growth hormone secretagogue receptor. Endocrinology 147:2306-2314.

Tremblay, F., M. Perreault, L. D. Klaman, J. F. Tobin, E. Smith, and R. E. Gimeno. 2007. Normal food intake and body weight in mice lacking the G protein-coupled receptor GPR39. Endocrinology 148:501-506.

Vazquez, M. J., C. R. Gonzalez, L. Varela, R. Lage, S. Tovar, S. Sangiao-Alvarellos, L. M. Williams, A. Vidal-Puig, R. Nogueiras, M. Lopez, and C. Dieguez. 2008.

Central resistin regulates hypothalamic and peripheral lipid metabolism in a nutritional-dependent fashion. Endocrinology 149:4534-4543.

Walter, I., B. Hegarty, and F. Seebacher. 2010. AMP-activated protein kinase controls metabolism and heat production during embryonic development in birds. J. Exp. Biol. 213:3167-3176.

Williams, D. L., H. J. Grill, D. E. Cummings, and J. M. Kaplan. 2003. Vagotomy dissociates short- and long-term controls of circulating ghrelin. Endocrinology 144:5184-5187.

Williams, D. L., H. J. Grill, D. E. Cummings, and J. M. Kaplan. 2006. Overfeeding-induced weight gain suppresses plasma ghrelin levels in rats. J. Endocrinol. Invest. 29:863-868.

Winder, W. W. 2001. Energy-sensing and signaling by AMP-activated protein kinase in skeletal muscle. J. Appl. Physiol. 91:1017-1028.

Woods, A., D. Azzout-Marniche, M. Foretz, S. C. Stein, P. Lemarchand, P. Ferre, F. Foufelle, and D. Carling. 2000. Characterization of the role of AMP-activated protein kinase in the regulation of glucose-activated gene expression using constitutively active and dominant negative forms of the kinase. Mol. Cell Biol. 20:6704-6711.

Woods, A., K. Dickerson, R. Heath, S. P. Hong, M. Momcilovic, S. R. Johnstone, M. Carlson, and D. Carling. 2005. Ca2+/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. Cell Metab. 2:21-33.

Woods, A., S. R. Johnstone, K. Dickerson, F. C. Leiper, L. G. Fryer, D. Neumann, U. Schlattner, T. Wallimann, M. Carlson, and D. Carling. 2003. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. Curr. Biol. 13:2004-2008.

Wortley, K. E., K. D. Anderson, K. Garcia, J. D. Murray, L. Malinova, R. Liu, M. Moncrieffe, K. Thabet, H. J. Cox, G. D. Yancopoulos, S. J. Wiegand, and M. W. Sleeman. 2004. Genetic deletion of ghrelin does not decrease food intake but influences metabolic fuel preference. Proc. Natl. Acad. Sci. U S A 101:8227-8232.

Wortley, K. E., J. P. del Rincon, J. D. Murray, K. Garcia, K. Iida, M. O. Thorner, and M. W. Sleeman. 2005. Absence of ghrelin protects against early-onset obesity. J. Clin. Invest. 115:3573-3578.

Wren, A. M., L. J. Seal, M. A. Cohen, A. E. Brynes, G. S. Frost, K. G. Murphy, W. S. Dhillo, M. A. Ghatei, and S. R. Bloom. 2001. Ghrelin enhances appetite and increases food intake in humans. J. Clin. Endocrinol. Metab. 86:5992.

Yamamoto, D., N. Ikeshita, R. Daito, E. H. Herningtyas, K. Toda, K. Takahashi, K. Iida, Y. Takahashi, H. Kaji, K. Chihara, and Y. Okimura. 2007. Neither intravenous nor intracerebroventricular administration of obestatin affects the secretion of GH, PRL, TSH and ACTH in rats. Regul. Pept. 138:141-144.

Yamanaka, A., C. T. Beuckmann, J. T. Willie, J. Hara, N. Tsujino, M. Mieda, M. Tominaga, K. Yagami, F. Sugiyama, K. Goto, M. Yanagisawa, and T. Sakurai. 2003. Hypothalamic orexin neurons regulate arousal according to energy balance in mice. Neuron 38:701-713.

Yang, C. S., C. K. Lam, M. Chari, G. W. Cheung, A. Kokorovic, S. Gao, I. Leclerc, G. A. Rutter, and T. K. Lam. 2010. Hypothalamic AMP-activated protein kinase regulates glucose production. Diabetes 59:2435-2443.

Yeh, L. A., K. H. Lee, and K. H. Kim. 1980. Regulation of rat liver acetyl-CoA carboxylase. Regulation of phosphorylation and inactivation of acetyl-CoA carboxylase by the adenylate energy charge. J. Biol. Chem. 255:2308-2314.

Zhang, J. V., C. Klein, P. G. Ren, S. Kass, L. ver Donck, D. Moechars, and A. J. Hsueh. 2007. Response to comment on``obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake". Science 315:766.

Zhang, J. V., P. G. Ren, O. Avsian-Kretchmer, C. W. Luo, R. Rauch, C. Klein, and A. J. Hsueh. 2005. Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. Science 310:996-999.

Zigman, J. M., Y. Nakano, R. Coppari, N. Balthasar, J. N. Marcus, C. E. Lee, J. E. Jones, A. E. Deysher, A. R. Waxman, R. D. White, T. D. Williams, J. L. Lachey, R. J. Seeley, B. B. Lowell, and J. K. Elmquist. 2005. Mice lacking ghrelin receptors resist the development of diet-induced obesity. J. Clin. Invest. 115:3564-3572.

Zizzari, P., R. Longchamps, J. Epelbaum, and M. T. Bluet-Pajot. 2007. Obestatin partially affects ghrelin stimulation of food intake and growth hormone secretion in rodents. Endocrinology 148:1648-1653.

CHAPTER 2

AMPK Involvement in Variation of Hypothalamic Energy Balance and Food Intake Regulation between Lines of Chickens Selected for High or Low Body Weight

(Submitted)

ABSTRACT The present study was designed to evaluate the molecular mechanisms associated with differences in body weight and body composition of two lines of chickens that had undergone long-term selection from a common founder population for high (HWS) or low (LWS) 56-day body weight. At selected ages, these lines differ in body weight by more than 10-fold. To associate with this different body weight, hypothalamic AMP-activated protein kinase (AMPK), which functions to maintain cellular energy balance as well as regulate food intake and energy homeostasis at the whole animal level, were investigated. LWS chickens had a higher level of AMPK activation, as evaluated through phosphorylation of AMPK α and acetyl-CoA carboxylase (ACC), than HWS chickens. This elevation could be caused by an upregulated upstream factor, calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK β). At the transcriptional level, data indicated concomitant upregulation of the α1 and γ1 subunit isoforms of AMPK in the hypothalamus of LWS chickens. The mRNA expressions of genes related to energy homeostasis and obesity, including ACC, carnitine palmitoyltransferase I (CPT1), fatty acid synthase (FAS), leptin receptor (LEPR), fat mass and obesity associated gene (FTO), and feeding-related neuropeptides, including neuropeptide Y (NPY), pro-opiomelanocortin (POMC) and orexin, were also investigated. There was greater mRNA expression of CPT1, LEPR and NPY and less

mRNA expression of ACC α, FAS, FTO, POMC and orexin in LWS than HWS chickens. Together, these data indicate that long-term selection for body weight affects hypothalamic regulation of energy balance and food intake. Alterations of AMPK activity could contribute to these changes.

Key Words: chicken, divergent selection, food intake, energy balance, AMPK

2.1. INTRODUCTION

The modern poultry meat industry involves intensive selection for increased efficiency of growth and development. Although the efficiency of production in broilers has been continuously improved by increasing food intake, it is necessary to restrict food consumption to maintain fecundity of broiler breeders. To continue to increase animal performance by devising strategies to both increase and decrease food intake, it is necessary to better understand how genetic selection for body weight has altered the mechanisms controlling food intake and energy balance.

The central nervous system (CNS), especially the hypothalamus, has an important role in the regulation of food intake and energy homeostasis. It has the ability to detect and integrate signals of energy balance including nutrients, neuropeptides, adipokines and circulating hormones to adjust food intake in response to changing energy requirements (Morton et al., 2006). AMP-activated protein kinase (AMPK), known as a cellular "energy sensor", is altered by hormonal and nutrient signals and can mediate the feeding response (Minokoshi et al., 2004). Several studies have highlighted the important role of hypothalamic AMPK in controlling body energy homeostasis through regulation of food

intake and energy expenditure, suggesting that high AMPK activity enhances orexigenic signals, whereas low AMPK activity suppress these signals (Kubota et al., 2007; Hayes et al., 2009; Lane and Cha, 2009; Florant et al., 2010).

The role of AMPK in metabolic regulation is that of fatty acid oxidation and synthesis. AMPK stimulates fatty acid oxidation by phosphorylating and inhibiting acetyl-CoA carboxylase (ACC), a key regulatory enzyme for fatty acid oxidation. This inhibition decreases the synthesis of malonyl-CoA, an inhibitor of carnitine palmitoyltransferase 1 (CPT1). CPT1 mediates the transport of fatty acid into the mitochondria for oxidation (Winder, 2001). Therefore, inhibition of ACC by AMPK results in increased fatty acid transport and subsequent oxidation. On the other hand, AMPK inhibits fatty acid synthesis by suppressing the expression of fatty acid synthase (FAS), a key enzyme for fatty acid synthesis (Woods et al., 2000). AMPK induced modulation of lipid metabolism in the hypothalamus is a major regulator of whole-body energy homeostasis (Lopez et al., 2010).

Hypothalamic AMPK regulation of food intake involves various appetite-related neuropeptides, such as neuropeptide Y (NPY) and pro-opiomelanocortin (POMC), which ultimately convey peripheral signals of energy balance to regions of the CNS to integrate and trigger a feeding response. Central expression of NPY, a well-known orexigenic neuropeptide, is inhibited by decreased AMPK activity (Minokoshi et al., 2004; Lee et al., 2005), whereas central expression of POMC, a well-described anorexigenic neuropeptide, is stimulated by decreased AMPK activity (Namkoong et al., 2005).

In the present study, we used lines of chickens that had undergone long-term divergent selection from a common White Rock base population for high (HWS) or low (LWS) body weight at 56 days of age (Marquez et al., 2010). These lines of chickens, which have different phenotypic and genotypic characteristics, differ in body weight by more than 10-fold at 8 weeks of age (Johansson et al., 2010). Our objective was to determine if the selection for body weight has changed hypothalamic expression of appetite-related neuropeptides involved in short-term food intake regulation, as well as that of genes related to fatty acid metabolism and obesity in these lines, and if these differences are due to altered AMPK pathway.

2.2. MATERIALS AND METHODS

2.2.1. Animals

The chickens used in this experiment were from two lines that have undergone 50 generations of selection for the single trait of high or low body weight at 56 days of age. The base population was formed by crossing 7 moderately inbred lines of White Plymouth Rocks and the selected lines have been maintained as pedigreed populations (Dunnington and Siegel, 1996; Marquez et al., 2010; Siegel and Wolford, 2003). Eggs from same-age parents from each line were incubated in the same machine. After hatch, chicks were placed as flocks in electric heated batteries with raised wire floors at 32±2°C. A mash diet (20% crude protein, 2684 kcal ME/kg) and water were provided *ad libitum*. Experimental procedures were approved by the Virginia Tech Animal Care and Use Committee.

2.2.2. Tissues Sampling

At 1, 14, 28, 42 and 56 days of age, 6 male birds from each line were weighed and sacrificed by cervical dislocation. The whole brain was removed from the cranial cavity and the preoptic-hypothalamic area was dissected from each brain using the landmarks of the optic chiasm rostrally, and the mammillary bodies caudally. Dissected hypothalamic tissues were quickly frozen in liquid nitrogen, and stored at -80℃ for later analysis.

2.2.3. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

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

Real-time RT-PCR was performed in 7500 Fast Real-Time PCR System (Applied Biosystems, Inc., Carlsbad, CA, USA). One μL of 4-fold diluted RT product was used for PCR in a final volume of 20 μL containing 10 μL 2×Fast SYBR Green Master Mix (Applied Biosystems, Inc.) and 0.5 μM primer mix (Table 2.1). Chicken glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as a reference gene for normalization purposes. The expression of GAPDH mRNA was similar for both lines. The thermal cycling conditions were: initial denaturation (20 s at 95°C), then a two-step amplification program (3 s at 95°C, 30 s at 60°C) was repeated 40 times. Melting curves were performed to insure a single specific PCR product for each gene. Real-time RT-PCR data were analyzed by the relative quantification ($\Delta\Delta C_T$) method (Livak and Schmittgen, 2001). All mRNA expression levels were expressed as fold-change compared to that of 1 day old HWS chicks.

Table 2. 1. Nucleotide sequences of specific primers.

Target gene	GenBank accession	PCR products (bp)	Primer sequences
[1]AMPK α1	NM_001039603	125	F: 5'-ATCTGTCTCGCCCTCATCCT-3'
			R: 5'-CCACTTCGCTCTTCTTACACCTT-3'
[2]AMPK α2	NM_001039605	128	F: 5'-CCAGCGAGTTCTACCTAGCCT-3'
			R: 5'-TGCCTTGGGACTGTCTGCTA-3'
[3]AMPK β1	NM_001039912	113	F: 5'-CCAGGAGCCCTATGTCTGTAAG-3'
			R: 5'-CAGGATCGCAAGAAATGCC-3'
[4]AMPK β2	NM_001044662	125	F: 5'-GCACTGCCCATCCCTCTAA-3'
			R: 5'-CGGCTGCCACGAAACAA-3'
[5]AMPK γ1	NM_001034827	118	F: 5'-AGAGGTCCCAAAGCCTGAGTT-3'
			R: 5'-GAAGATGCCCAGAGCCACA-3'
[6]AMPK γ2	NM_001030965	106	F: 5'-CACAAGCCCTACAGCACCG-3' R: 5'-TGAACCTCAGCCTTCACTATCCTA-3'
[7]AMPK γ3	NM_001031258	91	F: 5'-CCACGCTTCCTAAAGAAAACAG-3'
			R: 5'-CCGGCAGCATTAACAACG-3'
[8]ACC α	NM_205505	125	F: 5'-CCGAGAACCCAAAACTACCAG-3'
			R: 5'-GCCAGCAGTCTGAGCCACTA-3'
[9]CAMKK α	XM_001234324	97	F: 5'-CAGTTTGAAGGGAACGATGCC-3'

			R: 5'-CACTGAAACTTTTGCCCGTGT-3'
[10]CAMKK β	XM_415134	144	F: 5'-AGGACCAGGCTCGGTTCTAC-3'
			R: 5'-TGACACCAAAGTCGGCAATT-3'
[11]FAS	NM_205155	117	F: 5'-AAAGCAATTCGTCACGGACA-3'
			R: 5'-GGCACCATCAGGACTAAGCA-3'
[12]LKB 1	XM_418227	114	F: 5'-GCTGACCACCAATGGGACG-3'
			R: 5'-GGCTGGAATGCTGGCGAC-3'
[13]MO25 α	CR391562	93	F: 5'-CCCTAACAAGACGCAGCCTA-3'
			R: 5'-TCGGTCCTGTCATTCTGAAACT-3'
[14]MO25 β	NM_001006272	117	F: 5'-AAGAGGGTAACAGTTGCTCCG-3'
			R: 5'-CTTTCACTTATCTCCCACGTAATG-3'
[15]STRAD α	NM_001012844	149	F: 5'-TTTCCAAGCTCTTCAACCACC-3'
			R: 5'-CAATTCACTCATCCCGTCCATA-3'
[16]STRAD β	XM_421938	93	F: 5'-ACCCTGCCACGGACTTCTT-3'
			R: 5'-TTGCTTTTCTGGTTGGATTGAC-3'
[17]GAPDH	NM_204305	141	F: 5'-TGGCATCCAAGGAGTGAGC-3'
			R: 5'-GGGGAGACAGAAGGGAACAG-3'
[18]CPT1	AY675193	110	F: 5'-ACTATCAACGAGTCAGACACCACA-3'
			R: 5'-AAACACCGTAACCATCATCAGC-3'
[19]LEPR	NM_204323	87	F: 5'-GCATCTCTGCATCTCAGGAAAGA-3'
			R: 5'-GCAGGCTACAAACTAACAGATCCA-3'
[20]NPY	M87294	83	F: 5'-ATGAGGCTGTGGGTGTCGGT-3'
			R: 5'-TTGGAGGGGTACGCTTCTGC-3'
[21]POMC	NM_001031098	123	F: 5'-GGAGGAAAAGAAGGATGGAGG-3'
			R: 5'-AGAGTCATCAGCGGGGTCTG-3'
Orexin	NM_204185	86	F: 5'-GCTCCTGCCGTATCTACGACCT-3'
			R: 5'-CGGGATGCTCTTCCTCTTGC-3'
[22]FTO	XM_414084	124	F: 5'-TGAAGGTAGCGTGGGACATAGA-3'
			R: 5'-GGTGAAAAGCCAGCCAGAAC-3'

[1]AMPK α1 (AMP -activate kinase alpha 1 subunit), [2]AMPK α2 (AMP -activate kinase alpha 2 subunit), [3]AMPK β1 (AMP -activate kinase beta 1 subunit), [4]AMPK β2 (AMP -activate kinase beta 2 subunit), [5]AMPK γ1 (AMP -activate kinase gamma 1 subunit), [6]AMPK γ2 (AMP -activate kinase gamma 2 subunit), [7]AMPK γ3 (AMP -activate kinase gamma 3 subunit), [8]ACC α (acetyl-Coenzyme A carboxylase alpha), [9]CAMKK α (calcium/calmodulin-dependent protein kinase kinase 1), [10]CAMKK β (calcium/calmodulin-dependent protein kinase kinase 2), [11]FAS (fatty acid synthase), [12]LKB 1 (Serine/threonine-protein kinase 11), [13]MO25 α (Calcium-binding protein 39), [14]MO25 β (calcium binding protein 39-like), [15]STRAD α (STE20-related kinase adaptor alpha), [16]STRAD β (STE20-related kinase adaptor beta), [17]GAPDH (glyceraldehyde-3-phosphate dehydrogenase), [18]CPT1 (Carnitine palmitoyltransferase I), [19]LEPR (Leptin receptor), [20]NPY(*Neuropeptide Y*), [21]POMC (Pro-opiomelanocortin), [22]FTO (Fat mass and obesity associated gene).

2.2.4. Western Blot Analysis

Proteins were extracted with SDS lysis buffer from the preoptic-hypothalamic area (25 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol and 5% β-mercaptoethanol). Protein

extracts were separated on an 8.5% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Blots were blocked in 5% bovine serum albumin (BSA) dissolved in Tris-buffered saline with Tween-20 (TBST, 20 mmole Tris, pH 7.4, 150 mmole NaCl, and 0.1% Tween-20) for 180 min at 4 °C and then briefly washed two times for 5 min each in TBST. The blots were incubated overnight at 4 °C with primary antibodies against phosphor-AMPK α (α1 and α2, Thr^{172}) (1:500), phosphor-ACC (Ser^{79}) (1:250), ACC (1:250) and AMPK α (1:1000) in TBST with 1% BSA. All antibodies were from Cell Signaling Technology (Danvers, MA, USA). Each blot was washed twice for 5 min, and three times for 10 min, and then incubated at room temperature for 60 min with anti-rabbit IgG horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After the incubation, the blots were washed again and a chemiluminescent substrate, SuperSignal West Pico (Pierce, Rockford, IL, USA), was applied according to instructions of the manufacturer and the signal was detected using a Gel Doc XR System (Bio-Rad, Hercules, CA, USA). Densitometry analysis was performed using Quantity One (Bio-Rad, Hercules, CA, USA) image analysis software. The protein expression levels were expressed as a density ratio (pAMPK/AMPK, pACC/ACC).

2.2.5. Statistical Analysis

Data were analyzed using SAS 9.1.3 (SAS Institute Inc, Cary, NC, USA). The Shapiro-Wilk *W*-test was first used to examine the normal distribution. All data were analyzed by analysis of variance (ANOVA) using the general linear modeling procedure (GLM, SAS Institute Inc, Cary, NC, USA). The model included line, age and the line by age

interaction. Comparison between lines was also performed at each age using t-test for independent samples. All data were expressed as mean ± S.E.M. and the level of significance was set a priori at $P<0.05$.

2.3. RESULTS

2.3.1. Changes in Body Weight of HWS and LWS Lines of Chickens at Different Ages

Body weights of HWS and LWS chickens at different ages are presented in Fig. 2.1. There were significant age, line and age×line interaction effects for body weight. HWS chickens were significantly heavier than LWS chickens at all observed ages. The body weight difference between the two lines of chickens increased with ages. At 28 days of age, HWS chickens were 9-fold heavier than weight of the LWS chickens. By 56 days, the difference between lines was 11 fold.

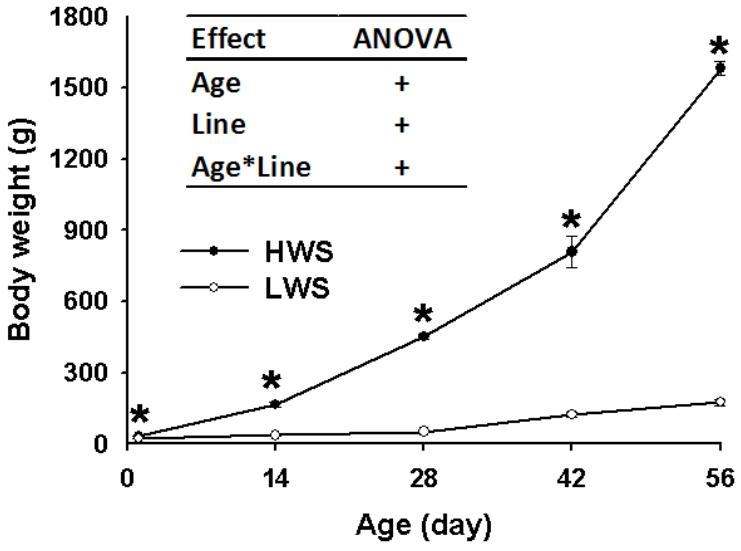

Fig. 2. 1. Body weight of HWS and LWS lines of chickens at 1, 14, 28, 42 and 56 days of age. Each bar represents mean ± S.E.M. (n=6). Asterisk sign (*) denotes significance

(P<0.05) in t-test for independent samples and plus sign (+) denotes significance (P<0.05) in ANOVA test.

2.3.2. mRNA Expression of Genes Encoding AMPK Subunits and Associated Proteins in Preoptic-hypothalamus

The mRNA expressions of AMPK subunits α, β and γ in the preoptic-hypothalamus of HWS and LWS chickens are illustrated in Fig. 2.2. There were significant age, line and age×line interaction effects for α1 mRNA expression, while age and age×line interaction were significant for α2. The interaction was a result of different line effects at different ages. The α1 mRNA level was significantly less at 28 days of age, whereas α2 was significantly less at the first day after hatch but greater at 28 days of age in HWS than in LWS chickens. The effects of age and line were significant for regulatory β1. HWS chickens had significantly lower β1 at 56 days of age. In contrast, β2 only had a significant age effect. Moreover, γ1 had significant age, line and age×line interaction effects. At 28 days of age, γ1 was significantly lower in HWS than in LWS chickens. There was only a significant age effect for γ2 and γ3 and line t-test was not significant at all ages.

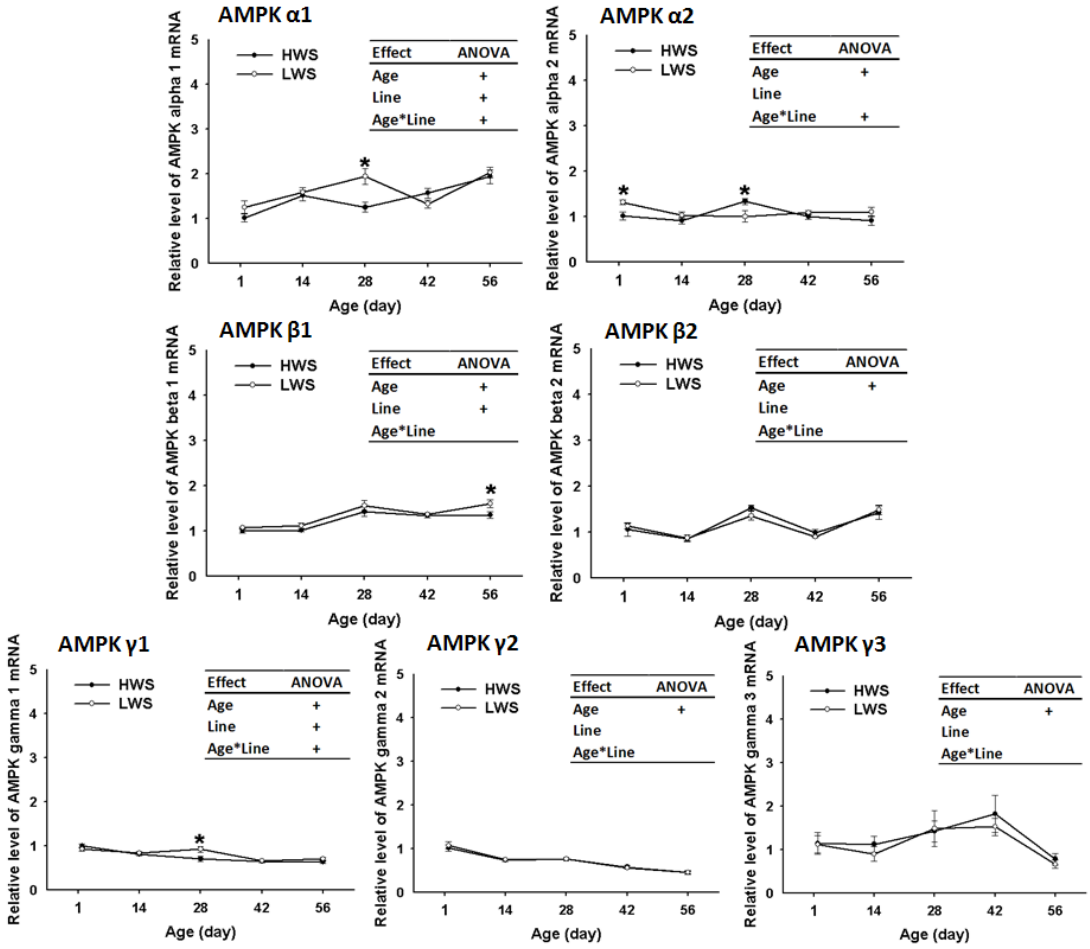

Fig. 2. 2. Gene expression of hypothalamic AMPK subunits in HWS and LWS lines of chickens at 1, 14, 28, 42, and 56 days of age. The data, which were expressed as fold-change compared to the 1-day-old HWS group, represent mean ± S.E.M. (n=6). Asterisk sign (*) denotes significance (P<0.05) in t-test for independent samples and plus sign (+) denotes significance (P<0.05) in ANOVA test.

mRNA expression of AMPK upstream factors Serine/threonine-protein kinase 11/STE20-related kinase adaptor/Calcium-binding protein 39 (LKB 1/STRAD/MO25) complex, calcium/calmodulin-dependent protein kinase kinase (CAMKK) and downstream factor ACC α in the preoptic-hypothalamus are presented in Fig. 2.3. Among LKB

1/STRAD/MO25 complex, no differences were found in LKB 1 mRNA expression. For regulatory MO25 α, there were significant age, line and age×line interaction effects, while for MO25 β, line and age×line interaction but not age effects were significant. MO25 α was significantly higher at 14, 28, and 42 days of age, whereas MO25 β was significantly lower at 28 and 56 days of age in HWS than LWS chickens. By contrast, there was no significant difference in mRNA expression of STRAD subunits except a significant age effect for STRAD α. The α and β subunits of another AMPK upstream factor CAMKK had significant age, line, and age×line effects. HWS chickens had significantly higher CAMKK α at 28 days of age, and significantly lower CAMKK β at 28, 42, and 56 days of age. Additionally, there were significant age and age×line interaction effects for the AMPK downstream target ACC α. Its expression was significantly higher at 28 days of age in HWS than LWS chickens.

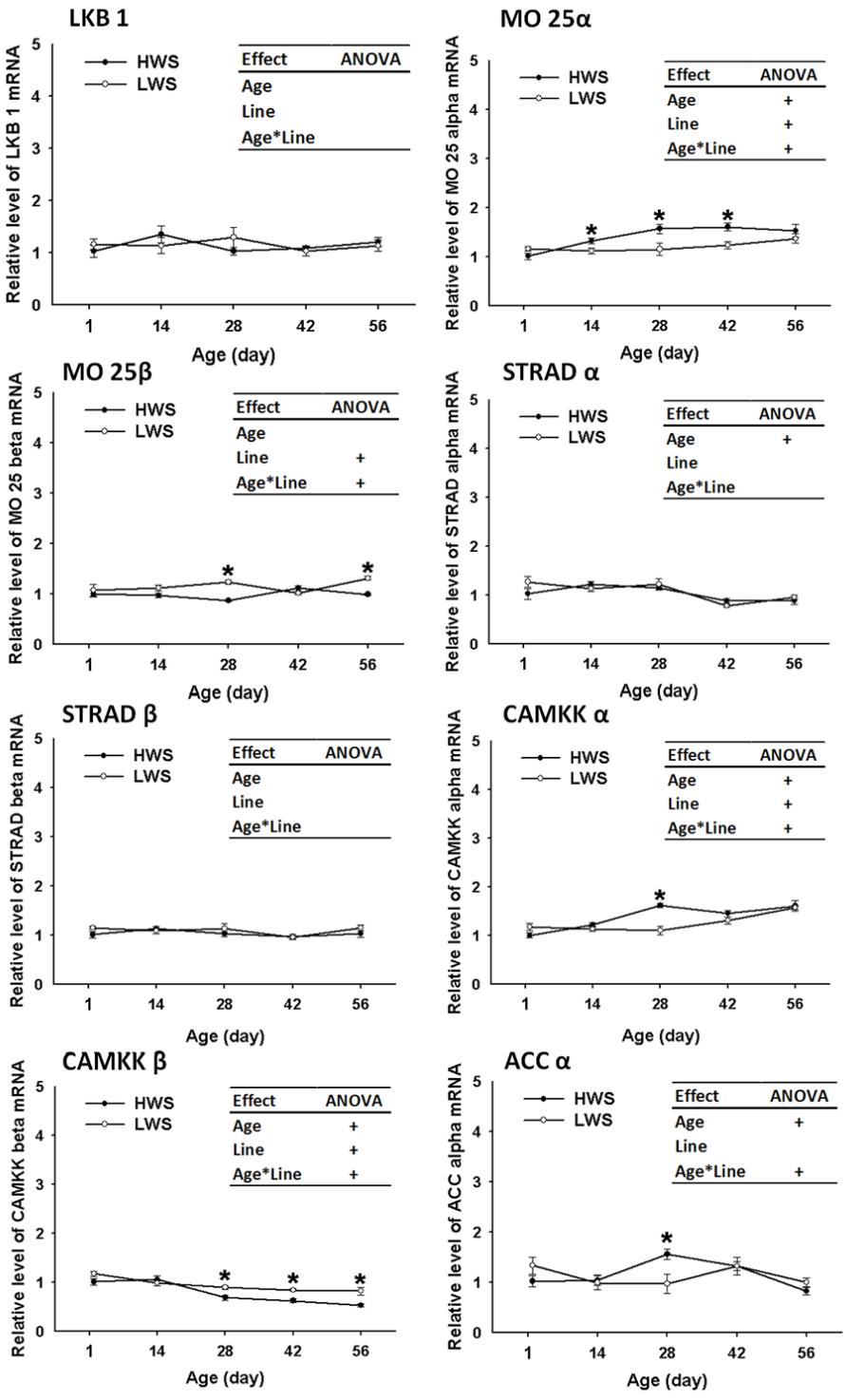

Fig. 2. 3. Gene expression of hypothalamic LKB1/STRAD/MO25 subunits, CAMKK subunits and ACC α in HWS and LWS lines of chickens at 1, 14, 28, 42, and 56 days of

age. The data, which were expressed as fold-change compared to the 1-day-old HWS group, represent mean ± S.E.M. (n=6). Asterisk sign (*) denotes significance (P<0.05) in t-test for independent samples and plus sign (+) denotes significance (P<0.05) in ANOVA test.

2.3.3. Phosphorylation of AMPK and ACC in Preoptic-hypothalamus

By Western blot, the phosphorylation of hypothalamic AMPK (Fig. 2.4A) and ACC (Fig. 2.4B) was determined in HWS and LWS chickens at different ages. The phosphorylation levels of the α1 and α2 catalytic subunits of AMPK in preoptic-hypothalamus were tested to compare with AMPK activity. Phosphorylation of ACC, a well characterized substrate of AMPK, was also used as an indicator of AMPK activity. There were significant age and line effects for phosphorylated AMPK and ACC in hypothalamus. HWS chickens had significantly lower phosphorylated AMPK at 28 days of age than LWS chickens. The phosphorylation of ACC was significantly lower in HWS than LWS chickens at 28 and 56 days of age.

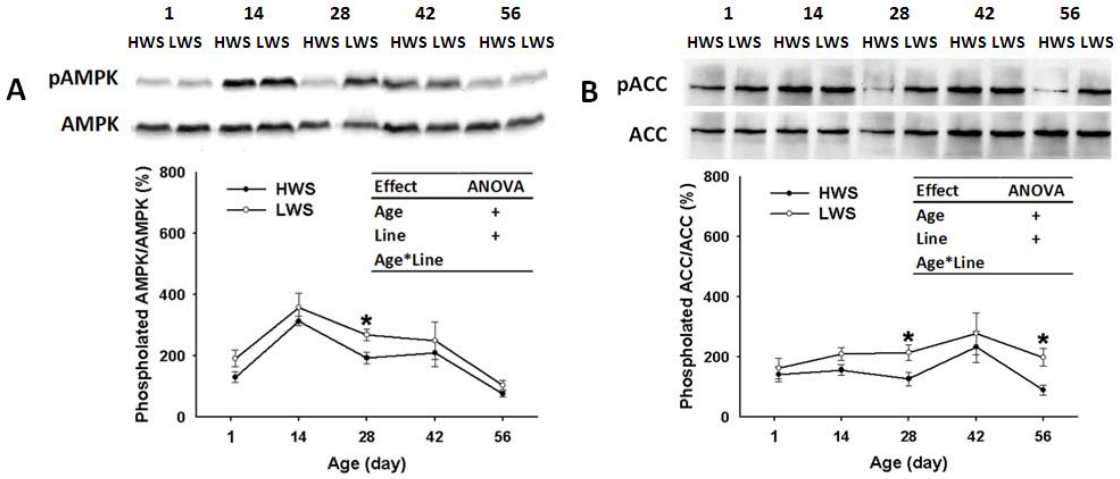

Fig. 2. 4. Phosphorylation of hypothalamic AMPK α and ACC in HWS and LWS lines of chickens at 1, 14, 28, 42, and 56 days of age. Representative Western Blot of phosphorylated AMPK α (pAMPK) and total AMPK α (AMPK) or phosphorylated ACC (pACC) and total ACC (ACC) in both lines of chickens are shown. The average levels of pAMPK or pACC in the preoptic-hypothalamus were expressed as pAMPK/AMPK or pACC/ACC (n=6). Asterisk sign (*) denotes significance (P<0.05) in t-test for independent samples and plus sign (+) denotes significance (P<0.05) in ANOVA test.

2.3.4. mRNA Expression of Genes Related to Fatty Acid Metabolism and Obesity in Preoptic-hypothalamus

The mRNA expressions of fatty acid metabolism related factors CPT1, FAS and obesity related factors leptin receptor (LEPR), and fat mass and obesity associated gene (FTO) in HWS and LWS chickens are illustrated in Fig. 2.5. There were significant age and age×line interaction effects for CPT1. There were significantly lower levels of CPT1 at 1 and 14 days of age in HWS than LWS chickens. In contrast, for FAS, age, line, and

age×line interaction effects were significant. FAS expression was significantly higher in HWS than LWS chickens at 14, 28, and 42 days of age. Moreover, age and line effects were significant for LEPR, while only an age effect was significant for FTO. HWS chickens had significantly less LEPR at 56 days of age than LWS chickens. The expression levels of FTO were significantly less the first day after hatch and greater at 42 days of age in HWS than LWS chickens.

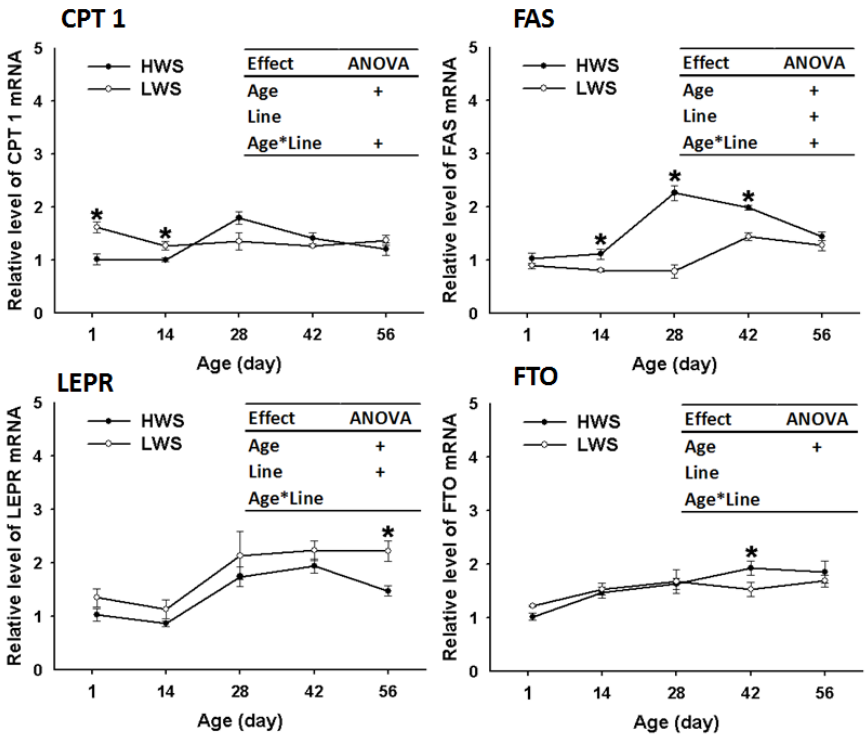

Fig. 2. 5. Gene expression of hypothalamic CPT1, FAS, LEPR and FTO in HWS and LWS lines of chickens at 1, 14, 28, 42, and 56 days of age. The data, which were expressed as fold-change compared to the 1-day-old HWS group, represent mean ± S.E.M. (n=6). Asterisk sign (*) denotes significance (P<0.05) in t-test for independent samples and plus sign (+) denotes significance (P<0.05) in ANOVA test.

2.3.5. mRNA Expression of Appetite Regulatory Neuropeptides in Preoptic-hypothalamus

The mRNA expression patterns of NPY, POMC, and orexin in the preoptic-hypothalamus of HWS and LWS chickens are shown in Fig. 2.6. There was a significant line effect for orexigenic neuropeptide NPY and its expression was significantly lower at 14, 28, and 42 days of age in HWS than LWS chickens. In contrast, the age, line, and age×line interaction effects were significant for anorexigenic neuropeptide POMC. HWS chickens had significantly greater POMC expression at 14, 28, 42, and 56 days of age. The orexigenic neuropeptide orexin also had significant age, line, and age×line effects. Its expression was significantly greater at 1, 14, and 28 days of age in HWS than LWS chickens.

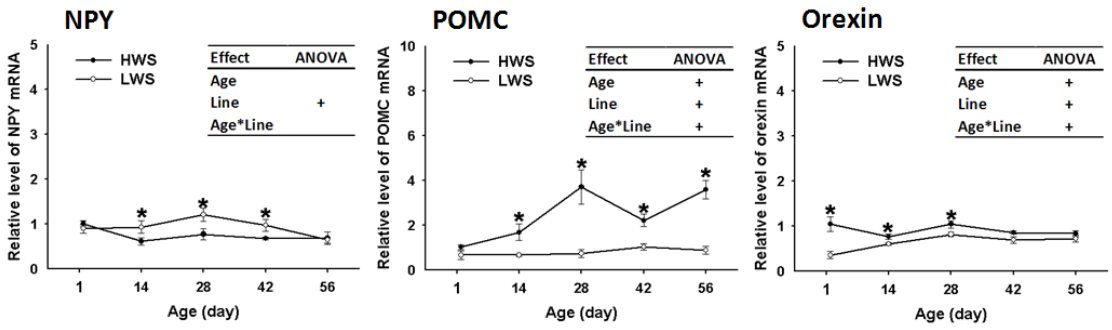

Fig. 2. 6. Gene expression of hypothalamic NPY, POMC and orexin in HWS and LWS lines of chickens at 1, 14, 28, 42, and 56 days of age. The data, which were expressed as fold-change compared to the 1-day-old HWS group, represent mean ± S.E.M. (n=6). Asterisk sign (*) denotes significance (P<0.05) in t-test for independent samples and plus sign (+) denotes significance (P<0.05) in ANOVA test.

2.4. DISCUSSION

The HWS and LWS lines were developed by a long-term divergent selection for high and low 56-day body weight from a common foundation population (Marquez et al., 2010). In our experiment, the difference in body weight of the line at 56 days of age was 11 fold. Thus the striking phenotypic differences between these lines provide excellent models for studies on the mechanisms regulating food intake, energy balance and body composition.

In the present study, the AMPK at both the gene and protein levels was determined to identify the molecular mechanisms underlying the variations in food intake and energy balance in HWS and LWS chickens. The gene expression of all seven isoforms of the three subunits of AMPK, previously reported in chicken embryonic brain (Proszkowiec-Weglarz and Richards, 2009), was measured in the preoptic-hypothalamus of HWS and LWS chickens. Selection for body weight affected the mRNA expression of the catalytic α1 and α2 and the regulatory β1 and γ1 AMPK subunits. Both α1 and α2 have catalytic domains, where AMPK becomes activated when phosphorylation takes place at threonine-172 (Ojuka et al., 2002). In the chicken hypothalamus, α1 is the main expressed isoform which may have a greater AMP-related response than α2 (Proszkowiec-Weglarz et al., 2006). The β subunits, which serve a scaffolding function, are necessary to form a functional αβγ complex (Hudson et al., 2003). The γ subunits serve an AMP-binding function and give AMPK the ability to sensitively detect shifts in the AMP/ATP ratio (Adams et al., 2004). The cooperation of these different subunits can allosterically activate the enzyme by up to 5-fold (Carling et al., 1987). Thus, the upregulation of γ1 mRNA level in the LWS chickens at 28 days of age would improve

AMPK's ability to detect AMP/ATP shift, likely implying a greater response of the kinase complex to AMP, which is consistent with the increase of catalytic α1 mRNA levels observed at this age's LWS chickens. This significant upregulation of α1 would lead to a greater proportion of α1 in total α subunits, which may account for the downregulation of α2 at this age.

A majority of AMPK activity is dependent on the phosphorylation of a threonine residue of the α subunits by an AMPK kinase (AMPKK), which is a complex with three proteins, the key catalytic unit LKB1 and two accessory units, termed STRAD and MO25 (Hawley et al., 2003; Woods et al., 2003). LKB1 activity is regulated by the pseudokinase STRAD and the adaptor protein MO25. The binding of LKB1 to STRAD and MO25 vastly enhances LKB1's activity and the LKB1/STRAD/MO25 complex represents the biologically active unit to phosphorylate and activate AMPK subfamily members (Boudeau et al., 2004). In our study, although MO25 α was significantly inhibited from 14 to 42 days of age, MO25 β was significantly stimulated at 28 and 56 days of age in LWS chickens. The key catalytic unit LKB1 and pseudokinase STRAD mRNA expressions were not altered by the selection for body weight. Adaptor protein MO25 modifies AMPK activity by stimulating the kinase activity of LKB1, which is the key unit in the LKB1/STRAD/MO25 complex. Thus, the changes in MO25 mRNA expression may not contribute to AMPK alteration without association of LKB1 and STRAD.

In addition to the LKB1 complex, another physiological activator of AMPK is CAMKK. Between the two isoforms, CAMKK β, rather than CAMKK α, is the predominant kinase

activating AMPK, although we cannot exclude a role for CAMKK α at this time (Hawley et al., 2005; Carling et al., 2008). Unlike the LKB1 complex, AMPK is phosphorylated and activated by CAMKK β in response to increased intracellular Ca^{2+} rather than increased AMP (Woods et al., 2005). Our results demonstrated that CAMKK β was significantly greater from 28 to 56 days of age, while CAMKK α was significantly lower at 28 days of age in LWS than HWS chickens. Considering the predominant role of CAMKK β in AMPK activation, the upregulation of CAMKK β in LWS chickens may contribute to the phosphorylation of AMPK, which is consistent with the greater AMPK activation observed in this line.

We assessed AMPK activation through phosphorylation levels of AMPK α and downstream factor ACC. ACC, a key regulatory enzyme of fatty acid oxidation, has been identified as a direct downstream factor of the AMPK pathway involved in fatty acid oxidation. AMPK activation can cause inhibition of ACC by phosphorylation, resulting in increased fatty acid transport and subsequent oxidation (Merrill et al., 1997). That LWS chickens had lower ACC α mRNA expression at 28 days of age and higher ACC phosphorylation level at 28 and 56 days of age, likely implies increased fatty acid oxidation. This is consistent with the greater AMPK phosphorylation at 28 days of age in LWS chickens. Thus, results on ACC α mRNA expression and phosphorylation of AMPK and ACC provide further evidence for the alteration of AMPK pathway by selection for body weight.

To further clarify the difference between HWS and LWS chickens in AMPK affected energy expenditure, the mRNA expression of fatty acid metabolism related factors CPT1,

FAS, and obesity related factors LEPR and FTO was measured in the preoptic-hypothalamus. CPT1 is a key enzyme for mitochondria fatty acid transport and its activation is inhibited by malonyl-CoA, which is converted from acetyl-CoA by ACC (Bonnefont et al., 2004), while FAS is a key lipogenic enzyme and its activation increases fatty acid synthesis (Smith et al., 2003). The mRNA expression of lipolytic enzyme CPT1 was markedly greater at 1 and 14 days of age, whereas the mRNA expression of lipogenic enzyme FAS was markedly less from 14 to 42 days of age in the hypothalamus of LWS than HWS chickens. The activation of AMPK can stimulate fatty acid oxidation and inhibit fatty acid synthesis in mammals (Gaidhu et al., 2009). Therefore, the greater AMPK activation observed in LWS chickens was consistent with their increased CPT1 and decreased FAS expression and suggests a similar role for AMPK in modulating fatty acid metabolism as in mammals. Moreover, higher LEPR mRNA expression at 56 days of age and lower FTO mRNA expression at 42 days of age in the hypothalamus were found in LWS chickens which exhibited a greater AMPK activation. Leptin, which acts as an adiposity signal, increases energy expenditure and reduces food intake by acting on LEPR in the hypothalamus (Williams et al., 2009). The upregulation of LEPR in LWS chickens suggests great activity of leptin in the hypothalamus, which is consistent with the hypophagia and low lipogenic characteristics. FTO is an obesity correlated gene and there is evidence that inactivation of FTO gene in mice increases energy expenditures (Fischer et al., 2009), whereas overexpression of FTO in mice leads to increased food intake and results in obesity (Church et al., 2010). Our findings agree with those from FTO deficient mice, as LWS chickens show decreased FTO expression in the hypothalamus, which is associated with slower growth

rate. Therefore, long-term divergent selection for body weight clearly altered fatty acid metabolism and obesity related factor expression in chickens.

To determine the differences between HWS and LWS chickens in appetite regulation, we measured the mRNA expression of several hypothalamic feeding-related neuropeptides, including orexigenic neuropeptides NPY and orexin, and anorexigenic neuropeptides POMC and CRH. The expression of NPY, the best-described orexigenic and anabolic effector, was significantly higher from 14 to 42 days of age in LWS than HWS chickens, whereas the expression of POMC, a key anorexigenic and catabolic effector, was significantly lower from 14 to 56 days of age in LWS than HWS chickens. These results are in good agreement with previous findings in dietary-induced obese mice (Bergen et al., 1999; Beck, 2006), which are associated with decreased NPY and increased POMC in the hypothalamus. This is consistent with the existence of a counter-regulatory mechanism, which involves the important "energy sensor" AMPK, to keep energy balance and limit obesity. By contrast, the expression of orexin, another orexigenic neuropeptide, was significantly lower from 1 to 28 days of age in LWS than HWS chickens. Although central administration of orexin to rodents acutely promotes appetite (Sakurai et al., 1998), long-term orexin overexpression promotes a negative energy balance and confers resistance to a high fat diet-induced obesity through improved leptin sensitivity (Funato et al., 2009). Thus, the long-term upregulation of orexin in HWS chickens may promote energy expenditure and contributes to negative feedback mechanisms involved in energy homeostasis.

In conclusion, the present study provided evidence that the mRNA expression of genes related to energy homeostasis and obesity, including ACC, CPT1, FAS, LEPR and FTO, and feeding-related neuropeptides, including NPY, POMC and orexin, have been altered by the long-term divergent selection for body weight. Compared to HWS chickens, LWS chickens had a higher level of AMPK phosphorylation, which may be caused by upregulated upstream factor CAMKK β. The greater activity of the AMPK complex in LWS chickens could trigger a greater rate of fatty acid oxidation by inhibiting ACC to activate CPT1 and a decreased rate of fatty acid synthesis by inactivating FAS. For obesity related factors, LEPR was upregulated while FTO was downregulated in LWS chickens, which is consistent with the hypophagia and low lipogenic characteristic of this genotype. HWS chickens had decreased NPY and increased POMC and orexin expressions, suggesting the existence of a counter-regulatory mechanism, triggered by downregulated AMPK activity, to maintain energy balance and limit obesity development. Taken together, our data suggest long-term selection for body weight affects hypothalamic regulation of energy balance and food intake. Alterations of AMPK activity could contribute to these changes.

2.5. REFERENCES

Adams, J., Z. P. Chen, B. J. Van Denderen, C. J. Morton, M. W. Parker, L. A. Witters, D. Stapleton, and B. E. Kemp. 2004. Intrasteric control of AMPK via the gamma1 subunit AMP allosteric regulatory site. Protein Sci. 13:155-165.

Beck, B. 2006. Neuropeptide Y in normal eating and in genetic and dietary-induced obesity. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 361:1159-1185.

Bergen, H. T., T. Mizuno, J. Taylor, and C. V. Mobbs. 1999. Resistance to diet-induced obesity is associated with increased proopiomelanocortin mRNA and decreased neuropeptide Y mRNA in the hypothalamus. Brain Res. 851:198-203.

Bonnefont, J. P., F. Djouadi, C. Prip-Buus, S. Gobin, A. Munnich, and J. Bastin. 2004. Carnitine palmitoyltransferases 1 and 2: biochemical, molecular and medical aspects. Mol. Aspects Med. 25:495-520.

Boudeau, J., J. W. Scott, N. Resta, M. Deak, A. Kieloch, D. Komander, D. G. Hardie, A. R. Prescott, D. M. van Aalten, and D. R. Alessi. 2004. Analysis of the LKB1-STRAD-MO25 complex. J. Cell Sci. 117:6365-6375.

Carling, D., M. J. Sanders, and A. Woods. 2008. The regulation of AMP-activated protein kinase by upstream kinases. Int. J. Obes. (Lond) 32 Suppl 4:S55-59.

Carling, D., V. A. Zammit, and D. G. Hardie. 1987. A common bicyclic protein kinase cascade inactivates the regulatory enzymes of fatty acid and cholesterol biosynthesis. FEBS Lett. 223:217-222.

Church, C., L. Moir, F. McMurray, C. Girard, G. T. Banks, L. Teboul, S. Wells, J. C. Bruning, P. M. Nolan, F. M. Ashcroft, and R. D. Cox. 2010. Overexpression of Fto leads to increased food intake and results in obesity. Nat. Genet. 42:1086-1092.

Dunnington, E. A., and P. B. Siegel. 1996. Long-term divergent selection for eight-week body weight in White Plymouth Rock chickens. Poult. Sci. 75:1168-1179.

Fischer, J., L. Koch, C. Emmerling, J. Vierkotten, T. Peters, J. C. Bruning, and U. Ruther. 2009. Inactivation of the Fto gene protects from obesity. Nature 458:894-898.

Florant, G. L., A. M. Fenn, J. E. Healy, G. K. Wilkerson, and R. J. Handa. 2010. To eat or not to eat: the effect of AICAR on food intake regulation in yellow-bellied marmots (Marmota flaviventris). J. Exp. Biol. 213:2031-2037.

Funato, H., A. L. Tsai, J. T. Willie, Y. Kisanuki, S. C. Williams, T. Sakurai, and M. Yanagisawa. 2009. Enhanced orexin receptor-2 signaling prevents diet-induced obesity and improves leptin sensitivity. Cell Metab. 9:64-76.

Gaidhu, M. P., S. Fediuc, N. M. Anthony, M. So, M. Mirpourian, R. L. Perry, and R. B. Ceddia. 2009. Prolonged AICAR-induced AMP-kinase activation promotes energy dissipation in white adipocytes: novel mechanisms integrating HSL and ATGL. J. Lipid Res. 50:704-715.

Hawley, S. A., J. Boudeau, J. L. Reid, K. J. Mustard, L. Udd, T. P. Makela, D. R. Alessi, and D. G. Hardie. 2003. Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. J. Biol. 2:28.

Hawley, S. A., D. A. Pan, K. J. Mustard, L. Ross, J. Bain, A. M. Edelman, B. G. Frenguelli, and D. G. Hardie. 2005. Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. Cell Metab. 2:9-19.

Hayes, M. R., K. P. Skibicka, K. K. Bence, and H. J. Grill. 2009. Dorsal hindbrain 5'-adenosine monophosphate-activated protein kinase as an intracellular mediator of energy balance. Endocrinology 150:2175-2182.

Hudson, E. R., D. A. Pan, J. James, J. M. Lucocq, S. A. Hawley, K. A. Green, O. Baba, T. Terashima, and D. G. Hardie. 2003. A novel domain in AMP-activated protein

kinase causes glycogen storage bodies similar to those seen in hereditary cardiac arrhythmias. Curr. Biol. 13:861-866.

Johansson, A. M., M. E. Pettersson, P. B. Siegel, and O. Carlborg. 2010. Genome-wide effects of long-term divergent selection. PLoS. Genet. 6:e1001188.

Kubota, N., W. Yano, T. Kubota, T. Yamauchi, S. Itoh, H. Kumagai, H. Kozono, I. Takamoto, S. Okamoto, T. Shiuchi, R. Suzuki, H. Satoh, A. Tsuchida, M. Moroi, K. Sugi, T. Noda, H. Ebinuma, Y. Ueta, T. Kondo, E. Araki, O. Ezaki, R. Nagai, K. Tobe, Y. Terauchi, K. Ueki, Y. Minokoshi, and T. Kadowaki. 2007. Adiponectin stimulates AMP-activated protein kinase in the hypothalamus and increases food intake. Cell Metab. 6:55-68.

Lane, M. D., and S. H. Cha. 2009. Effect of glucose and fructose on food intake via malonyl-CoA signaling in the brain. Biochem. Biophys. Res. Commun. 382:1-5.

Lee, K., B. Li, X. Xi, Y. Suh, and R. J. Martin. 2005. Role of neuronal energy status in the regulation of adenosine 5'-monophosphate-activated protein kinase, orexigenic neuropeptides expression, and feeding behavior. Endocrinology 146:3-10.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Lopez, M., L. Varela, M. J. Vazquez, S. Rodriguez-Cuenca, C. R. Gonzalez, V. R. Velagapudi, D. A. Morgan, E. Schoenmakers, K. Agassandian, R. Lage, P. B. Martinez de Morentin, S. Tovar, R. Nogueiras, D. Carling, C. Lelliott, R. Gallego, M. Oresic, K. Chatterjee, A. K. Saha, K. Rahmouni, C. Dieguez, and A. Vidal-

Puig. 2010. Hypothalamic AMPK and fatty acid metabolism mediate thyroid regulation of energy balance. Nat. Med. 16:1001-1008.

Marquez, G. C., P. B. Siegel, and R. M. Lewis. 2010. Genetic diversity and population structure in lines of chickens divergently selected for high and low 8-week body weight. Poult. Sci. 89:2580-2588.

Merrill, G. F., E. J. Kurth, D. G. Hardie, and W. W. Winder. 1997. AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. Am. J. Physiol. 273:E1107-1112.

Minokoshi, Y., T. Alquier, N. Furukawa, Y. B. Kim, A. Lee, B. Xue, J. Mu, F. Foufelle, P. Ferre, M. J. Birnbaum, B. J. Stuck, and B. B. Kahn. 2004. AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. Nature 428:569-574.

Morton, G. J., D. E. Cummings, D. G. Baskin, G. S. Barsh, and M. W. Schwartz. 2006. Central nervous system control of food intake and body weight. Nature 443:289-295.

Namkoong, C., M. S. Kim, P. G. Jang, S. M. Han, H. S. Park, E. H. Koh, W. J. Lee, J. Y. Kim, I. S. Park, J. Y. Park, and K. U. Lee. 2005. Enhanced hypothalamic AMP-activated protein kinase activity contributes to hyperphagia in diabetic rats. Diabetes 54:63-68.

Ojuka, E. O., T. E. Jones, L. A. Nolte, M. Chen, B. R. Wamhoff, M. Sturek, and J. O. Holloszy. 2002. Regulation of GLUT4 biogenesis in muscle: evidence for involvement of AMPK and Ca(2+). Am. J. Physiol. Endocrinol. Metab. 282:E1008-1013.

Proszkowiec-Weglarz, M., and M. P. Richards. 2009. Expression and activity of the 5'-adenosine monophosphate-activated protein kinase pathway in selected tissues during chicken embryonic development. Poult. Sci. 88:159-178.

Proszkowiec-Weglarz, M., M. P. Richards, R. Ramachandran, and J. P. McMurtry. 2006. Characterization of the AMP-activated protein kinase pathway in chickens. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 143:92-106.

Sakurai, T., A. Amemiya, M. Ishii, I. Matsuzaki, R. M. Chemelli, H. Tanaka, S. C. Williams, J. A. Richarson, G. P. Kozlowski, S. Wilson, J. R. Arch, R. E. Buckingham, A. C. Haynes, S. A. Carr, R. S. Annan, D. E. McNulty, W. S. Liu, J. A. Terrett, N. A. Elshourbagy, D. J. Bergsma, and M. Yanagisawa. 1998. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 92:1 page following 696.

Siegel, P. B., and J. H. Wolford. 2003. A review of some results of selection for juvenile body weight in chickens. Poult. Sci. 40:81-91.

Smith, S., A. Witkowski, and A. K. Joshi. 2003. Structural and functional organization of the animal fatty acid synthase. Prog. Lipid Res. 42:289-317.

Williams, K. W., M. M. Scott, and J. K. Elmquist. 2009. From observation to experimentation: leptin action in the mediobasal hypothalamus. Am. J. Clin. Nutr. 89:985S-990S.

Winder, W. W. 2001. Energy-sensing and signaling by AMP-activated protein kinase in skeletal muscle. J. Appl. Physiol. 91:1017-1028.

Woods, A., D. Azzout-Marniche, M. Foretz, S. C. Stein, P. Lemarchand, P. Ferre, F. Foufelle, and D. Carling. 2000. Characterization of the role of AMP-activated

protein kinase in the regulation of glucose-activated gene expression using constitutively active and dominant negative forms of the kinase. Mol. Cell. Biol. 20:6704-6711.

Woods, A., K. Dickerson, R. Heath, S. P. Hong, M. Momcilovic, S. R. Johnstone, M. Carlson, and D. Carling. 2005. Ca2+/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. Cell Metab. 2:21-33.

Woods, A., S. R. Johnstone, K. Dickerson, F. C. Leiper, L. G. Fryer, D. Neumann, U. Schlattner, T. Wallimann, M. Carlson, and D. Carling. 2003. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. Curr. Biol. 13:2004-2008.

CHAPTER 3

Different Feeding Responses to Food Intake Modulators in Lines of Chickens Selected for High or Low Body Weight

(Submitted)

ABSTRACT Central feeding responses to a range of food intake modulators were measured in two lines of chickens that had undergone 51-generations of selection for high (HWS) or low (LWS) 56-day body weight from a common founder population. Although intracerebroventricular (ICV) injections of chicken ghrelin strongly suppressed food intake in both lines, chickens from line LWS which are hypophagic, were more sensitive to central ghrelin than those from line HWS which are hyperphagic. Obestatin which caused a linear dose-dependent increase in food intake in line HWS had no effect in line LWS. For glucagon-like peptide-1 (GLP-1), chickens from line LWS responded to a lower dose with decreased food intake than those from line HWS. All doses of chicken glucagon-like peptide-2 (GLP-2) decreased food intake at a similar magnitude in line HWS, while only the highest dose (1.7 nmole) inhibited food intake in line LWS. Neuropeptide Y (NPY) caused a linear dose-dependent increase of food intake in LWS but not HWS chickens. For peptide YY (PYY), stimulatory feeding responses of HWS chickens were of a similar magnitude to all doses, while those from line LWS had an increased dose threshold of response (5 μg vs. 2.5 μg). A high dose (48 μg) of α-lipoic acid stimulated food intake in HWS but not LWS chickens. AICAR i.e. 5-amino-4-imidazolecarboxamide riboside caused a linear dose-dependent decrease in food intake in LWS but not HWS chickens. Compared to AICAR, 4-amino-5-imidazolecarboxamide

hydrochloride (AICA) had a more potent anorexigenic effect in both lines with differing dose thresholds (3 nmole in HWS vs. 6 nmole in LWS). For 6-[4-(2-piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine (Compound C), all doses tested resulted in a sustained increase in food intake in line LWS with the greatest effect being 12 μg. In line HWS, responses differed among doses with only the lowest dose (3 μg) being significantly higher than the control. Gonadotropin-inhibitory hormone (GnIH) and 4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid (C75) did not affect food intake in either line. Astressin had a transient (30 to 45 min) inhibitory effect on food intake in HWS but not LWS chickens. These results demonstrate that long-term selection for body weight has altered the brain's response to these modulators.

Key words: HWS, LWS, chicken, food intake, ICV

3.1. INTRODUCTION

Breeding programs and improved nutrition have led to dramatic increases in efficiency of modern broiler production. Presently, to maintain the well-being and fecundity of broiler breeders, it is necessary to restrict their food consumption. To devise strategies to alter food intake requires an understanding of how genetic selection for body weight has altered the brain's feeding response to different appetite modulators.

The central nervous system (CNS), especially the hypothalamus, plays a pivotal role in food intake regulation. The hypothalamus is critical in the detection and integration of afferent signals carried by nutrients, neuropeptides, adipokines, and circulating hormones from the gut and brainstem, as well as processing efferent signals to adjust food intake in

response to changing energy requirements (Morton et al., 2006). The gastrointestinal tract releases a variety of appetite-related peptide hormones including cholecystokinin (CCK), glucagon like peptide (GLP-1), oxyntomodulin, ghrelin, peptide YY (PYY) and pancreatic polypeptide (PP). These peptides which sensitively detect gut nutrient changes are transported to CNS by the bloodstream to influence food intake regulation (Morton et al., 2006). Different hypothalamic nuclei including arcuate nucleus (ARC), paraventricular nucleus (PVN), lateral hypothalamic area (LHA), dorsomedial nucleus (DMN), and ventromedial nucleus (VMN) are sites known to be intimately involved in food intake regulation (Simpson et al., 2009). Appetite-related neuropeptides secreted from these nuclei, such as neuropeptide Y (NPY), agouti-related peptide (AgRP), pro-opiomelanocortin (POMC), cocaine and amphetamine related transcript (CART), orexin, thyrotrophin releasing hormone (TRH) and corticotropin-releasing factor (CRF), convey peripheral signals of energy balance to regions of the CNS to integrate and trigger a feeding response (Simpson et al., 2009).

The present study used lines of chickens that had undergone 51-generations of divergent selection from a common White Rock base population for high (HWS) or low (LWS) body weight at 56 days of age (Marquez et al., 2010). These lines of chickens, which have different phenotypic and genotypic characteristics, differ in body weight by more than 10-fold at selection age (Johansson et al., 2010). Our objective was to determine if the divergent selection for body weight has altered central feeding responses to various appetite modulators.

3.2. MATERIALS AND METHODS

3.2.1. Animals

Eggs from age contemporary HWS and LWS parents were incubated in the same machine for each experiment. After hatch, chicks were vaccinated for Marek's disease and placed in electric heated batteries with raised wire floors at 32±2°C. A mash diet (20% crude protein and 2,684 kcal ME/kg) and water were provided *ad libitum*. At different ages HWS and LWS chickens were cannulated and transferred to individual cages measuring 17.6×26.4×17.6 cm. In these cages, each individual had its own feeder and waterer. Chickens were allowed a minimum of 3 days for recovery prior to test injection. Numbers of chickens, compounds, doses and ages tested are summarized in Table 3.1. Experimental procedures were approved by the Virginia Tech Animal Care and Use Committee.

Table 3. 1. Compounds tested by dosage and age in HWS and LWS chickens[1]

Compounds	Injection dose	Dose expressed in mole	HWS tested age (week)[2]	LWS tested age (week)[2]
Chicken ghrelin[3]	0, 0.1, 0.2 and 0.4 nmole	0, 0.1, 0.2 and 0.4 nmole	12	20
Chicken obestatin[3]	0, 0.016, 0.032 and 0.064 nmole	0, 0.016, 0.032 and 0.064 nmole	10	12
Chicken GLP-1[3]	0, 0.375, 0.75 and 1.5 nmole	0, 0.375, 0.75 and 1.5 nmole	5	22
Chicken GLP-2[3]	0, 0.425, 0.85 and 1.7 nmole	0, 0.425, 0.85 and 1.7 nmole	6	11
Human NPY[4]	0, 2.5, 5 and 10 μg	0, 0.585, 1.17 and 2.34 nmole	7	15
Human PYY[4]	0, 2.5, 5 and 10 μg	0, 0.58, 1.16 and 2.32 nmole	5	20
α-lipoic acid[5]	0, 12, 24 and 48 μg	0, 0.058, 0.116 and 0.233 μmole	7	15
Chicken GnIH[6]	0, 2, 4 and 8 μg	0, 1.41, 2.819 and 5.639 nmole	5	17
AICA[7]	0, 1.5, 3 and 6 μmole	0, 1.5, 3 and 6 μmole	9	11
AICAR[7]	0, 75, 150 and 300 μg	0, 0.29, 0.581 and 1.162 μmole	7	11
Compound C[7]	0, 3, 6 and 12 μg	0, 7.51, 15.019 and 30.038 nmole	6	15
C75[7]	0, 3, 6 and 12 μg	0, 11.797, 23.594 and 47.188 nmole	7	16
Astressin[7]	0, 1, 2 and 4 nmole	0, 1, 2 and 4 nmole	13	19

[1]The HWS and LWS chickens were from age contemporary 51-generation parents. In all experiments, the number for chickens tested was 16 in each line.
[2]Variation in age was in an attempt to match body weights of the two lines.
[3]Chicken ghrelin, chicken obestatin, chicken glucagon-like peptide-1 (GLP-1) and chicken glucagon-like peptide-2 (GLP-2) were synthesized by United States of Department of Agriculture (Washington, DC, USA) according to the peptide sequence of chicken ghrelin, obestatin, GLP-1 and GLP-2.

[4]Human neuropeptide Y (NPY) and human peptide YY (PYY) were bought from Bachem Americas, Inc. (Torrance, CA, USA).
[5]Alpha-lipoic acid was bought from Fluka BioChemika. (Buchs, Switzerland).
[6]Chicken gonadotropin-inhibitory hormone (GnIH) was bought from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA).
[7]4-Amino-5-imidazolecarboxamide hydrochloride (AICA), 5-amino-4-imidazole carboxamide riboside (AICAR), 6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine (Compound C), 4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid (C75) and astressin were bought from Sigma (St. Louis, MO, USA).

3.2.2. Method of Administration and Compounds Tested

Males from each line were anesthetized by intravenous injection of sodium pentobarbital (25mg/kg BW) and a 23-gauge thin-walled stainless steel guide cannula was stereotaxically implanted into the left lateral cerebral ventricle. Placement of the cannula into the ventricle was verified by the presence of cerebrospinal fluid in the guide cannula. All injections were made in a total volume of 10 μL using a Hamilton syringe with a 60-cm length of PE-20 tubing (Clay Adams, Parsippany, NJ 07054). Food and water intake was monitored at 15-min intervals from 0 to 180 min post-injection.

Appetite-related compounds were selected based on their regulatory effect on food intake in avian or mammalian species, and include a mix of orexigenic and anorexigenic actions. All compounds were administered with artificial cerebrospinal fluid (aCSF) as the vehicle, except Compound C which was dissolved in dimethyl sulfoxide (DMSO) + aCSF (20:80). Control groups received aCSF or, in the Compound C trial, DMSO + aCSF (20:80).

3.2.3. Experimental Design and Statistical Analysis

A replicated Latin square design was used in this experiment with chickens and days as blocking factors. HWS (n=16) or LWS (n=16) males were divided into 4 different

duplicate squares and received ICV injections of 4 doses of one of the compounds described above. Each male received all 4 doses throughout the 4-day experiment period, and all 4 doses were tested within each day. Cumulative food intake was analyzed with a repeated analysis of variance (ANOVA) every 15 minutes to 180 minutes using the general linear modeling procedure (GLM, SAS Institute Inc, Cary, NC 27513). Multiple comparisons among doses were made within each line, with Tukey's multiple range test used for comparing multiple means. Treatment effects were partitioned into linear and quadratic contrasts to determine the dose-response relationships. Significance was considered at $P<0.05$.

3.3. RESULTS

3.3.1. Ghrelin

Ghrelin at 0.4 nmole decreased food intake in HWS chickens at 45 min and from 90 to 165 min post-injection and the effect was linear from 75 to 105 min (Fig. 3.1A). All doses of ghrelin decreased food intake of LWS chickens with the effect linear and quadratic at all observed time points (Fig. 3.1B).

3.3.2. Obestatin

Obestatin increased food intake in HWS chickens from 45 to 135 min post-injection. This orexigenic effect was linearly from 60 to 120 min and quadratically from 90 to 105 min (Fig. 3.1C). However, obestatin had no effect on food intake in LWS chickens (Fig. 3.1D).

3.3.3. GLP-1

Although GLP-1 decreased food intake in both lines, dose responses differed. Only the highest dose 1.5 nmole GLP-1 decreased food intake in HWS chickens (Fig. 3.1E), while all doses of GLP-1 resulted in an inhibition of food intake with the most effective dose being 0.75 nmole in LWS chickens (Fig. 3.1F). The treatment effect was linear from 30 to 180 min for HWS, whereas for LWS chickens the effect was linear from 60 to 180 min and quadratic from 15 to 180 min.

3.3.4. Glucagon-Like Peptide-2 (GLP-2)

As with GLP-1, GLP-2 also decreased food intake in both lines. For HWS chickens, all doses of GLP-2 caused the same anorexigenic effect (Fig. 3.1G), while for LWS chickens, only the highest dose (1.7 nmole) effectively inhibited food intake (Fig. 3.1H). The treatment effect was linear from 30 to 180 min in HWS, and from 75 to 180 min in LWS chickens.

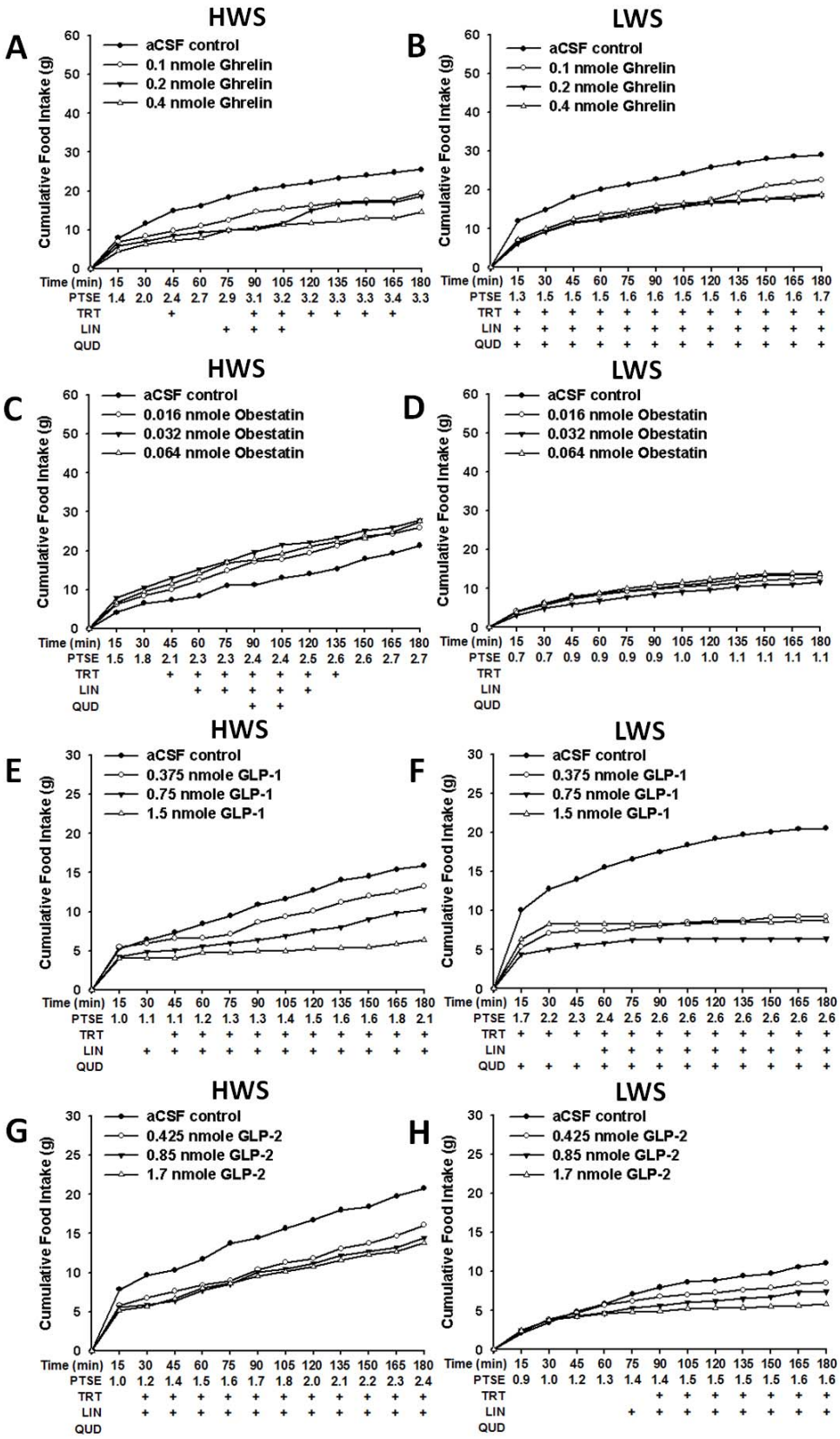
A
HWS
aCSF control
0.1 nmole Ghrelin
0.2 nmole Ghrelin
0.4 nmole Ghrelin
Cumulative Food Intake (g)
B
LWS
aCSF control
0.1 nmole Ghrelin
0.2 nmole Ghrelin
0.4 nmole Ghrelin
Cumulative Food Intake (g)
C
HWS
aCSF control
0.016 nmole Obestatin
0.032 nmole Obestatin
0.064 nmole Obestatin
Cumulative Food Intake (g)
D
LWS
aCSF control
0.016 nmole Obestatin
0.032 nmole Obestatin
0.064 nmole Obestatin
Cumulative Food Intake (g)
E
HWS
aCSF control
0.375 nmole GLP-1
0.75 nmole GLP-1
1.5 nmole GLP-1
Cumulative Food Intake (g)
F
LWS
aCSF control
0.375 nmole GLP-1
0.75 nmole GLP-1
1.5 nmole GLP-1
Cumulative Food Intake (g)
G
HWS
aCSF control
0.425 nmole GLP-2
0.85 nmole GLP-2
1.7 nmole GLP-2
Cumulative Food Intake (g)
H
LWS
aCSF control
0.425 nmole GLP-2
0.85 nmole GLP-2
1.7 nmole GLP-2
Cumulative Food Intake (g)

Fig. 3. 1. Cumulative food intake over 180 min of HWS and LWS male chickens following ICV injection of ghrelin, obestatin, GLP-1 and GLP-2. (A) ICV injection of ghrelin in 12-week old HWS chickens; (B) ICV injection of ghrelin in 20-week old LWS chickens; (C) ICV injection of obestatin in 10-week old HWS chickens; (D) ICV injection of obestatin in 12-week old LWS chickens; (E) ICV injection of GLP-1 in 5-week old HWS chickens; (F) ICV injection of GLP-1 in 22-week old LWS chickens; (G) ICV injection of GLP-2 in 6-week old HWS chickens; (H) ICV injection of GLP-2 in 11-week old LWS chickens. PTSE, pooled standard error; TRT, treatment effect; LIN, linear contrast; QUD, quadratic contrast; +, $P \leq 0.05$.

3.3.5. NPY

In LWS chickens, NPY caused a linear dose-dependent increase of food intake from 75 to 180 min (Fig. 3.2B). Whereas the highest dose 10 μg NPY had an orexigenic effect, lower doses did not affect food intake. In HWS chickens, none of the doses of NPY affected food intake (Fig. 3.2A).

3.3.6. PYY

PYY increased food intake from 105 to 180 min in HWS (Fig. 3.2C), and from 150 to 180 min in LWS chickens (Fig. 3.2D). In HWS chickens, all doses of PYY had a similar stimulation effect on food intake, while in LWS chickens, 5 and 10 but not 2.5 μg PYY increased food intake. The treatment effect was linear from 150 to 180 min and quadratic from 90 to 180 min for HWS chickens, whereas there was only linear from 150 to 180 for LWS chickens.

3.3.7. Alpha-lipoic Acid

In HWS chickens, α-lipoic acid caused a linear dose-dependent increase of food intake from 120 to 180 min (Fig. 3.2E). Only the highest dose (48 μg) α-lipoic acid increased food intake from 135 to 150 min, while lower doses had no effect. In LWS chickens, no dose of α-lipoic acid influenced food intake (Fig. 3.2F).

3.3.8. Gonadotropin-Inhibitory Hormone (GnIH)

Regardless of doses, GnIH did not affect food intake in either HWS or LWS chickens (Fig. 3.2G and 3.2H).

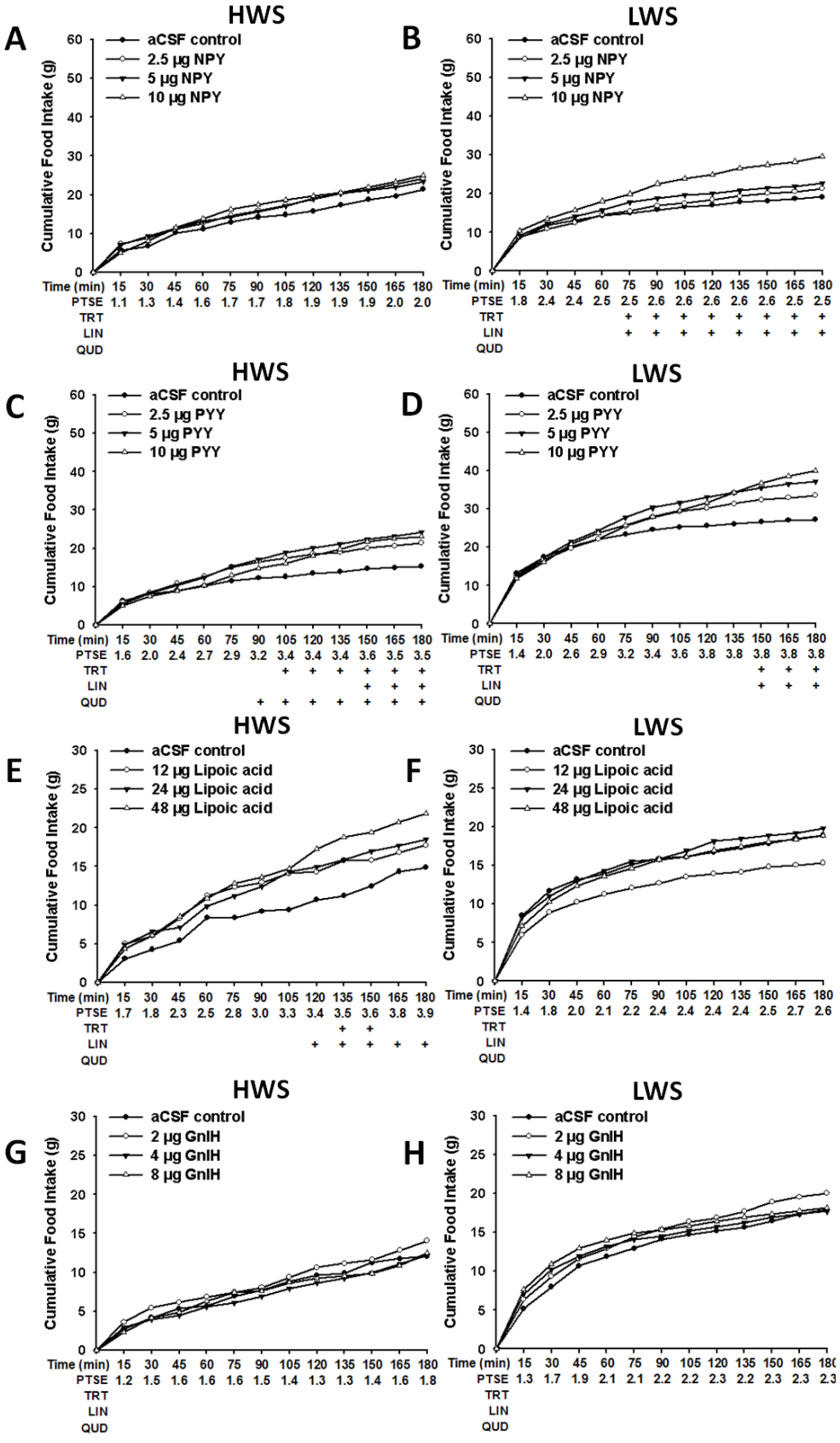
A
HWS
aCSF control
2.5 µg NPY
5 µg NPY
10 µg NPY
Cumulative Food Intake (g)
Time (min) 15 30 45 60 75 90 105 120 135 150 165 180
PTSE 1.1 1.3 1.4 1.6 1.7 1.7 1.8 1.9 1.9 1.9 2.0 2.0
TRT
LIN
QUD
B
LWS
aCSF control
2.5 µg NPY
5 µg NPY
10 µg NPY
Cumulative Food Intake (g)
Time (min) 15 30 45 60 75 90 105 120 135 150 165 180
PTSE 1.8 2.4 2.4 2.5 2.5 2.6 2.6 2.6 2.6 2.6 2.5 2.5
TRT + + + + + + + + + + + +
LIN + + + + + + + + + + + +
QUD
C
HWS
aCSF control
2.5 µg PYY
5 µg PYY
10 µg PYY
Cumulative Food Intake (g)
Time (min) 15 30 45 60 75 90 105 120 135 150 165 180
PTSE 1.6 2.0 2.4 2.7 2.9 3.2 3.4 3.4 3.4 3.6 3.5 3.5
TRT + + + + + + + + + + + +
LIN + + + + + + + + + + + +
QUD + + + + + + + + + + + +
D
LWS
aCSF control
2.5 µg PYY
5 µg PYY
10 µg PYY
Cumulative Food Intake (g)
Time (min) 15 30 45 60 75 90 105 120 135 150 165 180
PTSE 1.4 2.0 2.6 2.9 3.2 3.4 3.6 3.8 3.8 3.8 3.8 3.8
TRT + + + + + + + + + + + +
LIN + + + + + + + + + + + +
QUD
E
HWS
aCSF control
12 µg Lipoic acid
24 µg Lipoic acid
48 µg Lipoic acid
Cumulative Food Intake (g)
Time (min) 15 30 45 60 75 90 105 120 135 150 165 180
PTSE 1.7 1.8 2.3 2.5 2.8 3.0 3.3 3.4 3.5 3.6 3.8 3.9
TRT + + + + + + + + + + + +
LIN + + + + + + + + + + + +
QUD
F
LWS
aCSF control
12 µg Lipoic acid
24 µg Lipoic acid
48 µg Lipoic acid
Cumulative Food Intake (g)
Time (min) 15 30 45 60 75 90 105 120 135 150 165 180
PTSE 1.4 1.8 2.0 2.1 2.2 2.4 2.4 2.4 2.4 2.5 2.7 2.6
TRT
LIN
QUD
G
HWS
aCSF control
2 µg GnIH
4 µg GnIH
8 µg GnIH
Cumulative Food Intake (g)
Time (min) 15 30 45 60 75 90 105 120 135 150 165 180
PTSE 1.2 1.5 1.6 1.6 1.6 1.5 1.4 1.3 1.3 1.4 1.6 1.8
TRT
LIN
QUD
H
LWS
aCSF control
2 µg GnIH
4 µg GnIH
8 µg GnIH
Cumulative Food Intake (g)
Time (min) 15 30 45 60 75 90 105 120 135 150 165 180
PTSE 1.3 1.7 1.9 2.1 2.1 2.2 2.2 2.3 2.2 2.3 2.3 2.3
TRT
LIN
QUD

Fig. 3. 2. Cumulative food intake over 180 min of HWS and LWS male chickens following ICV injection of NPY, PYY, α lipoic acid and GnIH. (A) ICV injection of NPY in 7-week old HWS chickens; (B) ICV injection of NPY in 15-week old LWS chickens; (C) ICV injection of PYY in 5-week old HWS chickens; (D) ICV injection of PYY in 20-week old LWS chickens; (E) ICV injection of α lipoic acid in 7-week old HWS chickens; (F) ICV injection of α lipoic acid in 15-week old LWS chickens; (G) ICV injection of GnIH in 5-week old HWS chickens; (H) ICV injection of GnIH in 17-week old LWS chickens. PTSE, pooled standard error; TRT, treatment effect; LIN, linear contrast; QUD, quadratic contrast; +, $P \leq 0.05$.

3.3.9. 4-Amino-5-Imidazolecarboxamide Hydrochloride (AICA)

AICA decreased food intake at all observed time points in both lines, but with different thresholds of response. In HWS chickens, 3 and 6 μmole AICA had an anorexigenic effect whereas the lowest dose 1.5 μmole did not influence food intake (Fig. 3.3A). In LWS chickens, only the highest dose (6 μmole) AICA decreased food intake while lower doses had no effect (Fig. 3.3B). The treatment effect was linear from 60 to 180 min and quadratical from 15 to 30 min for line HWS whereas for line LWS the effect was linear at all time points.

3.3.10. 5-Amino-4-Imidazole Carboxamide Riboside (AICAR)

In LWS chickens, AICAR decreased food intake by 150 min post-injection and thereafter with the response being linear (Fig. 3.3D). The highest dose (300 μg) AICAR inhibited

food intake while there was no effect at the lower doses. In HWS chickens, AICAR did not affect food intake (Fig. 3.3C).

3.3.11. (6-(4-(2-piperidin-1-ylethoxy)phenyl))-3-pyridin-4-ylpyrazolo(1,5-a)pyrimidine (Compound C)

Compound C increased food intake in both lines with the effect linear at all observed time points in LWS. All doses of Compound C tested resulted in a sustained increase in food intake in line LWS with the greatest effect at 12 µg (Fig. 3.3F). In line HWS, however, responses differed among doses with only the lowest dose (3 µg) different from the control from 30 to 105 min post-injection (Fig. 3.3E).

3.3.12. 4-Methylene-2-Octyl-5-Oxotetrahydrofuran-3-Carboxylic Acid (*C75)*

None of the doses of C75 tested affected food intake in either line (Fig. 3.3G and 3.3H).

3.3.13. Astressin

Astressin decreased food intake in HWS but not LWS chickens. In HWS chickens, the highest dose (4 nmole) astressin effectively inhibited food intake from 30 to 45 min post-injection with the effect being linear at 30 min (Fig. 3.3I), whereas lower doses of astressin had no effect on food intake (Fig. 3.3J).

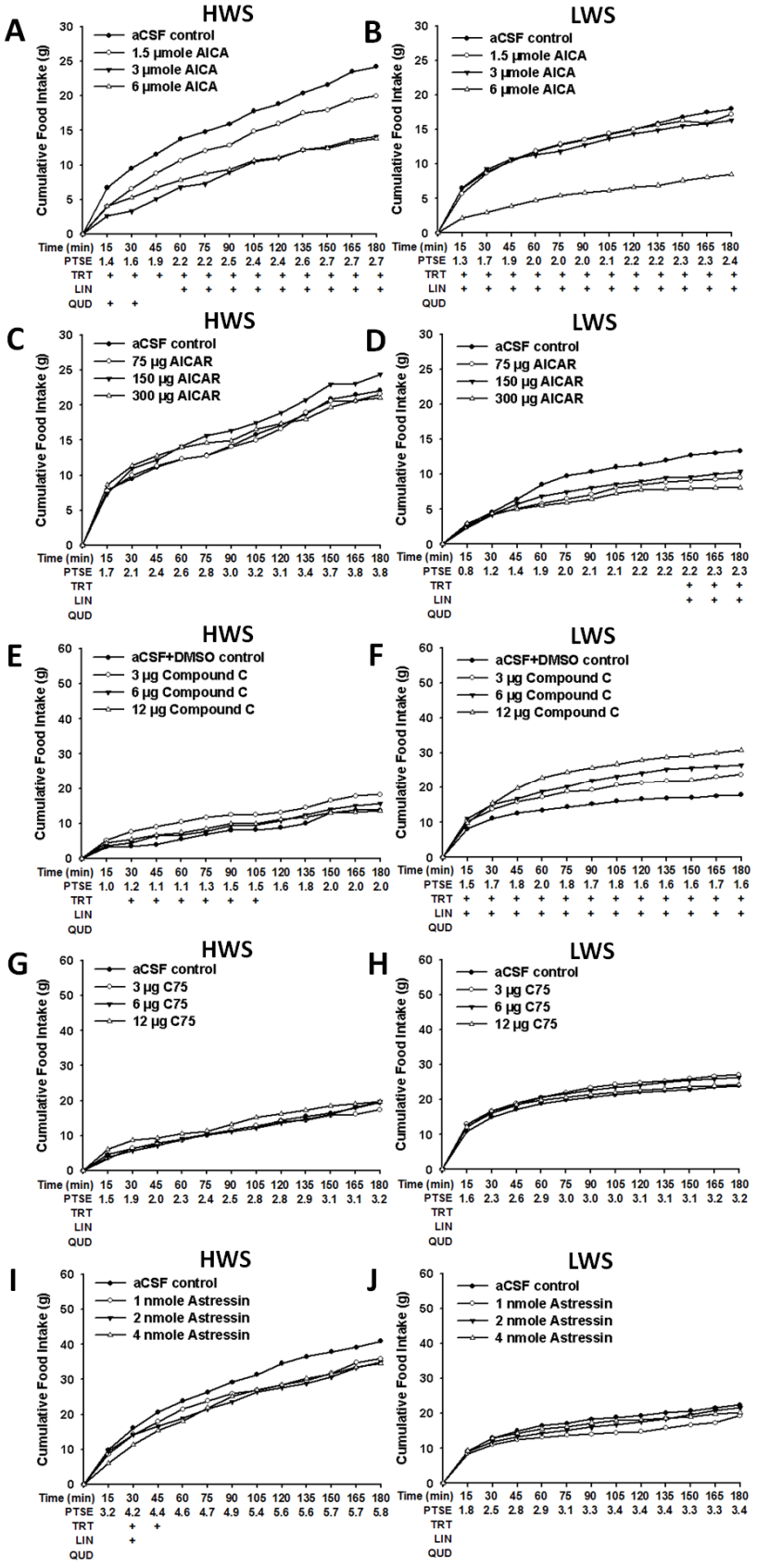
A
HWS
aCSF control
1.5 µmole AICA
3 µmole AICA
6 µmole AICA
B
LWS
aCSF control
1.5 µmole AICA
3 µmole AICA
6 µmole AICA
C
HWS
aCSF control
75 µg AICAR
150 µg AICAR
300 µg AICAR
D
LWS
aCSF control
75 µg AICAR
150 µg AICAR
300 µg AICAR
E
HWS
aCSF+DMSO control
3 µg Compound C
6 µg Compound C
12 µg Compound C
F
LWS
aCSF+DMSO control
3 µg Compound C
6 µg Compound C
12 µg Compound C
G
HWS
aCSF control
3 µg C75
6 µg C75
12 µg C75
H
LWS
aCSF control
3 µg C75
6 µg C75
12 µg C75
I
HWS
aCSF control
1 nmole Astressin
2 nmole Astressin
4 nmole Astressin
J
LWS
aCSF control
1 nmole Astressin
2 nmole Astressin
4 nmole Astressin
Cumulative Food Intake (g)
Time (min)
PTSE
TRT
LIN
QUD

Fig. 3. 3. Cumulative food intake over 180 min of HWS and LWS male chickens following ICV injection of AICA, AICAR, Compound C, C75 and astressin. (A) ICV injection of AICA in 9-week old HWS chickens; (B) ICV injection of AICA in 11-week old LWS chickens; (C) ICV injection of AICAR in 7-week old HWS chickens; (D) ICV injection of AICAR in 11-week old LWS chickens; (E) ICV injection of Compound C in 6-week old HWS chickens; (F) ICV injection of Compound C in 15-week old LWS chickens; (G) ICV injection of C75 in 7-week old HWS chickens; (H) ICV injection of C75 in 16-week old LWS chickens; (I) ICV injection of astressin in 13-week old HWS chickens; (J) ICV injection of astressin in 19-week old LWS chickens;PTSE, pooled standard error; TRT, treatment effect; LIN, linear contrast; QUD, quadratic contrast; +, $P \leq 0.05$.

3.4. DISCUSSION

Long-term selection for high or low body weight at 56 days of age has yielded chickens that differ considerably in food intake, growth, muscle accumulation, and fat accretion (Marquez et al., 2010). To determine if the response to selection has altered sensitivity of CNS to central or peripheral signals, we measured the central feeding response to a range of appetite modulators known to regulate food intake in mammals.

3.4.1. Different Feeding Responses of Mammals and Chickens to Ghrelin, Obestatin, AICA, AICAR, Compound C, PYY, Alpha-Lipoic Acid, Astressin, GnIH and C75

Although there are similarities in the neural regulation of food intake, the feeding responses to ghrelin, obestatin, AICA, AICAR, Compound C, PYY, α-lipoic acid, astressin, GnIH and C75 were different in mammals and chickens.

Ghrelin is a 28-amino acid peptide produced mainly by the stomach that is involved in the regulation of body weight and food intake in both mammals and chickens (Kojima et al., 1999). As opposed to the orexigenic effect of ghrelin in mammals (Nakazato et al., 2001), ICV injection of ghrelin strongly inhibits food intake in chickens regardless of age, sex, and time of injection or dosage (Saito et al., 2002; Saito et al., 2005; Chen et al., 2008). Our results were consistent with these reports in that ICV injections of chicken ghrelin strongly suppressed food intake in chickens. The anorexigenic feeding response of these lines to ICV injection of ghrelin was also consistent with our previous results from Cobb broilers and White Leghorn cockerels (Denbow, unpubished data). Additionally, LWS chickens, which are hypophagic, were more sensitive to central ghrelin than HWS chickens, which are hyperphagic. Ghrelin had a significant anorexigenic effect regardless of injection dosages in LWS chickens, suggesting a hyper-anorexic response in LWS chickens with a low threshold to ghrelin. In contrast, HWS chickens only responded to higher doses, while lower doses of ghrelin did not affect food intake. Thus, long-term selection for body weight has altered the brain's response to ghrelin and these correlated responses infer differential food intake regulatory mechanisms between the two lines.

Obestatin, a 23 amino acid peptide encoded by the same gene as ghrelin, was first reported to reduce food intake and body weight gain in rats and considered as a

physiological opponent of ghrelin (Zhang et al., 2005). Subsequent reports on the effects of obestatin on food intake, however, have been inconsistent. Studies supporting the previous reported anorexigenic effect of obestatin include Bresciani et al. (2006) and Green et al. (2007) whereas others have been unable to reproduce these results (e.g. Sibilia et al., 2006; Seoane et al., 2006; Gourcerol et al., 2007). Lagaud et al. (2007) reported that obestatin inhibited food intake and body weight gain in rodents with an unusual U-shaped dose-response relationship. This result may explain the difficulties in reproducing the effects of obestatin on feeding. As opposed to the anorexigenic effect of obestatin in mammals, obestatin caused a linear dose-dependent increase in food intake in HWS but not in LWS chickens. This result is in accordance with our previous results from White Leghorn cockerels (Denbow and McMurtry, unpublished data). Thus, although ghrelin and obestatin have important roles in appetite control in both mammals and chickens, their regulatory effects are opposite, suggesting different ghrelin/obestatin appetite-related neuropathways. The differential feeding responses to central obestatin of these two lines provides additional evidence for the alteration of appetite controlling mechanisms by long-term selection for body weight.

AICA is a reactant for the synthesis of heterocyclic compounds such as purines (Kuroda and Suzuki, 1993) and pyrimidines (Chern et al., 1992). The nucleoside of AICA i.e. AICAR, is readily taken up by cells and rapidly phosphorylated to 5-aminoimidazole-4-carboxamide-1-d-ribofuranosyl-5'-monophosphate (ZMP), which is an intermediate in the late steps of purine biosynthesis (Vincent et al., 1996). Although ZMP mimics all the activating effects of AMP on AMPK, it is 50-fold less potent than AMP at activating AMPK (Hardie, 2003). Once activated, AMPK stimulates food intake and decreases

energy expenditures by regulating the hypothalamic appetite-related neuron circuits (Minokoshi et al., 2004). In mammals, AICAR stimulates food intake and body weight gains, presumably by activating AMPK (Hu et al., 2005; Florant, et al., 2010). Here, we demonstrate that AICAR had a genetic line-dependent inhibitory effect on food intake because ICV injection of it caused a linear dose-dependent decrease in food intake in LWS but not HWS chickens. Compared to AICAR, AICA had a much more potent anorexigenic effect in both lines. The different feeding inhibition potencies of AICA and AICAR suggest that other kinase pathways or other biochemical mechanisms, separate from AICAR activated AMPK, may be involved in the anorexigenic effect of AICA in chickens. The reason why AMPK activator AICAR causes feeding inhibition instead of stimulation and if this inhibition is attributed to the regulation of AMPK still requires further clarification.

Compound C, a commonly used inhibitor of AMPK, can block the stimulatory effect of AICAR on AMPK activity and cause inhibition of food intake and body weight gain in mammals (Kim et al., 2004a; Hayes et al., 2009). We found that while Compound C increased food intake in both lines, they differed in dose responses. All doses of Compound C resulted in a significant sustained increase in food intake in line LWS with the greatest effect at 12 μg. In contrast, in line HWS responses differed among doses with only the lowest dose (3 μg) higher than the control. Although the orexigenic effect of Compound C, an antagonist of AMPK, in chickens is opposite to that reported in mammals, it is consistent with the anorexigenic effect of AICAR, an agonist of AMPK, in chickens. Additionally, ghrelin, an AMPK stimulator in mammals (Kola et al., 2005), has an inhibitory effect on food intake in chickens which is also opposite to the

stimulatory effect in mammals. These results suggest it is possible that there may be an opposite AMPK appetite regulation system between chickens and mammals.

The NPY family of peptides (including NPY, PYY and pancreatic polypeptide and their Y receptors) play essential roles in the regulation of food intake and energy homeostasis. PYY, a member of the NPY family, is a 36 amino acid protein released mainly from enterochromaffin (L) cells in the pancreas, small intestine, and colon in response to feeding (Taylor, 1985). It also has been observed in hypothalamic and pituitary tissues of human brain, which suggests a role of PYY as a neurotransmitter in appetite control and energy expenditure (Morimoto et al., 2008). Compared to NPY, a potent orexigenic peptide, PYY suppresses appetite and food intake (Batterham et al., 2002; Batterham et al., 2003), and delays gastric emptying in mammals (Playford et al., 1990). Conversely, ICV (Kuenzel et al., 1987) and in ovo administration (Coles et al., 1999) of PYY potently increased food intake and improved growth and feed conversion ratios of chicks. Our results were consistent with those as opposed to the anorexigenic effect of PYY in mammals, PYY increased food intake in both HWS and LWS chickens, but dose thresholds differed between them. That is while all doses of PYY had the same stimulation effect on food intake in HWS chickens, 10 and 5 μg but not lower doses of PYY exert orexigenic effect in LWS chickens. Thus, PYY has an orexigenic effect in chickens as opposed to mammals, and selection for body weight has changed the sensitivity of the stimulatory response to PYY.

Alpha-lipoic acid, an antioxidant found in a range of foods as well as in the human body reduced food intake, weight gain, and fatty acid synthesis by inhibiting 5' adenosine

monophosphate-activated protein kinase (AMPK) activity when administrated either IP, ICV or orally (Packer et al., 2001; Kim et al., 2004b; Koh et al., 2011). In chickens, most α-lipoic acid studies have focused on the effect on glucose and lipid metabolism, suggesting that α-lipoic acid stimulates the insulin sensitivity of tissues and fatty acid metabolism in both liver and adipose tissue (Hamano, 2002, 2006, 2007). As opposed to the weight loss and anorexigenic effects in mammals, our results showed that high dose (48 μg) of α-lipoic acid injection significantly stimulated food intake in HWS but not LWS chickens. Compared to the low dose threshold (3 μg) for the feeding inhibitory response in rats (Kim et al., 2004b), the effective dose for central α-lipoic acid feeding stimulatory response in HWS chickens is much higher (48 μg). Therefore, before drawing conclusions on the feeding response of central α-lipoic acid in chickens, it is necessary to clarify whether this orexigenic effect of high dose α-lipoic acid is a physiological or pharmacological effect.

In mammals, astressin, a CRF antagonist, reverses the anxiogenic effects caused by CRF stimulators including urocortin (Sajdyk and Gehlert, 2000) and prolacin-releasing peptide (Lawrence et al., 2004). Similarly, in chickens, anorexigenic modulators such as ghrelin, GLP-1 and stresscopin are mediated through the CRF pathway and their appetite downregulation effect is attenuated by astressin (Saito et al., 2005; Tachibana et al., 2006; Cline et al., 2009b). In 5-day-old HWS and LWS chicks, ICV injections of CRF effectively reduced food intake in both lines but with lower dose threshold in line LWS than HWS, suggesting selection for body weight has altered central CRF anorexigenic system (Cline et al., 2009a). Whereas the direct effect of astressin on food intake of chickens has not been clarified, our results showed that astressin decreased food intake in

HWS but not LWS chickens. That ICV injections of astressin, a nonselective CRF antagonist, decreased food intake instead of increasing it in HWS chickens suggests a need for further functional studies to clarify which signaling pathways or molecular mechanisms have been involved in its anorexigenic effect.

GnIH, an inhibitory regulator of reproduction released from the dorsomedial nucleus of the hypothalamus, acts as an endogenous orexigenic factor in the brain of chickens (Tachibana et al., 2005). Following this original report, an ICV injection of RF-amide-related peptide-3 (RFRP-3), a genetically related and functionally similar neuropeptide to GnIH in mammals, resulted in a similar orexigenic effect (Johnson et al., 2007; Johnson and Fraley, 2008; Murakami et al., 2008). Our results that GnIH did not affect food intake in either HWS or LWS chickens may be attributed to the different injection doses, genetic stock, and/or age differences in experimental protocols.

Cerulenin, a natural inhibitor of fatty acid synthase (FAS), and its synthetic analog, C75, potently reduce food intake and increase fatty acid oxidation and energy expenditure, leading to profound weight losses in mammals (Makimura et al., 2001; Wortman et al., 2003; Kim et al., 2004a; Tu et al., 2005). In chickens, intravenous administration of cerulenin reduced food intake and downregulated FAS and melanocortin receptors 1, 4, and 5, suggesting cerulenin may be mediated through the melanocortin system (Dridi et al., 2006). Although to our knowledge there are no previous reports on food regulation effects of C75 in chickens, we found that it did not affect food intake in either line. This result suggests that C75 may not have the same inhibitory effect on food intake as cerulenin in chickens.

3.4.2. Similar Feeding Response of Mammals and Chickens to GLP-1, GLP-2 and NPY

GLP-1, a posttranslational product of preproglucagon secreted from the intestine, inhibits food intake following both central and peripheral administration in mammals (Turton et al., 1996; Meeran et al., 1999; Schick et al., 2003). As in mammals, ICV injection of GLP-1 also strongly suppressed food intake and body weight in chickens (Bungo et al., 2001; Zhang et al., 2001; Tachibana et al., 2004). This anorexigenic effect is mediated by corticotrophin releasing factor (CRF) neurons in the brain and the sensitivity of the inhibitory response to GLP-1 differs between strains of chickens (Tachibana et al., 2006). We also observed that GLP-1 decreased food intake regardless of injection dosage in LWS chickens, suggesting a high central sensitivity for GLP-1 in this line with a low threshold. Conversely, HWS chickens only responded significantly to higher doses, while lower doses of GLP-1 had a tendency to inhibit food intake. Thus, the sensitivity of the inhibitory response to GLP-1 has been altered by long-term selection for body weight.

As GLP-1, GLP-2 is also a member of family of peptides derived from proglucagon that have played a role in food intake regulation. In mammals, central ICV injection of GLP-2 inhibits food intake (Tang-Christensen et al., 2000), while peripheral administration of GLP-2 does not influence food intake (Jeppesen et al., 2001; Sorensen et al., 2003). In contrast, neither ICV nor intraperitoneal (IP) administration of GLP-2 showed any effect on food intake, body temperature or locomotor activity in Japanese quail (Shousha et al., 2007). They used rat GLP-2, which shares only 52% amino acid identity with chicken GLP-2, and it may not have the ligand binding domain for activation in the avian system.

In our study, when chicken GLP-2 was ICV injected, all doses of GLP-2, as opposed to GLP-1, decreased food intake in HWS chickens, suggesting a lower threshold for central anorexigenic effect of GLP-2. Conversely, only higher doses of GLP-2 inhibited food intake in LWS chickens, while lower doses of GLP-2 had no effect. Thus, as in mammals, while ICV injection of GLP-2 strongly suppressed food intake, selection for body weight has altered the dose threshold for the inhibitory response.

A 36-amino acid neuropeptide, NPY is widely expressed in the central and peripheral nervous systems, and is related to feeding behavior, body composition, and energy homeostasis (Stanley et al., 1989; Kotz et al., 1998). Central administration of exogenous NPY (Hanson and Dallman, 1995; Raposinho et al., 2001), NPY agonists dexamethasone (White et al., 1994) or N-acetyl [Leu28, Leu31] NPY (24-36) (King et al., 1999) potently increased food intake as well as caused hormonal and metabolic changes that increase food efficiency and fat accretion in rats. Similarly, central administration of NPY strongly stimulated food intake and inhibited the hypothalamic-pituitary-thyroid axis to decrease energy expenditure in chicks through binding to hypothalamic Y1 and Y5 receptors (Kuenzel et al., 1987; Bungo et al., 2000; Fekete et al., 2002; Dodo et al., 2005). We observed that NPY had a linear dose-dependent increase in food intake in LWS chickens, while having no effect on food intake in HWS chickens. These results are opposite to the previous results utilizing 5-day-old HWS and LWS chicks (Cline et al., unpublished data), which showed that ICV injection of NPY increased food intake in HWS but not LWS chickens. These data suggest both line and age differences in hypothalamic response to NPY.

In summary, there are close interactions between the investigated compounds (Fig. 3.4), our data provide evidence that chickens have different feeding responses to many appetite modulators, and long-term selection for body weight has altered the brain's response to these modulators (Table 3.2). These differences may contribute to the differential food intake regulatory mechanisms and body weight in the HWS and LWS lines of chickens.

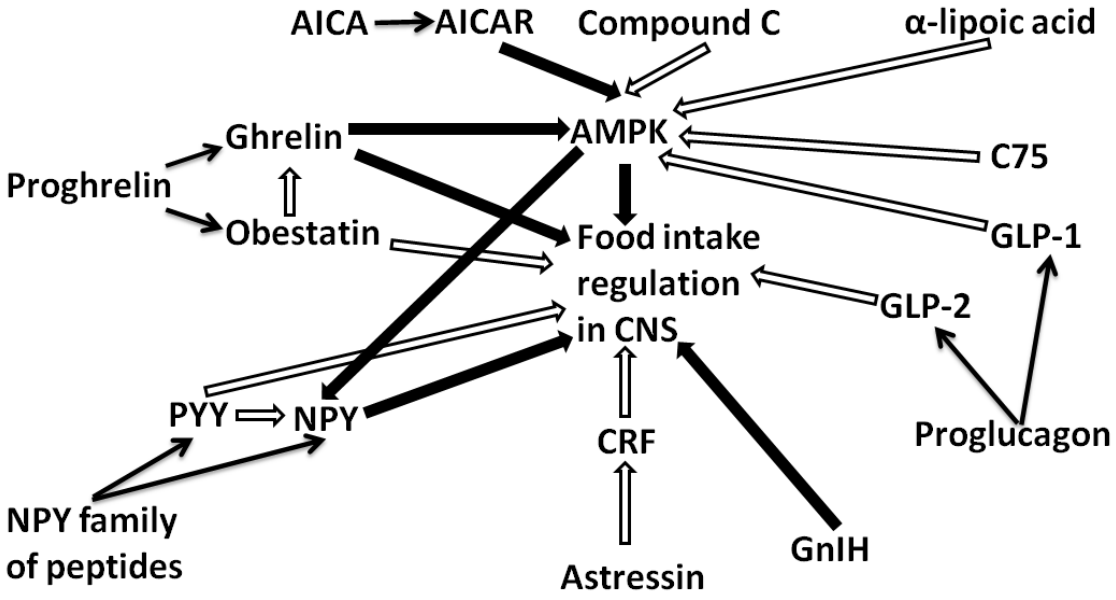

Fig. 3. 4. Food intake regulatory interactions between various appetite modulators in mammals. Line arrows represent peptide derivatives, black block arrows represent stimulation and white block arrows represent inhibition.

Table 3. 2. Different feeding responses to various appetite modulators in HWS and LWS chickens and mammals

Tested compounds	HWS	LWS	Mammals
Ghrelin			↑
Obestatin		—	↓ or —
AICA			No

AICAR	—	⇩	↑
Compound C	⇧	⇩	↓
PYY	⇩	⇧	↓
α-lipoic acid	⇧	—	↓
Astressin	⇩	—	No
GnIH	—	—	↑
C75	—	—	↓
GLP-1	⇩	⇩	↓
GLP-2	⇩	⇩	↓
NPY	—	⇧	↑

Up arrow (↑) represents stimulation, down arrow (↓) represents inhibition, line (—) represents no effect and "No" represents no report. The fill patterns of arrows represent dose threshold of HWS and LWS chickens for the appetite regulatory effect of the compounds: the arrow filled with light downward diagonal represents a lower threshold than blank arrow in the same row.

3.5. REFERENCES

Batterham, R. L., M. A. Cohen, S. M. Ellis, C. W. Le Roux, D. J. Withers, G. S. Frost, M. A. Ghatei, and S. R. Bloom. 2003. Inhibition of food intake in obese subjects by peptide YY3-36. N. Engl. J. Med. 349:941-948.

Batterham, R. L., M. A. Cowley, C. J. Small, H. Herzog, M. A. Cohen, C. L. Dakin, A. M. Wren, A. E. Brynes, M. J. Low, M. A. Ghatei, R. D. Cone, and S. R. Bloom. 2002. Gut hormone PYY(3-36) physiologically inhibits food intake. Nature 418:650-654.

Bresciani, E., D. Rapetti, F. Dona, I. Bulgarelli, L. Tamiazzo, V. Locatelli, and A. Torsello. 2006. Obestatin inhibits feeding but does not modulate GH and corticosterone secretion in the rat. J. Endocrinol. Invest. 29:RC16-18.

Bungo, T., R. Ando, S. Kawakami, A. Ohgushi, M. Shimojo, Y. Masuda, and M. Furuse. 2000. Central bombesin inhibits food intake and the orexigenic effect of neuropeptide Y in the neonatal chick. Physiol. Behav. 70:573-576.

Bungo, T., S. I. Kawakami, A. Ohgushi, K. Sashihara, N. Saito, K. Sugahara, S. Hasegawa, D. M. Denbow, and M. Furuse. 2001. Intracerebroventricular injection of fusaric acid attenuates the anorexia by glucagon-like peptide-1 in the neonatal chick. Pharmacol. Biochem. Behav. 70:251-255.

Chen, L. L., Q. Y. Jiang, H. Kaiya, X. T. Zhu, G. Shu, K. Kangawa, P. Gao, and Y. L. Zhang. 2008. Central administration of chicken ghrelin inhibits food intake in adult chickens. Pages 75 in 9th International Symposium on Avian Endocrinology. Leuven, Belgium.

Chern, J. W., C. C. Lee, Y. C. Liaw, and A. H. J. Wang. 1992. Heterocyclic-compounds .1. reactions of ortho-amino-carboxamide with beta-diketones- synthesis of imidazo[1,5-a]pyrimidine and pyrazolo[1,5-a]pyrimidine derivatives. heterocycles 34:1133-1145.

Cline, M. A., A. Y. Kuo, M. L. Smith, W. Nandar, B. C. Prall, P. B. Siegel, and D. M. Denbow. 2009a. Differential feed intake responses to central corticotrophin releasing factor in lines of chickens divergently selected for low or high body weight. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 152:130-134.

Cline, M. A., B. C. Prall, J. O. Rogers, and T. Tachibana. 2009b. Satiety induced by central stresscopin is mediated by corticotrophin-releasing factor receptors and hypothalamic changes in chicks. Pharmacol. Biochem. Behav. 92:663-669.

Coles, B. A., W. J. Croom, J. Brake, L. R. Daniel, V. L. Christensen, C. P. Phelps, A. Gore, and I. L. Taylor. 1999. In ovo peptide YY administration improves growth and feed conversion ratios in week-old broiler chicks. Poult. Sci. 78:1320-1322.

Dodo, K., T. Izumi, H. Ueda, and T. Bungo. 2005. Response of neuropeptide Y-induced feeding to mu-, delta- and kappa-opioid receptor antagonists in the neonatal chick. Neurosci. Lett. 373:85-88.

Dridi, S., C. Ververken, F. B. Hillgartner, L. Arckens, E. Van der Gucht, L. Cnops, E. Decuypere, and J. Buyse. 2006. FAS inhibitor cerulenin reduces food intake and melanocortin receptor gene expression without modulating the other (an)orexigenic neuropeptides in chickens. Am. J. Physiol. Regul. Integr. Comp. Physiol. 291:R138-147.

Fekete, C., S. Sarkar, W. M. Rand, J. W. Harney, C. H. Emerson, A. C. Bianco, A. Beck-Sickinger, and R. M. Lechan. 2002. Neuropeptide Y1 and Y5 receptors mediate the effects of neuropeptide Y on the hypothalamic-pituitary-thyroid axis. Endocrinology 143:4513-4519.

Florant, G. L., A. M. Fenn, J. E. Healy, G. K. Wilkerson, and R. J. Handa. 2010. To eat or not to eat: the effect of AICAR on food intake regulation in yellow-bellied marmots (Marmota flaviventris). J. Exp. Biol. 213:2031-2037.

Gourcerol, G., D. H. St-Pierre, and Y. Tache. 2007. Lack of obestatin effects on food intake: should obestatin be renamed ghrelin-associated peptide (GAP)? Regul. Pept. 141:1-7.

Green, B. D., N. Irwin, and P. R. Flatt. 2007. Direct and indirect effects of obestatin peptides on food intake and the regulation of glucose homeostasis and insulin secretion in mice. Peptides 28:981-987.

Hamano, Y. 2002. Influence of lipoic acid on lipid metabolism and beta-adrenergic response to intravenous or oral administration of clenbuterol in broiler chickens. Reprod. Nutr. Dev. 42:307-316.

Hamano, Y. 2006. Effects of dietary lipoic acid on plasma lipid, in vivo insulin sensitivity, metabolic response to corticosterone and in vitro lipolysis in broiler chickens. Br. J. Nutr. 95:1094-1101.

Hamano, Y. 2007. Continuous infusion of lipoic acid rapidly reduces plasma beta-hydroxybutyrate with elevation of non-esterified fatty acids in broiler chickens. Br. J. Nutr. 97:495-501.

Hanson, E. S., and M. F. Dallman. 1995. Neuropeptide Y (NPY) may integrate responses of hypothalamic feeding systems and the hypothalamo-pituitary-adrenal axis. J. Neuroendocrinol. 7:273-279.

Hardie, D. G. 2003. Minireview: the AMP-activated protein kinase cascade: the key sensor of cellular energy status. Endocrinology 144:5179-5183.

Hayes, M. R., K. P. Skibicka, K. K. Bence, and H. J. Grill. 2009. Dorsal hindbrain 5'-adenosine monophosphate-activated protein kinase as an intracellular mediator of energy balance. Endocrinology 150:2175-2182.

Hu, Z., Y. Dai, M. Prentki, S. Chohnan, and M. D. Lane. 2005. A role for hypothalamic malonyl-CoA in the control of food intake. J. Biol. Chem. 280:39681-39683.

Jeppesen, P. B., B. Hartmann, J. Thulesen, J. Graff, J. Lohmann, B. S. Hansen, F. Tofteng, S. S. Poulsen, J. L. Madsen, J. J. Holst, and P. B. Mortensen. 2001. Glucagon-like peptide 2 improves nutrient absorption and nutritional status in short-bowel patients with no colon. Gastroenterology 120:806-815.

Johansson, A. M., M. E. Pettersson, P. B. Siegel, and O. Carlborg. 2010. Genome-wide effects of long-term divergent selection. PLoS Genet. 6:e1001188.

Johnson, M. A., and G. S. Fraley. 2008. Rat RFRP-3 alters hypothalamic GHRH expression and growth hormone secretion but does not affect KiSS-1 gene expression or the onset of puberty in male rats. Neuroendocrinol. 88:305-315.

Johnson, M. A., K. Tsutsui, and G. S. Fraley. 2007. Rat RFamide-related peptide-3 stimulates GH secretion, inhibits LH secretion, and has variable effects on sex behavior in the adult male rat. Horm. Behav. 51:171-180.

Kim, E. K., I. Miller, S. Aja, L. E. Landree, M. Pinn, J. McFadden, F. P. Kuhajda, T. H. Moran, and G. V. Ronnett. 2004a. C75, a fatty acid synthase inhibitor, reduces food intake via hypothalamic AMP-activated protein kinase. J. Biol. Chem. 279:19970-19976.

Kim, M. S., J. Y. Park, C. Namkoong, P. G. Jang, J. W. Ryu, H. S. Song, J. Y. Yun, I. S. Namgoong, J. Ha, I. S. Park, I. K. Lee, B. Viollet, J. H. Youn, H. K. Lee, and K. U. Lee. 2004b. Anti-obesity effects of alpha-lipoic acid mediated by suppression of hypothalamic AMP-activated protein kinase. Nat. Med. 10:727-733.

King, P. J., P. S. Widdowson, H. N. Doods, and G. Williams. 1999. Regulation of neuropeptide Y release by neuropeptide Y receptor ligands and calcium channel antagonists in hypothalamic slices. J. Neurochem. 73:641-646.

Koh, E. H., W. J. Lee, S. A. Lee, E. H. Kim, E. H. Cho, E. Jeong, D. W. Kim, M. S. Kim, J. Y. Park, K. G. Park, H. J. Lee, I. K. Lee, S. Lim, H. C. Jang, K. H. Lee, and K. U. Lee. 2011. Effects of alpha-lipoic Acid on body weight in obese subjects. Am. J. Med. 124:85 e81-88.

Kojima, M., H. Hosoda, Y. Date, M. Nakazato, H. Matsuo, and K. Kangawa. 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402:656-660.

Kola, B., E. Hubina, S. A. Tucci, T. C. Kirkham, E. A. Garcia, S. E. Mitchell, L. M. Williams, S. A. Hawley, D. G. Hardie, A. B. Grossman, and M. Korbonits. 2005. Cannabinoids and ghrelin have both central and peripheral metabolic and cardiac effects via AMP-activated protein kinase. J. Biol. Chem. 280:25196-25201.

Kotz, C. M., J. E. Briggs, M. K. Grace, A. S. Levine, and C. J. Billington. 1998. Divergence of the feeding and thermogenic pathways influenced by NPY in the hypothalamic PVN of the rat. Am. J. Physiol. 275:R471-477.

Kuenzel, W. J., L. W. Douglass, and B. A. Davison. 1987. Robust feeding following central administration of neuropeptide Y or peptide YY in chicks, *Gallus domesticus*. Peptides 8:823-828.

Kuroda, T., and F. Suzuki. 1993. Facile Synthesis of 5,6,7,8-Tetrahydropyrido[2,1-B]Purin-10-One. Journal of Heterocyclic Chemistry 30:593-595.

Lagaud, G. J., A. Young, A. Acena, M. F. Morton, T. D. Barrett, and N. P. Shankley. 2007. Obestatin reduces food intake and suppresses body weight gain in rodents. Biochem. Biophys. Res. Commun. 357:264-269.

Lawrence, C. B., Y. L. Liu, M. J. Stock, and S. M. Luckman. 2004. Anorectic actions of prolactin-releasing peptide are mediated by corticotropin-releasing hormone receptors. Am. J. Physiol. Regul. Integr. Comp. Physiol. 286:R101-107.

Makimura, H., T. M. Mizuno, X. J. Yang, J. Silverstein, J. Beasley, and C. V. Mobbs. 2001. Cerulenin mimics effects of leptin on metabolic rate, food intake, and body weight independent of the melanocortin system, but unlike leptin, cerulenin fails to block neuroendocrine effects of fasting. Diabetes 50:733-739.

Marquez, G. C., P. B. Siegel, and R. M. Lewis. 2010. Genetic diversity and population structure in lines of chickens divergently selected for high and low 8-week body weight. Poult. Sci. 89:2580-2588.

Meeran, K., D. O'Shea, C. M. Edwards, M. D. Turton, M. M. Heath, I. Gunn, S. Abusnana, M. Rossi, C. J. Small, A. P. Goldstone, G. M. Taylor, D. Sunter, J. Steere, S. J. Choi, M. A. Ghatei, and S. R. Bloom. 1999. Repeated intracerebroventricular administration of glucagon-like peptide-1-(7-36) amide or exendin-(9-39) alters body weight in the rat. Endocrinology 140:244-250.

Minokoshi, Y., T. Alquier, N. Furukawa, Y. B. Kim, A. Lee, B. Xue, J. Mu, F. Foufelle, P. Ferre, M. J. Birnbaum, B. J. Stuck, and B. B. Kahn. 2004. AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. Nature 428:569-574.

Morimoto, R., F. Satoh, O. Murakami, K. Totsune, M. Saruta, T. Suzuki, H. Sasano, S. Ito, and K. Takahashi. 2008. Expression of peptide YY in human brain and pituitary tissues. Nutrition 24:878-884.

Morton, G. J., D. E. Cummings, D. G. Baskin, G. S. Barsh, and M. W. Schwartz. 2006. Central nervous system control of food intake and body weight. Nature 443:289-295.

Murakami, M., T. Matsuzaki, T. Iwasa, T. Yasui, M. Irahara, T. Osugi, and K. Tsutsui. 2008. Hypophysiotropic role of RFamide-related peptide-3 in the inhibition of LH secretion in female rats. J. Endocrinol. 199:105-112.

Nakazato, M., N. Murakami, Y. Date, M. Kojima, H. Matsuo, K. Kangawa, and S. Matsukura. 2001. A role for ghrelin in the central regulation of feeding. Nature 409:194-198.

Packer, L., K. Kraemer, and G. Rimbach. 2001. Molecular aspects of lipoic acid in the prevention of diabetes complications. Nutrition 17:888-895.

Playford, R. J., J. Domin, J. Beacham, K. B. Parmar, K. Tatemoto, S. R. Bloom, and J. Calam. 1990. Preliminary report: role of peptide YY in defence against diarrhoea. Lancet 335:1555-1557.

Raposinho, P. D., D. D. Pierroz, P. Broqua, R. B. White, T. Pedrazzini, and M. L. Aubert. 2001. Chronic administration of neuropeptide Y into the lateral ventricle of C57BL/6J male mice produces an obesity syndrome including hyperphagia, hyperleptinemia, insulin resistance, and hypogonadism. Mol. Cell Endocrinol.185:195-204.

Saito, E. S., H. Kaiya, T. Tachibana, S. Tomonaga, D. M. Denbow, K. Kangawa, and M. Furuse. 2005. Inhibitory effect of ghrelin on food intake is mediated by the corticotropin-releasing factor system in neonatal chicks. Regul. Pept. 125:201-208.

Saito, E. S., H. Kaiya, T. Takagi, I. Yamasaki, D. M. Denbow, K. Kangawa, and M. Furuse. 2002. Chicken ghrelin and growth hormone-releasing peptide-2 inhibit food intake of neonatal chicks. Eur. J. Pharmacol. 453:75-79.

Sajdyk, T. J., and D. R. Gehlert. 2000. Astressin, a corticotropin releasing factor antagonist, reverses the anxiogenic effects of urocortin when administered into the basolateral amygdala. Brain Res. 877:226-234.

Schick, R. R., J. P. Zimmermann, T. vorm Walde, and V. Schusdziarra. 2003. Peptides that regulate food intake: glucagon-like peptide 1-(7-36) amide acts at lateral and medial hypothalamic sites to suppress feeding in rats. Am. J. Physiol. Regul. Integr. Comp. Physiol. 284:R1427-1435.

Seoane, L. M., O. Al-Massadi, Y. Pazos, U. Pagotto, and F. F. Casanueva. 2006. Central obestatin administration does not modify either spontaneous or ghrelin-induced food intake in rats. J. Endocrinol. Invest. 29:RC13-15.

Shousha, S., K. Nakahara, T. Nasu, T. Sakamoto, and N. Murakami. 2007. Effect of glucagon-like peptide-1 and -2 on regulation of food intake, body temperature and locomotor activity in the Japanese quail. Neurosci Lett. 415:102-107.

Sibilia, V., E. Bresciani, N. Lattuada, D. Rapetti, V. Locatelli, V. De Luca, F. Dona, C. Netti, A. Torsello, and F. Guidobono. 2006. Intracerebroventricular acute and

chronic administration of obestatin minimally affect food intake but not weight gain in the rat. J. Endocrinol. Invest. 29:RC31-34.

Simpson, K. A., N. M. Martin, and S. R. Bloom. 2009. Hypothalamic regulation of food intake and clinical therapeutic applications. Arq. Bras. Endocrinol. Metabol. 53:120-128.

Sorensen, L. B., A. Flint, A. Raben, B. Hartmann, J. J. Holst, and A. Astrup. 2003. No effect of physiological concentrations of glucagon-like peptide-2 on appetite and energy intake in normal weight subjects. Int. J. Obes. Relat. Metab. Disord. 27:450-456.

Stanley, B. G., K. C. Anderson, M. H. Grayson, and S. F. Leibowitz. 1989. Repeated hypothalamic stimulation with neuropeptide Y increases daily carbohydrate and fat intake and body weight gain in female rats. Physiol. Behav. 46:173-177.

Tachibana, T., K. Hirofuji, M. Matsumoto, M. Furuse, S. Hasegawa, F. Yoshizawa, and K. Sugahara. 2004. The hypothalamus is involved in the anorexic effect of glucagon-like peptide-1 in chicks. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 137:183-188.

Tachibana, T., M. Sato, D. Oikawa, and M. Furuse. 2006. Involvement of CRF on the anorexic effect of GLP-1 in layer chicks. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 143:112-117.

Tachibana, T., M. Sato, H. Takahashi, K. Ukena, K. Tsutsui, and M. Furuse. 2005. Gonadotropin-inhibiting hormone stimulates feeding behavior in chicks. Brain Res. 1050:94-100.

Tang-Christensen, M., P. J. Larsen, J. Thulesen, J. Romer, and N. Vrang. 2000. The proglucagon-derived peptide, glucagon-like peptide-2, is a neurotransmitter involved in the regulation of food intake. Nat. Med. 6:802-807.

Taylor, I. L. 1985. Distribution and release of peptide YY in dog measured by specific radioimmunoassay. Gastroenterology 88:731-737.

Tu, Y., J. N. Thupari, E. K. Kim, M. L. Pinn, T. H. Moran, G. V. Ronnett, and F. P. Kuhajda. 2005. C75 alters central and peripheral gene expression to reduce food intake and increase energy expenditure. Endocrinology 146:486-493.

Turton, M. D., D. O'Shea, I. Gunn, S. A. Beak, C. M. Edwards, K. Meeran, S. J. Choi, G. M. Taylor, M. M. Heath, P. D. Lambert, J. P. Wilding, D. M. Smith, M. A. Ghatei, J. Herbert, and S. R. Bloom. 1996. A role for glucagon-like peptide-1 in the central regulation of feeding. Nature 379:69-72.

Vincent, M. F., F. Bontemps, and G. Van den Berghe. 1996. Substrate cycling between 5-amino-4-imidazolecarboxamide riboside and its monophosphate in isolated rat hepatocytes. Biochem. Pharmacol. 52:999-1006.

White, B. D., R. G. Dean, G. L. Edwards, and R. J. Martin. 1994. Type II corticosteroid receptor stimulation increases NPY gene expression in basomedial hypothalamus of rats. Am. J. Physiol. 266:R1523-1529.

Wortman, M. D., D. J. Clegg, D. D'Alessio, S. C. Woods, and R. J. Seeley. 2003. C75 inhibits food intake by increasing CNS glucose metabolism. Nat. Med. 9:483-485.

Zhang, J. V., P. G. Ren, O. Avsian-Kretchmer, C. W. Luo, R. Rauch, C. Klein, and A. J. Hsueh. 2005. Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. Science 310:996-999.

Zhang, R., T. Nakanishi, A. Ohgushi, R. Ando, T. Yoshimatsu, D. M. Denbow, and M. Furuse. 2001. Interaction of corticotropin-releasing factor and glucagon-like peptide-1 on behaviors in chicks. Eur. J. Pharmacol. 430:73-78.

CHAPTER 4

AICAR and Compound C Regulate Food Intake Independently of AMP-activated Protein Kinase in Lines of Chickens Selected for High or Low Body Weight

(Accepted)

ABSTRACT AMP-activated protein kinase (AMPK) functions to maintain cellular and body energy balance. Our aim was to investigate the effect of intracerebroventricular (ICV) administration of AMPK stimulator AICAR and AMPK inhibitor Compound C on food intake in lines of chickens that had undergone long-term selection from a common founder population for high (HWS) or low (LWS) body weight. AICAR caused a quadratic dose-dependent decrease in food intake in LWS but not HWS chicks. Compound C caused a quadratic dose-dependent increase in food intake in HWS but not in LWS chicks. Key aspects of the AMPK pathway, including upstream kinases mRNA expression, AMPK subunit α mRNA expression and phosphorylation, and a downstream target acetyl CoA carboxylase (ACC) phosphorylation were not affected by either AICAR or Compound C in either line. The exception was a significant inhibitory effect of AICAR on ACC phosphorylation ratio due to increased total ACC protein content without changing phosphorylated ACC protein levels. Thus, the anorexigenic effect of AICAR in LWS chicks and orexigenic effect of Compound C in HWS chicks resulted from activation or inhibition of other kinase pathways separate from AMPK. These results suggest genetic variation in feeding response for central AICAR and Compound C in chickens, which may contribute to the different body weights between the HWS and LWS lines.

Key words: AICAR, AMPK, chick, Compound C, food intake

4.1. INTRODUCTION

AMP-activated protein kinase (AMPK), known as a cellular "energy sensor", plays a central role in hypothalamic appetite regulation (Andersson et al., 2004; Kim et al., 2004; Minokoshi et al., 2004). AMPK functions to maintain cellular energy balance by turning off anabolic pathways and turning on the ATP-producing pathway when the AMP/ATP ratio increases (Viollet et al., 2006). Thus, AMPK is involved in the regulation of whole body energy homeostasis whose activity in hypothalamic neurons is altered by hormonal and nutrient signals that mediate the feeding response (Kahn et al., 2005; Lee et al., 2005).

Aminoimidazole-4-carboxamide riboside (AICAR), a commonly used AMPK activator, is an intermediate in the generation of inosine monophosphate, which can be easily taken up by cells and rapidly phosphorylated to 5-aminoimidazole-4-carboxamide-1-d-ribofuranosyl-5'-monophosphate (ZMP). ZMP mimics all the activating effects of AMP on AMPK, although it is 50-fold less potent than AMP at activating AMPK (Hardie, 2003). Once activated, AMPK reduces glucose production in the liver and increases fatty acid oxidation (Kahn et al., 2005). Its activity is inhibited by a widely used antagonist 6-[4-(2-piperidin-1-yl-ethoxy)-phenyl)]- 3-pyridin-4-yl-pyrazolo[1,5-a] pyrimidine, which is also known as Compound C. Compound C has been shown to prevent AICAR-induced phosphorylation of AMPK and block the antilipolytic effect of AICAR on adipocytes in rats (Anthony et al., 2009). Moreover, the phosphorylation effect of AICAR and

metformin on acetyl CoA carboxylase (ACC), a direct downstream target of AMPK, is also inhibited by Compound C (King et al., 2006).

Hypothalamic AMPK activity contributes to body energy homeostasis through regulation of food intake and energy expenditure. AICAR causes AMPK activation which stimulates food intake and long-term body weight gain (Andersson et al., 2004; Florant et al., 2010), while Compound C caused AMPK suppression inhibits food intake and long-term body weight gain (Kim et al., 2004; Hayes et al., 2009). Glucose, leptin and insulin, each inhibit AMPK activity in the hypothalamus and decrease food intake in mice (Minokoshi et al., 2004; Gao et al., 2007; Lane and Cha, 2009), whereas ghrelin and adiponectin stimulate the activity of AMPK in the hypothalamus and increase food intake in rats (Kola et al., 2005; Kubota et al., 2007). Clearly, feeding-inhibiting factors appear to decrease hypothalamic AMPK activity and feeding-stimulating factors increase it. A better understanding of how the AMPK pathway is involved in food intake and body weight gain may contribute to pharmacologically control of appetite.

A functional AMPK pathway also exists in chickens with characteristics similar to the corresponding pathway in mammals (Proszkowiec-Weglarz et al., 2006). As in mammals, AMPK plays an important role in food intake regulation and energy balance. In 3 week-old broiler chickens, fasting significantly changed mRNA expression of lipogenic genes that were accompanied by upregulation of AMPK α phosphorylation levels (Proszkowiec-Weglarz et al., 2009). In chicken embryos, heat production, glucose utilization, and liver and skeletal muscle mitochondrial capacity were increased by chronic injection of AICAR (Walter et al., 2010). Selection for body fatness in chickens

regulated muscle glycogen and breast meat quality by altering AMPK activity (Sibut et al., 2008). Recent studies in our lab also demonstrated that long-term selection for body weight in broiler chickens has changed the feeding response to central ghrelin, and an altered hypothalamic AMPK system may contribute to this differentiation (Xu et al., 2011).

In the modern poultry industry, chickens are intensively selected for either egg or meat production, resulting in changes in body weight and composition. A better understanding of how genetic selection for body weight has altered the mechanisms controlling food intake is necessary to devise strategies to both increase and decrease food intake. The animal model used in the present experiment involved lines of chickens that had undergone 50 generations of divergent selection from a common White Rock base population for high (HWS) or low (LWS) body weight at 56 days of age (Marquez et al., 2010). The base population was formed by crossing seven moderately inbred lines of White Plymouth Rocks and the selected lines have been maintained as pedigreed populations (Dunnington and Siegel, 1996; Siegel and Wolford, 2003; Marquez et al., 2010). These lines of chickens differ both at the phenotypic and genotypic levels (Johansson et al., 2010; Marquez et al., 2010) with a difference in body weights at selection age of more than 10-fold. By using these lines, it has been reported that in mature HWS and LWS chickens, AICAR caused a linear decrease of food intake in LWS while having no effect in HWS chickens. Compound C increased food intake in HWS with the effect being significant with the 3μg dose and linearly significant at 3, 6 and 12 μg doses in LWS chickens (Xu et al., 2009). The aim of our study was to compare food intake of 5-day-old HWS and LWS chicks in response to both an AMPK activator

(AICAR) and inhibitor (Compound C). The objective was to determine if selection for differences in body weight, a polygenic trait, altered the brain's feeding response to AICAR and Compound C by changing the AMPK pathway.

4.2. MATERIALS AND METHODS

4.2.1. Animals

Eggs from same-age parents from each line were incubated in the same machine for each experiment. After hatch, chicks were placed as flocks in electric heated batteries with raised wire floors for 2 days, and then transferred to individual cages in a room at 32±2°C. A mash diet (20% crude protein, 2684 kcal ME/kg) and water were provided *ad libitum*. The individual cages were designed to allow visual and auditory but not physical contact with other chicks. All trials were conducted at 5 days post-hatching, which allowed time for chicks to adjust to this environment and for yolk absorption. Data were collected from both lines simultaneously and injections followed the sequence: HWS, LWS, HWS, and so forth. Experimental procedures were approved by the Virginia Tech Animal Care and Use Committee.

4.2.2. Peptides and ICV Injection

AICAR (Calbiochem, La Jolla, CA, USA) and Compound C (Sigma, St.Louis, MO, USA) were respectively dissolved in artificial cerebrospinal fluid (aCSF) or dimethyl sulfoxide (DMSO) with 0.1% Evans Blue dye to facilitate the localization of injection site. The ICV injection of 5 μL of each substance was made using a microsyringe according to the method of Davis et al (1979). The procedure does not induce

physiological stress (Furuse et al., 1999). At the end of each experiment, chicks were sacrificed by cervical dislocation and then decapitated. The injection site verified by frontal plane section. Data from individuals without dye present in the lateral ventricle were deleted. Sex was determined visually by dissection. Numbers of chicks used in analyses are provided in the legend of each figure.

4.2.3. Experiment 1: Food and water intake

Forty-eight 5-day-old chicks from each line were fasted for 180 min, then weighed (average weight HWS=50.6±1.03g and LWS=24.0±0.42g) and randomly assigned to receive either 0 (aCSF only), 75, 150, and 300 μg AICAR by ICV injection. In a separate trial, chicks were subjected to the same procedure but received 0 (DMSO only), 3, 6, and 12 μg Compound C by ICV injection. After injection, chicks were returned to their cages and given free access to food and water. Cumulative intake was recorded every 30 min through 180 min post-injection by weighing (± 0.01g) individual food and water containers. Water and food intake were expressed as percent body weight to adjust the differences in body weight between HWS and LWS chicks. Data was analyzed at each time period by analysis of variance (ANOVA) using the general linear modeling procedure (GLM, SAS Institute Inc, Cary, NC, USA). Effect of sex and interaction of sex with other variables were first tested and none was significant. Thus the model was reduced to include line, dose and the line by dose interaction. When interaction was significant, multiple comparisons among doses were made within each line and Tukey's multiple range test was used. Treatment effects were partitioned into linear and quadratic

contrasts to determine the dose-response relationships. Significance was considered at $P<0.05$.

4.2.4. Experiment 2: Expression of Genes Encoding AMPK Subunits and Associated Proteins in Hypothalamus

Twelve male chicks from each line were fasted 180 min, then weighed and randomly assigned to receive either 0 or 150 μg AICAR by ICV injection. A separate trial followed the same procedure with chicks given 0 or 6 μg Compound C by ICV injection. After injection, chicks were given free access to both food and water to mimic the conditions in Experiment 1. The food intake data were analyzed as soon as it was collected. Two hours after injection, the treatment effects were significant for both AICAR and Compound C. So at this time, chicks were decapitated and the preoptic-hypothalamic area was dissected from each brain using the landmarks of the optic chiasm rostrally, and the mammillary bodies caudally. Dissected hypothalamic tissues were quickly frozen in liquid nitrogen, and stored at -80 °C for later analysis.

Preoptic-hypothalamic tissues were homogenized in Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and total RNA extracted according to the manufacturer's instructions. Total RNA concentration was then quantified by measuring the absorbance at 260 nm. Fifty μg of total RNA was treated with DNase-I (Promega, Madison, WI, USA) to eliminate contaminating genomic DNA. Purified DNA-free RNA was dissolved in RNase-free water and immediately used as templates in reverse transcription. Two μg of total RNA were incubated at 42°C for 1 h in a 25 μL mixture containing 200 Units Moloney Murine Leukemia Virus reverse transcriptase (M-MLV)

(Promega, Madison, WI, USA), 1×M-MLV reaction buffer (Promega, Madison, WI, USA), 25 Units RNase inhibitor (Promega, Madison, WI, USA), 4 μg Oligo dT primer (Eurofins MWG Operon, Huntsville, AL, USA) and 0.5 mM dNTP (Promega, Madison, WI, USA). The reaction was terminated by heating at 95°C for 5 min and quickly cooling on ice. The cDNA (RT products) were aliquoted and stored at - 20°C.

Real-time RT-PCR was performed in 7500 Fast Real-Time PCR System (Applied Biosystems, Inc., Carlsbad, CA, USA). One μL of 4-fold diluted RT product was used for PCR in a final volume of 20 μL containing 10 μL 2×Fast SYBR Green Master Mix (Applied Biosystems, Inc.) and 0.5 μM primer mix (Table 4.1). Tested genes include AMP-activated kinase alpha 1 subunit (AMPK α1), AMP-activated kinase alpha 2 subunit (AMPK α2), AMP-activated kinase beta 1 subunit (AMPK β1), AMP-activated kinase beta 2 subunit (AMPK β2), AMP-activated kinase gamma 1 subunit (AMPK γ1), AMP-activated kinase gamma 2 subunit (AMPK γ2), AMP-activated kinase gamma 3 subunit (AMPK γ3), acetyl-Coenzyme A carboxylase alpha (ACC α), calcium/calmodulin-dependent protein kinase kinase 1 (CAMKK α), calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK β), fatty acid synthase (FAS), Serine/threonine-protein kinase 11 (LKB 1), Calcium-binding protein 39 (MO25 α), calcium binding protein 39-like (MO25 β) and STE20-related kinase adaptor alpha (STRAD α). Chicken glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as a reference gene for normalization purposes. The expression of GAPDH mRNA was similar for both lines. Thermal cycling conditions were: initial denaturation (20 s at 95°C), then a two-step amplification program (3 s at 95°C, 30 s at 60°C) repeated 40 times. Real-time RT-PCR data were analyzed by the relative quantification ($\Delta\Delta C_T$)

method (Livak and Schmittgen, 2001). Data were analyzed by ANOVA using GLM (SAS Institute Inc, Cary, NC, USA). The model included line, treatment (trt) and the line by trt interaction. Treatment effect was also analyzed in each line using t-test for independent samples. All data were expressed as mean ± S.E.M. and the level of significance was set a priori at $P<0.05$. All mRNA expression levels were expressed as fold-change compared to the LWS aCSF group.

Table 4. 1. Nucleotide sequences of specific primers.

Target gene	GenBank accession	PCR products (bp)	Primer sequences
[1]AMPK α1	**NM_001039603**	125	F: 5'-ATCTGTCTCGCCCTCATCCT-3'
			R: 5'-CCACTTCGCTCTTCTTACACCTT-3'
[2]AMPK α2	**NM_001039605**	128	F: 5'-CCAGCGAGTTCTACCTAGCCT-3'
			R: 5'-TGCCTTGGGACTGTCTGCTA-3'
[3]AMPK β1	**NM_001039912**	113	F: 5'-CCAGGAGCCCTATGTCTGTAAG-3'
			R: 5'-CAGGATCGCAAGAAATGCC-3'
[4]AMPK β2	**NM_001044662**	125	F: 5'-GCACTGCCCATCCCTCTAA-3'
			R: 5'-CGGCTGCCACGAAACAA-3'
[5]AMPK γ1	**NM_001034827**	118	F: 5'-AGAGGTCCCAAAGCCTGAGTT-3'
			R: 5'-GAAGATGCCCAGAGCCACA-3'
[6]AMPK γ2	**NM_001030965**	106	F: 5'-CACAAGCCCTACAGCACCG-3'
			R: 5'-TGAACCTCAGCCTTCACTATCCTA-3'
[7]AMPK γ3	**NM_001031258**	91	F: 5'-CCACGCTTCCTAAAGAAAACAG-3'
			R: 5'-CCGGCAGCATTAACAACG-3'
[8]ACC α	**NM_205505**	125	F: 5'-CCGAGAACCCAAAACTACCAG-3'
			R: 5'-GCCAGCAGTCTGAGCCACTA-3'
[9]CAMKK α	**XM_001234324**	97	F: 5'-CAGTTTGAAGGGAACGATGCC-3'
			R: 5'-CACTGAAACTTTTGCCCGTGT-3'
[10]CAMKK β	**XM_415134**	144	F: 5'-AGGACCAGGCTCGGTTCTAC-3'
			R: 5'-TGACACCAAAGTCGGCAATT-3'
[11]FAS	**NM_205155**	117	F: 5'-AAAGCAATTCGTCACGGACA-3'
			R: 5'-GGCACCATCAGGACTAAGCA-3'
[12]LKB 1	**XM_418227**	114	F: 5'-GCTGACCACCAATGGGACG-3'
			R: 5'-GGCTGGAATGCTGGCGAC-3'
[13]MO25 α	**CR391562**	93	F: 5'-CCCTAACAAGACGCAGCCTA-3'
			R: 5'-TCGGTCCTGTCATTCTGAAACT-3'

[14]MO25 β	**NM_001006272**	117	F: 5'-AAGAGGGTAACAGTTGCTCCG-3'
			R: 5'-CTTTCACTTATCTCCCACGTAATG-3'
[15]STRAD α	**NM_001012844**	149	F: 5'-TTTCCAAGCTCTTCAACCACC-3'
			R: 5'-CAATTCACTCATCCCGTCCATA-3'
[16]GAPDH	**NM_204305**	141	F: 5'-TGGCATCCAAGGAGTGAGC-3'
			R: 5'-GGGGAGACAGAAGGGAACAG-3'

[1]AMPK α1 (AMP -activate kinase alpha 1 subunit), [2]AMPK α2 (AMP -activate kinase alpha 2 subunit), [3]AMPK β1 (AMP -activate kinase beta 1 subunit), [4]AMPK β2 (AMP -activate kinase beta 2 subunit), [5]AMPK γ1 (AMP -activate kinase gamma 1 subunit), [6]AMPK γ2 (AMP -activate kinase gamma 2 subunit), [7]AMPK γ3 (AMP -activate kinase gamma 3 subunit), [8]ACC α (acetyl-Coenzyme A carboxylase alpha), [9]CAMKK α (calcium/calmodulin-dependent protein kinase kinase 1), [10]CAMKK β (calcium/calmodulin-dependent protein kinase kinase 2), [11]FAS (fatty acid synthase), [12]LKB 1 (Serine/threonine-protein kinase 11), [13]MO25 α (Calcium-binding protein 39), [14]MO25 β (calcium binding protein 39-like), [15]STRAD α (STE20-related kinase adaptor alpha), [16]GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

4.2.5. Experiment 3: Expression of pAMPK and pACC Proteins in Hypothalamus

In Experiment 3, 12 male chicks from each line received the same treatment as Experiment 2. Two hours after injection, they were decapitated and the preoptic-hypothalamic area dissected from each brain. Proteins were extracted with SDS lysis buffer (25 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol and 5% β-mercaptoethanol). Protein extracts were separated on a 8.5% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Blots were blocked in 5% bovine serum albumin (BSA) dissolved in Tris-buffered saline with Tween-20 (TBST, 20 mmole Tris, pH 7.4, 150 mmole NaCl, and 0.1% Tween-20) for 180 min at 4°C and then briefly washed two times for 5 min each in TBST. The blots were incubated overnight at 4°C with primary antibodies against phosphor-AMPK α (α1 and α2, Thr^{172}) (1:500), phosphor-ACC (Ser^{79}) (1:250), ACC (1:250), AMPK α (1:1000) and β-actin (1:1000) in TBST with 1% BSA. All antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Each blot was washed twice for 5 min, three times for 10 min and then incubated with anti-rabbit IgG horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at room temperature for 60 min. Finally, the blots were

washed and detected by enhanced chemiluminescence (ECL). A chemiluminescent substrate, SuperSignal West Pico (Pierce, Rockford, IL, USA), was applied according to instructions of the manufacturer and the signal was detected using a Gel Doc XR System (Bio-Rad, Hercules, CA, USA). Densitometry analysis was performed using Quantity One (Bio-Rad, Hercules, CA, USA) image analysis software. Phosphate and total protein expression levels were expressed as a density ratio (pAMPK/β-actin, AMPK/β-actin, pACC/β-actin, ACC/β-actin, pAMPK/AMPK, pACC/ACC). The statistical model was the same as that for Experiment 2.

4.3. RESULTS

4.3.1. Experiment 1: Food and water intake

There was a significant line by AICAR dose interaction for food intake. When the effect of AICAR dose on food intake was analyzed separately for each line, there was no effect in HWS chicks (Fig. 4.1A) while for LWS chicks there was a quadratic dose-dependent decrease at 120 min postinjection and thereafter (Fig. 4.1B). The 150 μg level of AICAR had a significant suppression effect on food intake by 150 min postinjection; chicks treated with this dose of AICAR consumed very little food after the initial food intake period before 30 min post-injection. Water intake was not affected by ICV injection of AICAR in either line (data not shown).

The interaction between line and Compound C dose was significant. Thus, as with AICAR, the Compound C dose effect on food intake was analyzed separately within each line. Compound C had a quadratic dose-dependent increase in food intake in HWS chicks

by 90 min postinjection (Fig. 4.1C) with the 6 µg level significantly stimulating food intake by 150 min postinjection. Other doses of Compound C had an attenuated orexigenic effect. For LWS chicks, ICV injection of Compound C did not affect food intake (Fig. 4.1D). Water intake was not affected by ICV injection of Compound C in either line (data not shown).

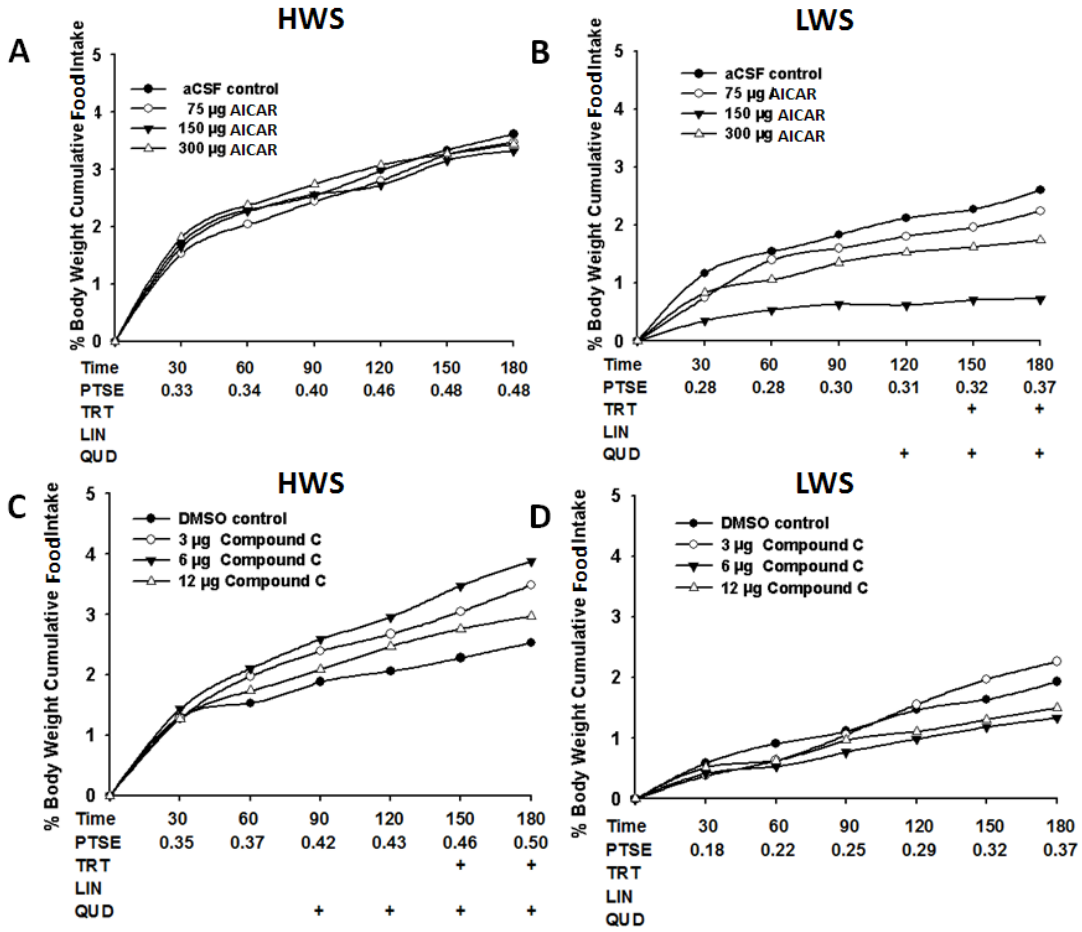

Fig. 4. 1. Cumulative food intake expressed as percent body weight of 5-day HWS and LWS lines of chicks following ICV injection of aminoimidazole-4-carboxamide riboside (AICAR) or 6-[4-(2-piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrazolo[1,5-a] pyrimidine (Compound C) (n=12 chicks/group). (A) HWS chicks injected with AICAR;

(B) LWS chicks injected with AICAR; (C) HWS chicks injected with Compound C; (D) LWS chicks injected with Compound C. LIN, linear contrast; PTSE, pooled standard error; TRT, treatment effect; QUD, quadratic contrast; +, $P<0.05$. Experiment 1.

4.3.2. Experiment 2: Expression of Genes Encoding AMPK subunits and Associated Proteins in Hypothalamus

The most effective doses of AICAR 150 μg and Compound C 6 μg were ICV injected in 5-day-old HWS and LWS chicks (Fig. 4.2). AICAR decreased food intake in LWS but not HWS chicks at 120 min. On the contrary, Compound C increased food intake in HWS but not in LWS chicks at 120 min. Thus all brains were collected at 120 min after injection for later mRNA and protein analysis.

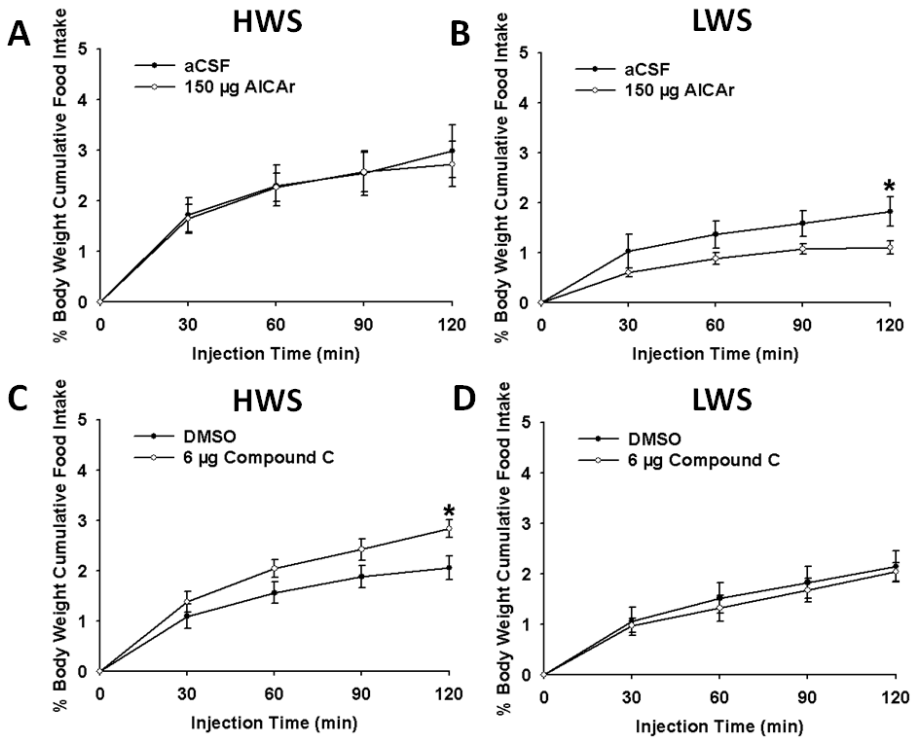

Fig. 4. 2. Cumulative food intake expressed as percent body weight of 5-day HWS and LWS lines of chicks following ICV injection of 150 µg AICAR or 6 µg Compound C (n=12 chicks/group). (A) HWS chicks injected with AICAR; (B) LWS chicks injected with AICAR; (C) HWS chicks injected with Compound C; (D) LWS chicks injected with Compound C. *, *P<0.05.*

The effects of 150 µg AICAR ICV injection on AMPK subunits mRNA expression in the preoptic-hypothalamus of 5-day-old HWS and LWS chicks are illustrated in Fig. 4.3. Significant line and line×trt effects for AMPK β1 mRNA expression existed. AICAR significantly increased the hypothalamic AMPK β1 subunit mRNA level in HWS, but not LWS chicks. Additionally, AICAR had a significant stimulatory effect on hypothalamic AMPK γ1 and γ3 subunit mRNA levels in LWS, but not HWS chicks. The trt effect was significant for AMPK γ1. Expressions of other subunits including α1, α2, β2 and γ2 were

not influenced by AICAR. AICAR ICV injection caused an effective increase in hypothalamic STRAD α mRNA levels in both lines with a significant trt effect (Fig. 4.4). However, the mRNA expressions of other upstream factors in AMPK pathway, including LKB1, MO 25α, MO 25β, CAMKK α and CAMKK β, were not affected by AICAR ICV injection in either line. Moreover, the mRNA levels of AMPK downstream factors i.e. ACC α and FAS did not change after AICAR ICV injection in either line.

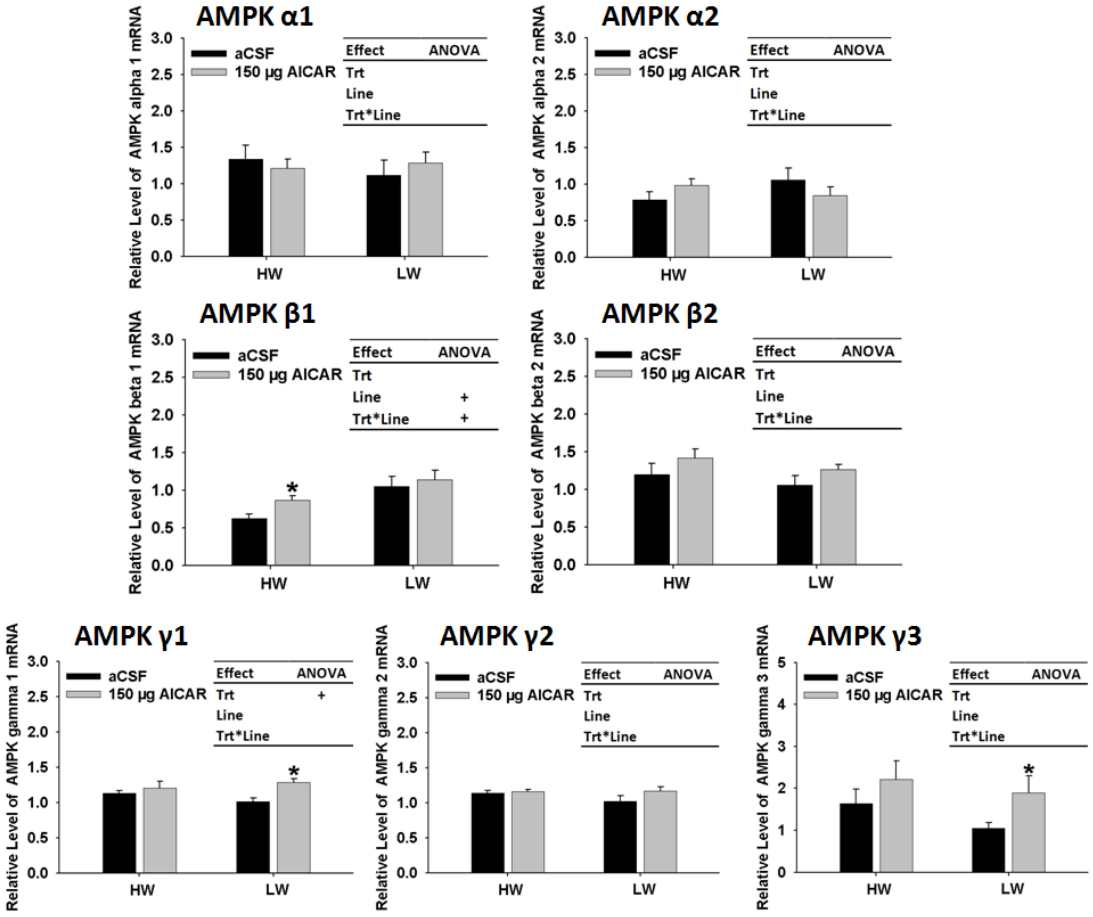

Fig. 4. 3. Gene expression of hypothalamic AMPK subunits in 5-day HWS and LWS lines of chicks following ICV injection of 150 µg aminoimidazole-4-carboxamide riboside (AICAR). The data expressed as fold-change compared to the LWS aCSF group,

represent mean ± SEM (n=6 chicks/group). Asterisk sign (*) denotes significance (P<0.05) in t-test for independent samples and plus sign (+) denotes significance (P<0.05) in ANOVA test.

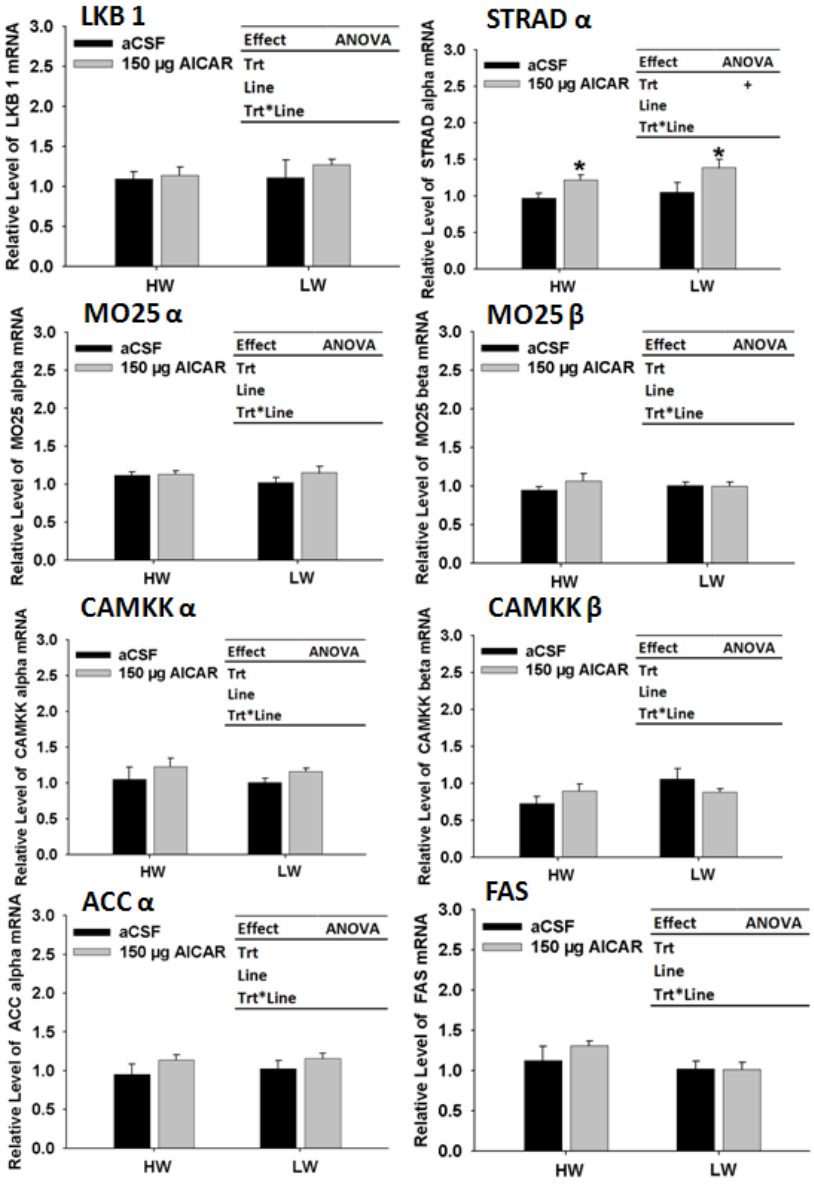

Fig. 4. 4. Gene expression of hypothalamic LKB1/STRAD/MO 25 subunits, CAMKK subunits, ACC α and FAS in 5-day HWS and LWS lines of chicks following ICV injection of 150 µg aminoimidazole-4-carboxamide riboside (AICAR). The data

expressed as fold-change compared to the LWS aCSF group, represent mean ± SEM (n=6 chicks/group). Asterisk sign (*) denotes significance (P<0.05) in t-test for independent samples and plus sign (+) denotes significance (P<0.05) in ANOVA test.

Compound C had no effect on mRNA expressions of any of the genes measured in either line with the exception of a significant inhibitory effect for AMPK γ2 subunit (Fig. 4.5 and Fig. 4.6) in HWS chicks. Significant line and line×trt effects for AMPK γ2 were demonstrated, and the line effect was significant for FAS.

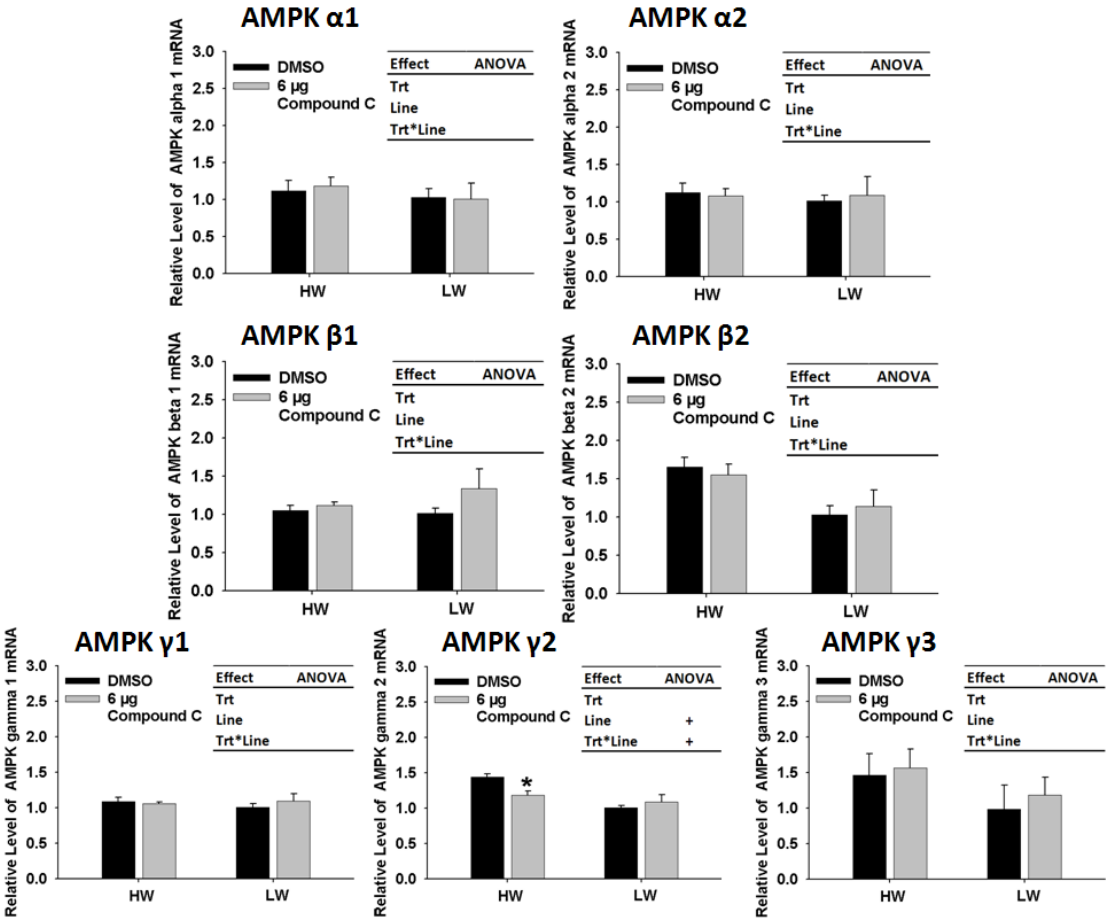

Fig. 4. 5. Gene expression of hypothalamic AMPK subunits in 5-day HWS and LWS lines of chicks following ICV injection of 6 µg 6-[4-(2-piperidin-1-yl-ethoxy)-phenyl)]-

3-pyridin-4-yl-pyrazolo[1,5-a] pyrimidine (Compound C). The data expressed as fold-change compared to the LWS aCSF group, represent mean ± SEM (n=6 chicks/group). Asterisk sign (*) denotes significance (P<0.05) in t-test for independent samples and plus sign (+) denotes significance (P<0.05) in ANOVA test.

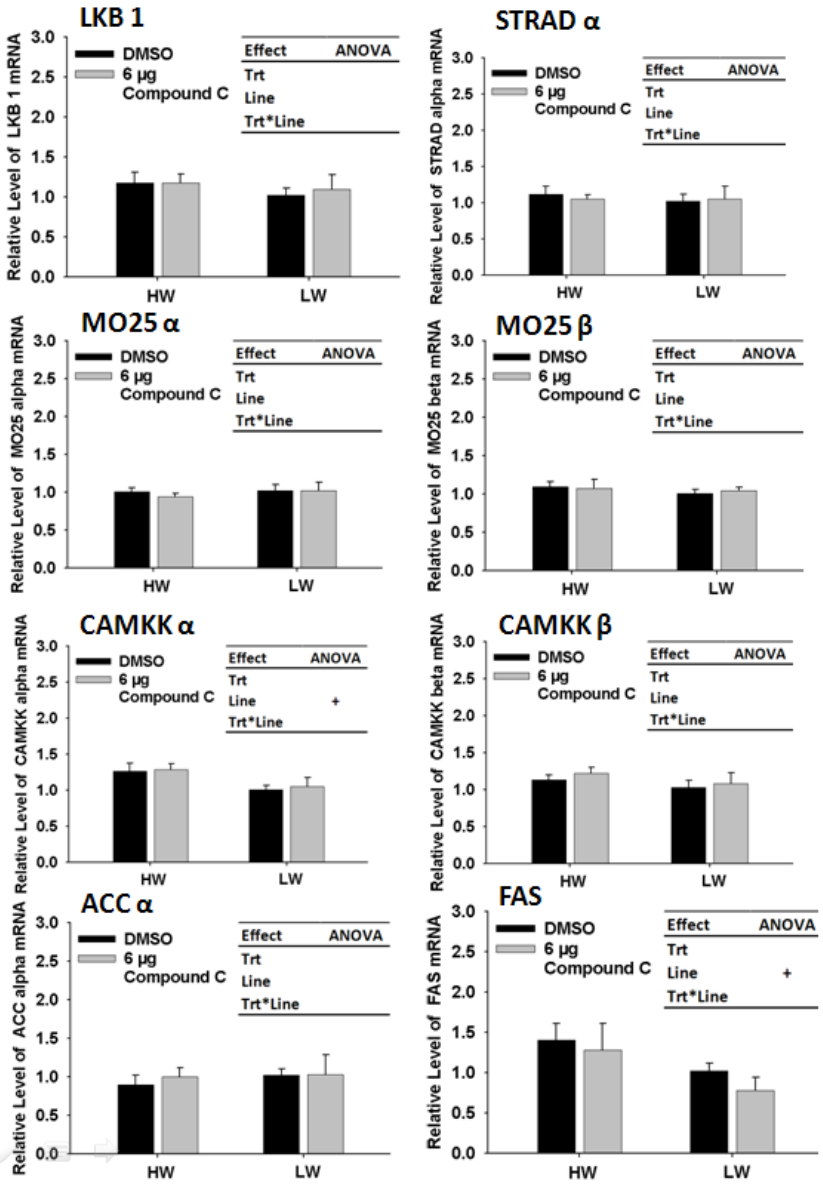

Fig. 4. 6. Gene expression of hypothalamic LKB1/STRAD/MO 25 subunits, CAMKK subunits, ACC α and FAS in 5-day HWS and LWS lines of chicks following ICV

injection of 6 μg (6-[4-(2-piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrazolo[1,5-a] pyrimidine (Compound C). The data expressed as fold-change compared to the LWS aCSF group, represent mean ± SEM (n=6 chicks/group). Asterisk sign (*) denotes significance (P<0.05) in t-test for independent samples and plus sign (+) denotes significance (P<0.05) in ANOVA test.

4.3.3. Experiment 3: Expression of pAMPK and pACC Proteins in Hypothalamus

AMPK activity was monitored by measuring phosphorylation ratios of AMPK α and ACC in the hypothalamus (Fig. 4.7). The phosphorylation ratio of hypothalamic AMPK α was not affected by ICV injection of AICAR in either line (Fig. 4.7A), which was in contrast to significantly lower levels of phosphorylated ACC ratio in the hypothalamus than in the aCSF control chicks in both lines (Fig. 4.7B). ANOVA test also showed significant trt and line effects for ACC phosphorylation ratio. Additionally, total ACC (ACC/ β-actin) was significantly upregulated by AICAR with trt effect significant in both lines (Fig. 4.7C). For Compound C, there was no significant trt or line×trt interaction effect. The ICV injection of Compound C did not affect either the phosphorylation ratios of hypothalamic AMPK and ACC or total ACC in either line (Fig. 4.7D, 4.7E and 4.7F). The protein levels of pAMPK (pAMPK/β-acin), AMPK (AMPK/β-acin) or pACC (pACC/β-acin) were not altered by either AICAR or Compound C in either lines (data not shown).

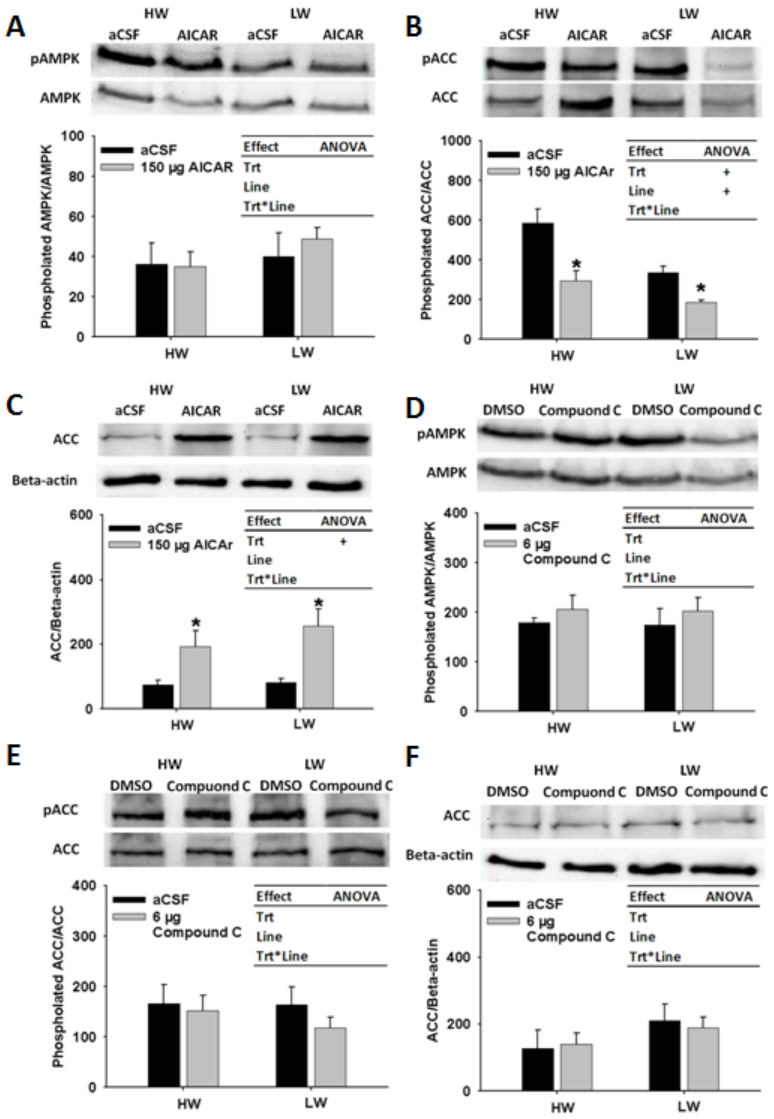

Fig. 4. 7. Effects of ICV injection of aminoimidazole-4-carboxamide riboside (AICAR) or (6-[4-(2-piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrazolo[1,5-a] pyrimidine (Compound C) on phosphorylation ratio of hypothalamic AMPK α or ACC and total ACC in 5-day HWS and LWS lines of chicks. Representative Western blots of phosphorylated AMPK α (pAMPK α) and total AMPK α (AMPK α) or phosphorylated ACC (pACC) and total ACC (ACC) as well as total ACC and beta-actin in both lines of chicks are shown. The average levels of pAMPK, pACC and total ACC in the

hypothalamus after a single ICV injection of AICAR or Compound C were expressed as pAMPK/AMPK, pACC/ACC and ACC/β-actin, respectively. (A) Phosphorylation ratio of AMPK α after injection of AICAR; (B) Phosphorylation ratio of ACC after injection of AICAR; (C) Total ACC after injection of AICAR; (D) Phosphorylation ratio of AMPK α after injection of Compound C; (E) Phosphorylation ratio of ACC after injection of Compound C; (F) Total ACC after injection of Compound C. The data expressed as a percent of the control group, represent the mean ± SEM (n=6 chicks/group). Asterisk sign (*) denotes significance (P<0.05) in t-test for independent samples and plus sign (+) denotes significance (P<0.05) in ANOVA test.

4.4. DISCUSSION

4.4.1. AICAR

AICAR is a most commonly used AMPK activator in a number of cellular systems (Pold et al., 2005; Fan et al., 2009; Thomson et al., 2009), and its effects on lipid and glucose metabolism have relevancy in appetite and metabolism regulation and in diabetes. In mammalian species, AICAR stimulates food intake and body weight gains, presumably by activating AMPK (Andersson et al., 2004; Hu et al., 2005; Florant, et al., 2010). Here, we demonstrate that while AICAR also plays a role in food intake regulation in chicks, it is line dependent. ICV injection of AICAR caused a quadratic dose-dependent decrease in food intake in LWS but not in HWS chicks. The differential response of these weight selected lines to ICV injection of AICAR was consistent with that observed for mature HWS and LWS chickens (Xu et al., 2009). Opposed to the orexigenic effect in mammals, AICAR significantly decreased food intake in LWS chicks, suggesting a dose-dependent

anorexic response while HWS chicks did not respond to any of the doses of AICAR investigated. Thus, long-term selection for high or low body weight from a common base population has altered the brain's response to AICAR and these comparisons imply a modified AICAR downstream pathway facilitating anorexigenic effect of AICAR.

In mammalian species, AICAR's orexigenic effect is mediated by activating the hypothalamic AMPK system, which then stimulates fatty acid oxidation and inhibits fatty acid synthesis to increase food intake (Andersson et al., 2004; Hu et al., 2005; Florant et al., 2010). In avian species, there is still no evidence for central AICAR effect on food intake. Our study demonstrated line specific feeding response to central AICAR may involve hypothalamic AMPK system. When different AMPK subunit gene expression and α subunit threonine 172 phosphorylation were tested to determine if the anorexigenic effect of AICAR was mediated by the AMPK system, we found that ICV injection of AICAR increased hypothalamic AMPK β1 subunit mRNA level in HWS chicks, whereas AICAR stimulated hypothalamic AMPK γ1 and γ3 subunits mRNA expression in LWS chicks. However, neither AMPK α1 and α2 mRNA expressions nor AMPK α phosphrylation was affected by AICAR in either line. So although selection for body weight has altered the hypothalamic response to AICAR, this feeding response differentiation may not involve the AMPK system.

AMPK is a heterotrimer comprising a catalytic α subunit with associated β and γ subunits. The γ subunits contain particular cystathionine beta synthase (CBS) domains that bind the nucleotides AMP and ATP to sensitively detect shifts in the AMP/ATP ratio (Adams et al., 2004). Binding of AMP to the γ subunit exposes the catalytic domain

found on the α subunit and promotes net phosphorylation at threonine-172 in the activation loop, causing more than 100-fold activation (Hawley et al., 1996). The β subunits provide scaffolds for binding α and γ subunits together to form a functional αβγ complex that is regulated by AMP (Hudson et al., 2003). The cooperation of these different subunits leads to the phosphorylation of AMPK, which correlates with AMPK activity. In our lines, the ICV injection of AICAR caused different stimulatory effects on mRNA expression of AMPK regulatory subunits β and γ, but not on mRNA expression or phosphrylation of catalytic subunit α. It suggests that AICAR may inhibit food intake via a mechanism independent of AMPK activity. Because regulatory subunits β and γ are capable of leading to catalytic activity only when complexed with the α subunits, the availability of the catalytic subunits is a key determinant of AMPK activity.

To further clarify the effect of ICV injection of AICAR on the AMPK system in HWS and LWS chicks, we evaluated the mRNA expressions of AMPK upstream AMPK kinase (AMPKK), a complex of three proteins, LKB1, STRAD and MO25. LKB1 mediates AMPK activation in response to an increase in the AMP/ATP ratio, and requires binding of MO25 and STRAD for activity. The LKB1-STRAD-MO25 complex represents the biologically active unit to phosphorylate and activate AMPK (Hawley et al., 2003; Woods et al., 2003). Our results demonstrate that although AICAR caused an effective increase in hypothalamic STRAD α expression in both lines, expressions of LKB1, MO 25α and MO 25β were not affected in either line. Regulatory subunit STRAD α modifies AMPK activity by stimulating the kinase activity of LKB1, which is a key regulator of the AMPK pathway. Thus, the upregulation of STRAD α may not contribute to activation of AMPK without association of LKB1. ICV injection of AICAR may not activate the

AMPK pathway by only stimulating STRAD α in chicks. In addition to the LKB1 complex, another possible physiological activator of AMPK is CAMKK, especially the CAMKK β isoform. CAMKK β phosphorylates and activates AMPK in response to increased intracellular Ca^{2+} (Hawley et al., 2005; Woods et al., 2005; Carling et al., 2008). In our study, the mRNA expressions of CAMKK α and β were not affected by ICV injection of AICAR, providing additional evidence that AICAR decreases food intake via a mechanism independent of AMPK activity.

Activation of AMPK can divert metabolism of fatty acid synthesis towards oxidation and promote a lipolytic response in mammals (Gaidhu et al., 2009). Essential bioactive enzymes in fatty acid metabolism, including malonyl-CoA, ACC and FAS, are involved in this AMPK downstream regulation (Merrill et al., 1997; Foretz et al., 1998). When we investigated whether the anorexigenic effect of AICAR was dependent on regulating AMPK downstream factors ACC and FAS, we found that although AICAR ICV injection did not change the mRNA expression of ACC α and FAS, it significantly increased total ACC without changing pACC protein levels, causing downregulation of the ACC phosphorylation ratio. ACC is a key regulator for the metabolism of fatty acids, and its activity is inhibited by phosporylation (Munday et al., 1988). Decreased phosphorylation leads to activation of ACC that catalyzes the production of malonyl-CoA, which is considered an inhibitor of feeding. Thus hypothalamic ACC phosphorylation downregualtion caused by increased total ACC has a role in regulation of food intake in AICAR-treated chicks. There may be other neuropathways involved in AICAR appetite regulation than AMPK triggered ACC phosphorylation which contributed to the

differences between HWS and LWS chicks in their feeding response to AICAR ICV injection.

4.4.2. Compound C

Compound C, a commonly used inhibitor of AMPK, can block the stimulation effect of AICAR on AMPK activity and cause inhibition of food intake and body weight gain in mammals (Kim et al., 2004; Hayes et al., 2009). In our study, Compound C caused a quadratic dose-dependent increase in food intake in HWS chicks but had no effect in LWS chicks. The orexigenic effect of Compound C in HWS chicks was consistent with our previous results (Xu et al., 2009), which showed that Compound C increased food intake in both mature HWS and LWS chickens, suggesting that there is not only a line but also an age difference in hypothalamic response to Compound C.

To determine whether the line difference in hypothalamic response to Compound C was associated with an altered AMPK pathway, we tested AMPK and related gene expression and phosphorylation ratio of AMPK α and ACC in Compound C treated chicks in both lines. Compound C did not influence the expression of any of these genes or the phosphorylation ratio of AMPK α and ACC, except for a significant inhibitory effect on AMPK γ2 subunit mRNA expression in HWS chicks. These data suggest that the AMPK pathway was not altered by Compound C, because key aspects of the AMPK pathway i.e. upstream kinases expression, AMPK α expression and phosphorylation ratio, and a downstream target ACC phosphorylation ratio were not affected by Compound C. The orexigenic effect of Compound C in HWS chicks was not mediated by the AMPK

pathway, and may be due to the activation or inhibition of the other pathways or other biochemical mechanisms separate from AMPK.

In summary, ICV injections of AICAR and Compound C had different food intake regulation effects in two lines of chickens that had undergone long-term divergent selection for high or low body weight. AICAR decreased food intake in chicks from the LWS but not HWS line. In contrast, Compound C increased food intake in HWS but not LWS chicks. Compared to the opposite food regulation effect of AICAR and Compound C in mammals, the anorexigenic effect of AICAR and orexigenic effect of Compound C in chicks were not caused by the alteration of the AMPK system and needs further clarification. Although it is well known that AICAR is widely considered as an agonist for AMPK, its physiological effects are not exclusively AMPK-dependent. For example, AICAR administration in neural stem cells in culture induced astroglial differentiation via activating the JAK/STAT3 pathway independently of AMPK (Zang et al., 2008). Similarly, Compound C has been reported to stimulate ceramide production in MCF7 breast carcinoma cells without inhibiting AMPK (Jin et al., 2009). Therefore, it is possible that AICAR and Compound C regulate food intake in chicks by activating or inhibiting other kinase pathways or other biochemical mechanisms separate from AMPK. These results suggest a genetic variation in feeding response for central AICAR and Compound C. This variation did not involve AMPK and may be associated with other kinase pathways.

4.5. REFERENCES

Adams, J., Z. P. Chen, B. J. Van Denderen, C. J. Morton, M. W. Parker, L. A. Witters, D. Stapleton, and B. E. Kemp. 2004. Intrasteric control of AMPK via the gamma1 subunit AMP allosteric regulatory site. Protein Sci. 13:155-165.

Andersson, U., K. Filipsson, C. R. Abbott, A. Woods, K. Smith, S. R. Bloom, D. Carling, and C. J. Small. 2004. AMP-activated protein kinase plays a role in the control of food intake. J. Biol. Chem. 279:12005-12008.

Anthony, N. M., M. P. Gaidhu, and R. B. Ceddia. 2009. Regulation of visceral and subcutaneous adipocyte lipolysis by acute AICAR-induced AMPK activation. Obesity (Silver Spring) 17:1312-1317.

Carling, D., M. J. Sanders, and A. Woods. 2008. The regulation of AMP-activated protein kinase by upstream kinases. Int. J. Obes. (Lond) 32 Suppl. 4:S55-59.

Davis, J. L., D. T. Masuoka, L. K. Gerbrandt, and A. Cherkin. 1979. Autoradiographic distribution of L-proline in chicks after intracerebral injection. Physiol. Behav. 22:693-695.

Dunnington, E. A., and P. B. Siegel. 1996. Long-term divergent selection for eight-week body weight in white Plymouth rock chickens. Poult. Sci. 75:1168-1179.

Fan, X., Y. Ding, S. Brown, L. Zhou, M. Shaw, M. C. Vella, H. Cheng, E. C. McNay, R. S. Sherwin, and R. J. McCrimmon. 2009. Hypothalamic AMP-activated protein kinase activation with AICAR amplifies counterregulatory responses to hypoglycemia in a rodent model of type 1 diabetes. Am. J. Physiol. Regul. Integr. Comp. Physiol. 296:R1702-1708.

Florant, G. L., A. M. Fenn, J. E. Healy, G. K. Wilkerson, and R. J. Handa. 2010. To eat or not to eat: the effect of AICAR on food intake regulation in yellow-bellied marmots (Marmota flaviventris). J. Exp. Biol. 213:2031-2037.

Foretz, M., D. Carling, C. Guichard, P. Ferre, and F. Foufelle. 1998. AMP-activated protein kinase inhibits the glucose-activated expression of fatty acid synthase gene in rat hepatocytes. J. Biol. Chem. 273:14767-14771.

Furuse, M., R. Ando, T. Bungo, R. Ao, M. Shimojo, and Y. Masuda. 1999. Intracerebroventricular injection of orexins does not stimulate food intake in neonatal chicks. Br. Poult. Sci. 40:698-700.

Gaidhu, M. P., S. Fediuc, N. M. Anthony, M. So, M. Mirpourian, R. L. Perry, and R. B. Ceddia. 2009. Prolonged AICAR-induced AMP-kinase activation promotes energy dissipation in white adipocytes: novel mechanisms integrating HSL and ATGL. J. Lipid Res. 50:704-715.

Gao, S., K. P. Kinzig, S. Aja, K. A. Scott, W. Keung, S. Kelly, K. Strynadka, S. Chohnan, W. W. Smith, K. L. Tamashiro, E. E. Ladenheim, G. V. Ronnett, Y. Tu, M. J. Birnbaum, G. D. Lopaschuk, and T. H. Moran. 2007. Leptin activates hypothalamic acetyl-CoA carboxylase to inhibit food intake. Proc. Natl. Acad. Sci. U S A 104:17358-17363.

Hardie, D. G. 2003. Minireview: the AMP-activated protein kinase cascade: the key sensor of cellular energy status. Endocrinology 144:5179-5183.

Hawley, S. A., J. Boudeau, J. L. Reid, K. J. Mustard, L. Udd, T. P. Makela, D. R. Alessi, and D. G. Hardie. 2003. Complexes between the LKB1 tumor suppressor,

STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. J. Biol. 2:28.

Hawley, S. A., M. Davison, A. Woods, S. P. Davies, R. K. Beri, D. Carling, and D. G. Hardie. 1996. Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. J. Biol. Chem. 271:27879-27887.

Hawley, S. A., D. A. Pan, K. J. Mustard, L. Ross, J. Bain, A. M. Edelman, B. G. Frenguelli, and D. G. Hardie. 2005. Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. Cell Metab. 2:9-19.

Hayes, M. R., K. P. Skibicka, K. K. Bence, and H. J. Grill. 2009. Dorsal hindbrain 5'-adenosine monophosphate-activated protein kinase as an intracellular mediator of energy balance. Endocrinology 150:2175-2182.

Hu, Z., Y. Dai, M. Prentki, S. Chohnan, and M. D. Lane. 2005. A role for hypothalamic malonyl-CoA in the control of food intake. J. Biol. Chem. 280:39681-39683.

Hudson, E. R., D. A. Pan, J. James, J. M. Lucocq, S. A. Hawley, K. A. Green, O. Baba, T. Terashima, and D. G. Hardie. 2003. A novel domain in AMP-activated protein kinase causes glycogen storage bodies similar to those seen in hereditary cardiac arrhythmias. Curr. Biol. 13:861-866.

Jin, J., T. D. Mullen, Q. Hou, J. Bielawski, A. Bielawska, X. Zhang, L. M. Obeid, Y. A. Hannun, and Y. T. Hsu. 2009. AMPK inhibitor Compound C stimulates ceramide production and promotes Bax redistribution and apoptosis in MCF7 breast carcinoma cells. J. Lipid Res. 50:2389-2397.

Johansson, A. M., M. E. Pettersson, P. B. Siegel, and O. Carlborg. 2010. Genome-wide effects of long-term divergent selection. PLoS Genet. 6:e1001188.

Kahn, B. B., T. Alquier, D. Carling, and D. G. Hardie. 2005. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metab. 1:15-25.

Kim, E. K., I. Miller, S. Aja, L. E. Landree, M. Pinn, J. McFadden, F. P. Kuhajda, T. H. Moran, and G. V. Ronnett. 2004. C75, a fatty acid synthase inhibitor, reduces food intake via hypothalamic AMP-activated protein kinase. J. Biol. Chem. 279:19970-19976.

King, T. D., L. Song, and R. S. Jope. 2006. AMP-activated protein kinase (AMPK) activating agents cause dephosphorylation of Akt and glycogen synthase kinase-3. Biochem. Pharmacol. 71:1637-1647.

Kola, B., E. Hubina, S. A. Tucci, T. C. Kirkham, E. A. Garcia, S. E. Mitchell, L. M. Williams, S. A. Hawley, D. G. Hardie, A. B. Grossman, and M. Korbonits. 2005. Cannabinoids and ghrelin have both central and peripheral metabolic and cardiac effects via AMP-activated protein kinase. J. Biol. Chem. 280:25196-25201.

Kubota, N., W. Yano, T. Kubota, T. Yamauchi, S. Itoh, H. Kumagai, H. Kozono, I. Takamoto, S. Okamoto, T. Shiuchi, R. Suzuki, H. Satoh, A. Tsuchida, M. Moroi, K. Sugi, T. Noda, H. Ebinuma, Y. Ueta, T. Kondo, E. Araki, O. Ezaki, R. Nagai, K. Tobe, Y. Terauchi, K. Ueki, Y. Minokoshi, and T. Kadowaki. 2007. Adiponectin stimulates AMP-activated protein kinase in the hypothalamus and increases food intake. Cell Metab. 6:55-68.

Lane, M. D., and S. H. Cha. 2009. Effect of glucose and fructose on food intake via malonyl-CoA signaling in the brain. Biochem. Biophys. Res. Commun. 382:1-5.

Lee, K., B. Li, X. Xi, Y. Suh, and R. J. Martin. 2005. Role of neuronal energy status in the regulation of adenosine 5'-monophosphate-activated protein kinase, orexigenic neuropeptides expression, and feeding behavior. Endocrinology 146:3-10.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Marquez, G. C., P. B. Siegel, and R. M. Lewis. 2010. Genetic diversity and population structure in lines of chickens divergently selected for high and low 8-week body weight. Poult. Sci. 89:2580-2588.

Merrill, G. F., E. J. Kurth, D. G. Hardie, and W. W. Winder. 1997. AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. Am. J. Physiol. 273:E1107-1112.

Minokoshi, Y., T. Alquier, N. Furukawa, Y. B. Kim, A. Lee, B. Xue, J. Mu, F. Foufelle, P. Ferre, M. J. Birnbaum, B. J. Stuck, and B. B. Kahn. 2004. AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. Nature 428:569-574.

Munday, M. R., D. Carling, and D. G. Hardie. 1988. Negative interactions between phosphorylation of acetyl-CoA carboxylase by the cyclic AMP-dependent and AMP-activated protein kinases. FEBS Lett. 235:144-148.

Pold, R., L. S. Jensen, N. Jessen, E. S. Buhl, O. Schmitz, A. Flyvbjerg, N. Fujii, L. J. Goodyear, C. F. Gotfredsen, C. L. Brand, and S. Lund. 2005. Long-term AICAR administration and exercise prevents diabetes in ZDF rats. Diabetes 54:928-934.

Proszkowiec-Weglarz, M., M. P. Richards, B. D. Humphrey, R. W. Rosebrough, and J. P. McMurtry. 2009. AMP-activated protein kinase and carbohydrate response element binding protein: a study of two potential regulatory factors in the hepatic lipogenic program of broiler chickens. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 154:68-79.

Proszkowiec-Weglarz, M., M. P. Richards, R. Ramachandran, and J. P. McMurtry. 2006. Characterization of the AMP-activated protein kinase pathway in chickens. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 143:92-106.

Sibut, V., E. Le Bihan-Duval, S. Tesseraud, E. Godet, T. Bordeau, E. Cailleau-Audouin, P. Chartrin, M. J. Duclos, and C. Berri. 2008. Adenosine monophosphate-activated protein kinase involved in variations of muscle glycogen and breast meat quality between lean and fat chickens. J. Anim. Sci. 86:2888-2896.

Siegel, P. B., and J. H. Wolford. 2003. A review of some results of selection for juvenile body weight in chickens. Poult. Sci. 40:81-91.

Thomson, D. M., J. D. Brown, N. Fillmore, S. K. Ellsworth, D. L. Jacobs, W. W. Winder, C. A. Fick, and S. E. Gordon. 2009. AMP-activated protein kinase response to contractions and treatment with the AMPK activator AICAR in young adult and old skeletal muscle. J. Physiol. 587:2077-2086.

Viollet, B., M. Foretz, B. Guigas, S. Horman, R. Dentin, L. Bertrand, L. Hue, and F. Andreelli. 2006. Activation of AMP-activated protein kinase in the liver: a new strategy for the management of metabolic hepatic disorders. J. Physiol. 574:41-53.

Walter, I., B. Hegarty, and F. Seebacher. 2010. AMP-activated protein kinase controls metabolism and heat production during embryonic development in birds. J. Exp. Biol. 213:3167-3176.

Woods, A., K. Dickerson, R. Heath, S. P. Hong, M. Momcilovic, S. R. Johnstone, M. Carlson, and D. Carling. 2005. Ca2+/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. Cell Metab. 2:21-33.

Woods, A., S. R. Johnstone, K. Dickerson, F. C. Leiper, L. G. Fryer, D. Neumann, U. Schlattner, T. Wallimann, M. Carlson, and D. Carling. 2003. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. Curr. Biol. 13:2004-2008.

Xu, P., P. B. Siegel, and D. M. Denbow. 2009. AICAr and compound C influence on feed and water intake in lines of chickens selected for high or low body weight. Pages 76 in Annual Graduate Student Assembly Research Symposium, Blacksburg, VA.

Xu, P., P. B. Siegel, and D. M. Denbow. 2011. Genetic selection for body weight in chickens has altered responses of the brain's AMPK system to food intake regulation effect of ghrelin, but not obestatin. Behav. Brain Res. 221: 216-226.

Zang, Y., L. F. Yu, T. Pang, L. P. Fang, X. Feng, T. Q. Wen, F. J. Nan, L. Y. Feng, and J. Li. 2008. AICAR induces astroglial differentiation of neural stem cells via

activating the JAK/STAT3 pathway independently of AMP-activated protein kinase. J. Biol. Chem. 283:6201-6208.

CHAPTER 5

Genetic Selection for Body Weight in Chickens has Altered Responses of the Brain's AMPK System to Food Intake Regulation Effect of Ghrelin, but not Obestatin (Accepted)

This paper was trying to determing if genetic selection for body weight has altered the central response to ghrelin and obestatin and if this alteration was due to altered AMPK system.

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Research report

Genetic selection for body weight in chickens has altered responses of the brain's AMPK system to food intake regulation effect of ghrelin, but not obestatin☆

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ABSTRACT

The effects of ghrelin and obestatin regulation of food intake are different in mammals and chickens. We investigated central effects of ghrelin and obestatin in lines of chickens selected 50 generations for high (HWS) or low (LWS) body weight. We hypothesized that the effect of ghrelin and obestatin on food intake in 5-day-old chicks is mediated by the AMP-activated protein kinase (AMPK) system and selection for body weight alters the brain's response to ghrelin and obestatin by changing the neuronal AMPK system. Although intracerebroventricular (ICV) ghrelin injection decreased food intake in both lines, the threshold for the anorexigenic effect of central ghrelin was lower in LWS than HWS chicks. Obestatin caused a linear dose-dependent increase in food intake in HWS but not LWS chicks. ICV injection of 0.4 nmol ghrelin inhibited hypothalamic AMPK related gene expression and phosphorylation of AMPK α and acetyl-CoA carboxylase (ACC) with the magnitude of inhibition different in the two lines. In contrast, ICV injection of 4 nmol obestatin did not affect mRNA expression of AMPK system or phosphorylation of AMPK and ACC in either line. These data support the premise of a lower threshold for anorexigenic effect of central ghrelin in LWS than HWS chicks, and this difference may be associated with differential hypothalamic AMPK signaling. Additionally, the hypothalamic mRNA level of ghrelin was significantly higher in LWS than HWS, which may have also contributed to the different threshold response to ghrelin in these two lines. The expression of the ghrelin receptor was also higher in the LWS line, but not until 56 days of age. In summary, selection for body weight has resulted in differences in the central ghrelin and obestatin system, and an altered brain AMPK system may contribute to the different neuronal response to ghrelin, but not obestatin.

1. Introduction

Ghrelin, a natural ligand of the orphan growth hormone secretagogue receptor type 1a (GHS-R1a), was first discovered in gastric extracts of the rat stomach in 1999 [1]. Since then, ghrelin has been identified in a wide array of vertebrate species and its structure is highly conserved [2]. Ghrelin, which is mainly produced in the stomach, is also expressed in other tissues such as the small intestine, lung, heart, pancreas, pituitary and arcuate nucleus of the hypothalamus in mammals [1]. As suggested by its wide-spread distribution, ghrelin plays a significant role in the release of growth hormone (GH) as well as in other physiological events, including regulation of food intake, gastric motility, glucose homeostasis, cardiovascular, pulmonary and immune function [3–5].

Beside ghrelin, other bioactive peptides are also encoded by the ghrelin gene. The C-terminal peptide of the rat ghrelin precursor contains another 23 amino acid bioactive peptide called obestatin [6]. Obestatin has subtle but opposite physiological effects to ghrelin [4,6,7]. As opposed to ghrelin, a number of experiments have shown anorectic effects after both peripheral and central administration of obestatin [8–10]. Studies involving these two active products of the ghrelin gene suggest a dynamic network with multiple effector elements which are dependent on each other.

Avian ghrelin consists of 26 amino acids and possesses a 54% sequence identity with human ghrelin [11]. In avian species, ghrelin is expressed in many tissues including brain, lung, spleen and intestine with highest expression mainly in the proventricular [11,12]. Chicken ghrelin has the same GH-releasing effect as rat or human ghrelin [13], however, the food intake regulatory effect of ghrelin in avians is opposite to that in mammals. Intracerebroventricular (ICV), intravenous (IV) and subcutaneous (SC) injection of ghrelin stimulated feeding in rats, whereas ICV injection of ghrelin strongly suppressed feeding in chickens regardless of age, sex, and time or dosage of injection [14,15]. An obestatin-like sequence was also found in the avian ghrelin precursor protein, which suggests an

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obestatin-like peptide exists in birds [16]. However, it was predicted that this obestatin-like peptide did not have the C-terminal amide structure which was reported [6] as essential for bioactivity of obestatin. So far there is still no evidence for a biological action for obestatin in birds. Chicken or rat ghrelin injected ICV showed the same anorexic effect in neonatal chicks, and growth hormone-releasing peptide-2 also inhibited food intake [17]. Different from the stimulatory effects on food intake mediated by arcuate nucleus (ARC) neuropeptides such as Neuropeptide Y (NPY) and orexin in mammals, the inhibitory effect of ghrelin may be mediated by corticotropin-regulating factor (CRF) [15]. These results indicate that the mechanisms underlying the food intake regulation activity of ghrelin in birds are different from those in mammals. An understanding of the molecular basis for the anorexigenic effect of ghrelin in birds is needed to clarify this discrepancy between mammals and birds.

AMP-activated protein kinase (AMPK) provides a candidate target capable of mediating the food regulation effect of ghrelin and associated obestatin. AMPK is a multisubunit enzyme that is considered as a major regulator of fatty acid synthesis pathway due to its function to phosphorylate and inactivate key enzymes such as acetyl-CoA carboxylase (ACC) [18]. AMPK is also involved in the regulation of whole body energy homeostasis, whose activity in hypothalamic neurons is altered by hormonal and nutrient signals that mediate the feeding response [19,20]. Glucose, leptin and insulin, which inhibit AMPK activity in the hypothalamus, decrease food intake in mice [21], whereas ghrelin and adiponectin stimulates AMPK activity in the hypothalamus and increases food intake in rats [22]. Clearly, feeding-inhibiting factors appear to decrease hypothalamic AMPK activity and feeding-stimulating factors increase it.

In the modern poultry industry, chickens are intensively selected for either egg or meat production. These selections have resulted in changes in body weight and body composition. To better understand how genetic selection for body weight has altered the mechanisms controlling food intake is necessary in order to devise strategies to both increase and decrease food intake. The animal model used in the present experiment involved lines of chickens that had undergone long-term divergent selection from a common White Rock base population for high (HWS) or low (LWS) body weight at 56 days of age [23]. These lines of chickens differ both at the phenotypic and genotypic levels [23,24] with a difference in body weights at selection age by more than 10-fold. They also exhibit hypophagia (LWS) and hyperphagia (HWS) characteristics [25]. Our hypothesis is that the effect of ghrelin and obestatin on food intake in chicks is mediated by the AMPK system and that selection for body weight alters the brain's response to ghrelin and obestatin by changing the neuronal AMPK system.

2. Materials and methods

2.1. Animals

The chickens used in this experiment were from two lines that have undergone 50 generations of selection for the single trait of high or low body weight at 56 days of age. The base population was formed by crossing seven moderately inbred lines of White Plymouth Rock and the selected lines have been maintained as pedigreed populations throughout the selection program [23,25,26]. Eggs from same-age parents from each line were incubated in the same machine for each experiment. After hatch, chicks were placed in electric heated batteries with raised wire floors at 32 ± 2 °C. A mash diet (20% crude protein, 2684 kcal ME/kg) and water were provided *ad libitum*. For the ICV injection experiment, chicks were transferred to individual cages in a temperature control room (32 ± 2 °C) after 2 days in the electric heated batteries. The individual cages were designed to allow visual and auditory, but not physical contact, with other chicks. All ICV trials were conducted at 5 days post-hatch, which allowed time for chicks to adjust to the cages. Data were collected from both lines simultaneously and injections followed the sequence: HWS, LWS, HWS, and so forth. For ontogenic testing, chicks were raised in electric heated batteries until the sampling day. Experimental procedures were approved by the Virginia Tech Animal Care and Use Committee.

2.2. Peptides and intracerebroventricular (ICV) injection

Ghrelin (chicken) and obestatin (chicken) were synthesized by United States Department of Agriculture (Washington, DC, USA) according to the peptide sequence of chicken ghrelin and obestatin. The peptides were dissolved in artificial cerebrospinal fluid (aCSF) with 0.1% Evans Blue dye to facilitate the localization of injection site. The ICV injection of 5 μL of each substance was made using a microsyringe according to the method of Davis et al. [27]. The procedure does not induce physiological stress [28]. Chicks from both lines were assigned to treatments at random. At the end of each experiment, the chicks were decapitated and the injection site was verified by frontal plane section. Data from individuals without dye present in the lateral ventricle were deleted. Sex was determined visually by dissection. Numbers of chicks used in analyses are provided in the legend of each figure.

2.3. Experiment 1: food and water intake after ICV injection

Chicks from each line were fasted for 180 min, then weighed and randomly assigned to receive either 0 (aCSF only), 0.1, 0.2 or 0.4 nmol chicken ghrelin by ICV injection. In another separate trial, chicks were subjected to the same procedure but received 0, 0.25, 1 or 4 nmol obestatin by ICV injection. After injection, chicks were returned to their cages and given free access to food and water. Their cumulative intake were recorded every 30 min through 180 min post-injection by weighing (±0.01 g) individual food and water containers. Water and food intake were expressed as percent body weight to adjust the differences between HWS and LWS chicks and analyzed at each time period by analysis of variance (ANOVA) using the general linear modeling procedure (GLM, SAS Institute Inc, Cary, NC, USA). Full model includes sex, line, dose and interaction between them. Because the effect of sex and interactions involved sex were not significant, the reduced model included line, dose and the line by dose interaction. Multiple comparisons among doses were also made within each line and Tukey's multiple range test was used for comparing multiple means. Treatment effects were partitioned into linear and quadratic contrasts to determine the dose–response relationships and significance was considered at $P < 0.05$.

2.4. Experiment 2: expression of genes encoding AMPK subunits and associated proteins in hypothalamus after ICV injection

Male chicks from each line were fasted 180 min before injection, weighed and randomly assigned to receive either 0 or 0.4 nmol chicken ghrelin by ICV injection. Another separate trial followed the same procedure with doses being 0 or 4 nmol obestatin administration by ICV injection. After injection, chicks were given free access to both food and water to mimic the conditions in Experiment 1. Sixty min after injection, the chicks were decapitated and the preoptic-hypothalamic area was dissected from each brain using the landmarks of the optic chiasm rostrally, and the mammillary bodies caudally. Dissected hypothalamic tissues were quickly frozen in liquid nitrogen, and stored at −80 °C for later analysis.

Preoptic-hypothalamic tissues were homogenized in Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and total RNA was extracted according to the manufacturer's instruction. Total RNA concentration was then quantified by measuring the absorbance at 260 nm. 50 μg of total RNA was treated with DNase-I (Promega, Madison, WI, USA) to eliminate the contaminating genomic DNA. Purified DNA-free RNA was dissolved in RNase-free water and immediately used as templates in reverse transcription. 2 μg of total RNA were incubated at 42 °C for 1 h in a 25 μL mixture containing 200 units Moloney Murine Leukemia Virus reverse transcriptase (M-MLV) (Promega, Madison, WI, USA), 1× M-MLV reaction buffer (Promega, Madison, WI, USA), 25 units RNase inhibitor (Promega, Madison, WI, USA), 4 μg Oligo dT primer (Eurofins MWG Operon, Huntsville, AL, USA) and 0.5 mM dNTP (Promega, Madison, WI, USA). The reaction was terminated by heating at 95 °C for 5 min and quickly cooled on ice. The cDNA (RT products) were aliquoted and stored at − 20 °C.

Real-time RT-PCR was performed in 7500 Fast Real-Time PCR System (Applied Biosystems, Inc., Carlsbad, CA, USA). 1 μL of 4-fold diluted RT product was used for PCR in a final volume of 20 μL containing 10 μL 2× Fast SYBR Green Master Mix (Applied Biosystems, Inc.) and 0.5 μM primer mix (Table 1). Chicken glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as a reference gene for normalization purposes. The expression of GAPDH mRNA was similar for both lines. The thermal cycling conditions were: initial denaturation (20 s at 95 °C), then a two-step amplification program (3 s at 95 °C, 30 s at 60 °C) was repeated 40 times. Real-time RT-PCR data were analyzed by the relative quantification ($\Delta\Delta C_T$) method [29]. The statistical model for ANOVA was the same as that for Experiment 1. All mRNA expression levels were expressed as fold-change compared to the LWS aCSF group.

2.5. Experiment 3: expression of pAMPK and pACC proteins in hypothalamus after ICV injection

Male chicks from each line received the same treatment as in Experiment 2. 60 min after injection, chicks were decapitated and the preoptic-hypothalamic area was dissected from each brain. Proteins were extracted with SDS lysis buffer (25 mM Tris–HCl, pH 6.8, 2.3% SDS, 10% glycerol and 5% β-mercaptoethanol). Pro-

Table 1
Nucleotide sequences of specific primers.

Target gene	GenBank accession	PCR products (bp)	Primer sequences
[1]AMPK α1	NM_001039603	125	F: 5′-ATCTGTCTCGCCCTCATCCT-3′ R: 5′-CCACTTCGCTCTTCTTACACCTT-3′
[2]AMPK α2	NM_001039605	128	F: 5′-CCAGCGAGTTCTACCTAGCCT-3′ R: 5′-TGCCTTGGGACTGTCTGCTA-3′
[3]AMPK β1	NM_001039912	113	F: 5′-CCAGGAGCCCTATGTCTGTAAG-3′ R: 5′-CAGGATCGCAAGAAATGCC-3′
[4]AMPK β2	NM_001044662	125	F: 5′-GCACTGCCCATCCCTCTAA-3′ R: 5′-CGGCTGCCACGAAACAA-3′
[5]AMPK γ1	NM_001034827	118	F: 5′-AGAGGTCCCAAAGCCTGAGTT-3′ R: 5′-GAAGATGCCCAGAGCCACA-3′
[6]AMPK γ2	NM_001030965	106	F: 5′-CACAAGCCCTACAGCACCG-3′ R: 5′-TGAACCTCAGCCTTCACTATCCTA-3′
[7]AMPK γ3	NM_001031258	91	F: 5′-CCACGCTTCCTAAAGAAAACAG-3′ R: 5′-CCGGCAGCATTAACAACG-3′
[8]ACC α	NM_205505	125	F: 5′-CCGAGAACCCAAAACTACCAG-3′ R: 5′-GCCAGCAGTCTGAGCCACTA-3′
[9]CAMKK α	XM_001234324	97	F: 5′-CAGTTTGAAGGGAACGATGCC-3′ R: 5′-CACTGAAACTTTTGCCCGTGT-3′
[10]CAMKK β	XM_415134	144	F: 5′-AGGACCAGGCTCGGTTCTAC-3′ R: 5′-TGACACCAAAGTCGGCAATT-3′
[11]FAS	NM_205155	117	F: 5′-AAAGCAATTCGTCACGGACA-3′ R: 5′-GGCACCATCAGGACTAAGCA-3′
[12]LKB 1	XM_418227	114	F: 5′-GCTGACCACCAATGGGACG-3′ R: 5′-GGCTGGAATGCTGGCGAC-3′
[13]MO25 α	CR391562	93	F: 5′-CCCTAACAAGACGCAGCCTA-3′ R: 5′-TCGGTCCTGTCATTCTGAAACT-3′
[14]MO25 β	NM_001006272	117	F: 5′-AAGAGGGTAACAGTTGCTCCG-3′ R: 5′-CTTTCACTTATCTCCCACGTAATG-3′
[15]STRAD α	NM_001012844	149	F: 5′-TTTCCAAGCTCTTCAACCACC-3′ R: 5′-CAATTCACTCATCCCGTCCATA-3′
[16]GAPDH	NM_204305	141	F: 5′-TGGCATCCAAGGAGTGAGC-3′ R: 5′-GGGGAGACAGAAGGGAACAG-3′
Ghrelin	NM_001001131	101	F: 5′-GAAACTGCTCTGGCTGGCTCT-3′ R: 5′-CCTCGGCGATGTAATCTTGC-3′
[17]GHS-R1a	NM_204394	85	F: 5′-TTATCAGGGACAAGAACAACAAGC-3′ R: 5′-TGAAAAGGCAACCAGCAGAGTA-3′

[1]AMPK α1 (AMP-activate kinase alpha 1 subunit), [2]AMPK α2 (AMP-activate kinase alpha 2 subunit), [3]AMPK β1 (AMP-activate kinase beta 1 subunit), [4]AMPK β2 (AMP-activate kinase beta 2 subunit), [5]AMPK γ1 (AMP-activate kinase gamma 1 subunit), [6]AMPK γ2 (AMP-activate kinase gamma 2 subunit), [7]AMPK γ3 (AMP-activate kinase gamma 3 subunit), [8]ACC α (acetyl-coenzyme A carboxylase alpha), [9]CAMKK α (calcium/calmodulin-dependent protein kinase kinase 1), [10]CAMKK β (calcium/calmodulin-dependent protein kinase kinase 2), [11]FAS (fatty acid synthase), [12]LKB 1 (serine/threonine-protein kinase 11), [13]MO25 α (calcium-binding protein 39), [14]MO25 β (calcium binding protein 39-like), [15]STRAD α (STE20-related kinase adaptor alpha), [16]GAPDH (glyceraldehyde-3-phosphate dehydrogenase), [16]GHS-R1a (growth hormone secretagogue receptor type 1a).

teins extracts were separated on a 8.5% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Blots were blocked in 5% bovine serum albumin (BSA) dissolved in Tris-buffered saline with Tween-20 (TBST, 20 mmol Tris, pH 7.4, 150 mmol NaCl, and 0.1% Tween-20) for 180 min at 4°C and then briefly washed two times for 5 min each in TBST. The blots were incubated overnight at 4°C with primary antibodies against phosphor-AMPKα (α1 and α2, Thr^{172}) (1:500), phosphor-ACC (Ser^{79}) (1:250), ACC (1:250) and AMPK α (1:1000) in TBST with 1% BSA. All antibodies were from Cell Signaling Technology (Danvers, MA, USA). Blots were washed five times for 10 min each and then incubated with anti-rabbit IgG horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at room temperature for 60 min. The blots were washed five times for 10 min each. A chemiluminescent substrate SuperSignal West Pico (Pierce, Rockford, IL, USA) was applied according to instructions of the manufacture and the signal was detected using a Gel Doc XR System (Bio-Rad, Hercules, CA, USA). Densitometry analysis was performed using Quantity One (Bio-Rad, Hercules, CA, USA) image analysis software. The phosphate protein expression levels were expressed as a density ratio (pAMPK/AMPK, pACC/ACC). The statistical model was similar to that for Experiment 2.

2.6. Experiment 4: ontogenic changes of ghrelin and growth hormone secretagogue receptor type 1a (GHS-R1a) mRNA expression in the hypothalamus

At 1, 14, 28, 42 and 56 days of age, six male birds from each line were sacrificed by cervical dislocation. The whole brain was removed and the preoptic–hypothalamic area was dissected from each brain. Dissected hypothalamic tissues were quickly frozen in liquid nitrogen, and stored at −80°C for later analysis. The mRNA expression of ghrelin and ghrelin receptor GHS-R1a were tested using the same method described in Experiment 2. The primer sequences for ghrelin and GHS-R1a are showed in Table 1. All mRNA expression levels was expressed as fold-change compared to that of HWS 1-day-old chicks. Data were analyzed by analysis of variance (ANOVA) using the general linear modeling procedure (GLM, SAS Institute Inc., Cary, NC, USA). The model included line, age and the line by age interaction. Comparison between lines was also performed at each age using a t-test for independent samples. Data were expressed as the mean ± SEM and the level of significance was set at $P < 0.05$.

3. Results

3.1. Experiment 1: Food and water intake after ICV injection

The interaction of line by ghrelin dose was significant for food intake, because the lines responded differently to ghrelin dose (Fig. 1). Thus, the effect of ghrelin dose on food intake was analyzed within each line. Although ICV injection of ghrelin decreased food intake in both lines, the pattern of response differed. In HWS chicks, there was a linear dose-dependent decrease in food consumption at all observation times (Fig. 1A). The highest dose of ghrelin injection had a sustaining suppression effect on food intake with food consumed very little after 60 min post-injection. Lower doses of ghrelin injection showed a dose-dependent attenuation in the magnitude of food intake suppression. For LWS chicks, the suppression effect of ghrelin on food intake was significant from 90 min post-injection (Fig. 1B). All doses of ghrelin were significantly effective at reducing food intake and the effect was linearly at this time period. Water intake was not affected by ICV injection of ghrelin in either line (data not shown).

The interaction between line and obestatin dose was significant, thus as with ghrelin, the obestatin dose effect on food intake within each line was analyzed separately. Obestatin caused a linear dose-dependent increase in food intake in HWS chicks from 30 to 120 min

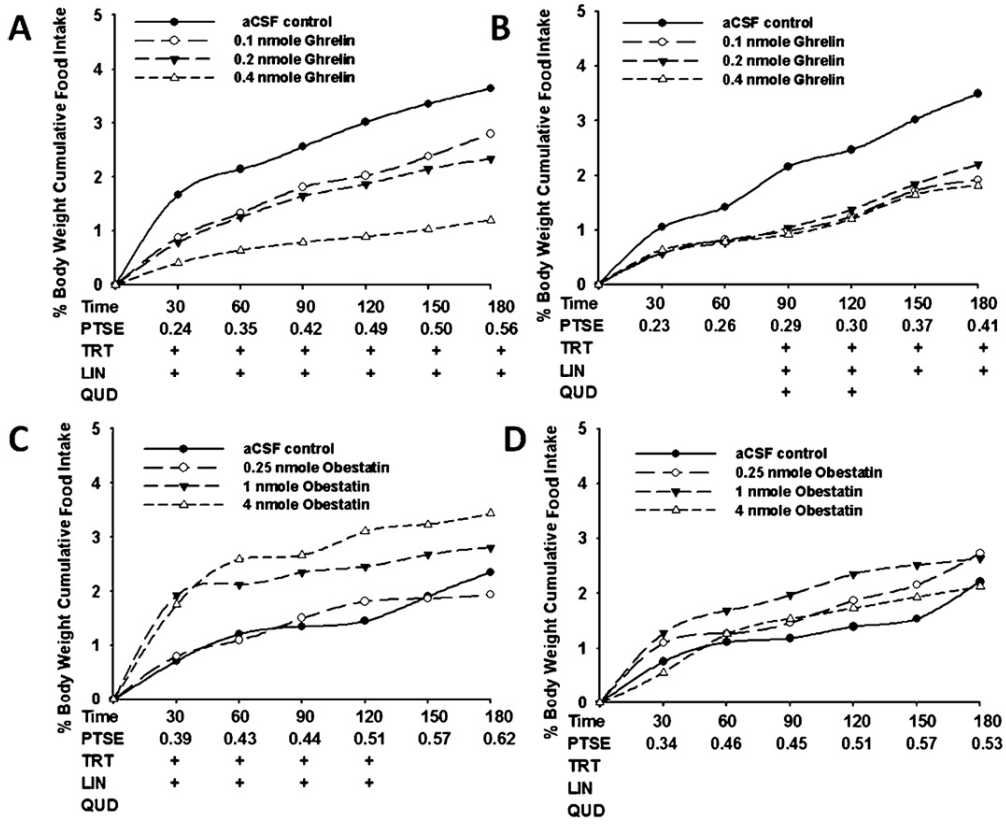

Fig. 1. Cumulative food intake expressed as percent body weight of 5-day HWS and LWS lines of chicks following ICV injection of ghrelin or obestatin ($n = 12$ chicks/group). (A) HWS chicks injected with ghrelin; (B) LWS chicks injected with ghrelin; (C) HWS chicks injected with obestatin; (D) LWS chicks injected with obestatin. LIN, linear contrast; PTSE, pooled standard error; TRT, treatment effect; QUD, quadratic contrast; + $P < 0.05$ (Experiment 1).

post-injection (Fig. 1C). The highest dose of obestatin effectively stimulated food intake during this time period, while lower doses of obestatin had an attenuated orexigenic effect. In LWS line, ICV injection of obestatin did not change food intake (Fig. 1D). Water intake was not affected by ICV injection of obestatin in either line (data not shown).

3.2. Experiment 2: expression of genes encoding AMPK subunits and associated proteins in hypothalamus after ICV injection

The effects of 0.4 nmol ghrelin ICV injection on AMPK subunits mRNA expression in the preoptic-hypothalamus of 5-day-old HWS and LWS lines of chicks are illustrated in Fig. 2. Ghrelin significantly downregulated hypothalamic AMPK α1, α2, β1 and β2 subunits mRNA levels in LWS line, but not in HWS line resulting in a line by dose interaction. Additionally, ghrelin had a significant inhibitory effect on hypothalamic AMPK γ1 and γ2 subunits mRNA levels in HWS, but not LWS chicks. Ghrelin-treated HWS and LWS chicks were generally characterized by a lower LKB1/STRAD/MO 25 complex gene expression (Fig. 3). This downregulation effect was significant for STRAD α and MO 25α mRNA levels, but not for LKB1 and MO 25 β mRNA levels. Compared to preinjection levels, there was a significant inhibitory effect on the mRNA levels of the CAMKK α and β subunits by ghrelin in LWS but not HWS chicks. The mRNA levels of ACC α and FAS significantly increased after ghrelin ICV injection in LWS but not HWS chicks.

Obestatin did not affect the expression of any of these genes in either line with the exceptions of a significant inhibitory effect for AMPK β2 (Fig. 4), MO 25β and CAMKK α subunit (Fig. 5) in HWS line of chicks.

3.3. Experiment 3: expression of pAMPK and pACC proteins in hypothalamus after ICV injection

There was an acute effect of ghrelin on the phosphorylation of hypothalamic AMPK α in HWS and LWS chicks (Fig. 6A). The phosphorylation levels of the α1 and α2 catalytic subunits of AMPK in the hypothalamus were tested to compare with AMPK activity. In both HWS and LWS chicks, ghrelin ICV injection resulted in significantly lower levels of phosphorylated AMPK in the hypothalamus than in the vehicle-treated control chicks. A single injection of ghrelin also significantly down-regulated the levels of phosphorylated ACC in the hypothalamus of LWS, but not HWS chicks (Fig. 6B).

Fig. 6C and D shows the acute effect of obestatin ICV injection on the phosphorylation of hypothalamic AMPK and ACC in HWS and LWS chicks. There was a consistent line effect for the phosphorylation of AMPK and ACC (HWS > LWS), but the treatment and line by treatment interaction was not significant. The ICV injection of obestatin did not affect the phosphorylation of hypothamamic AMPK and ACC in either line.

3.4. Experiment 4: ontogenic changes of ghrelin and GHS-R1a mRNA expression in the hypothalamus

The mRNA expression of ghrelin and ghrelin receptor GHS-R1a in the preoptic-hypothalamus of HWS and LWS lines of chickens are illustrated in Fig. 7. There was a significant line, age and line and age interaction for ghrelin and GHS-R1a. The interaction resulted form a different line effect at the different ages. Ghrelin mRNA expression was significantly less in the HWS than LWS chickens at 1, 28 and 56 days of age. Additionally, the expression of GHS-R1a was significantly lower in the HWS than LWS chickens at 56 days of age.

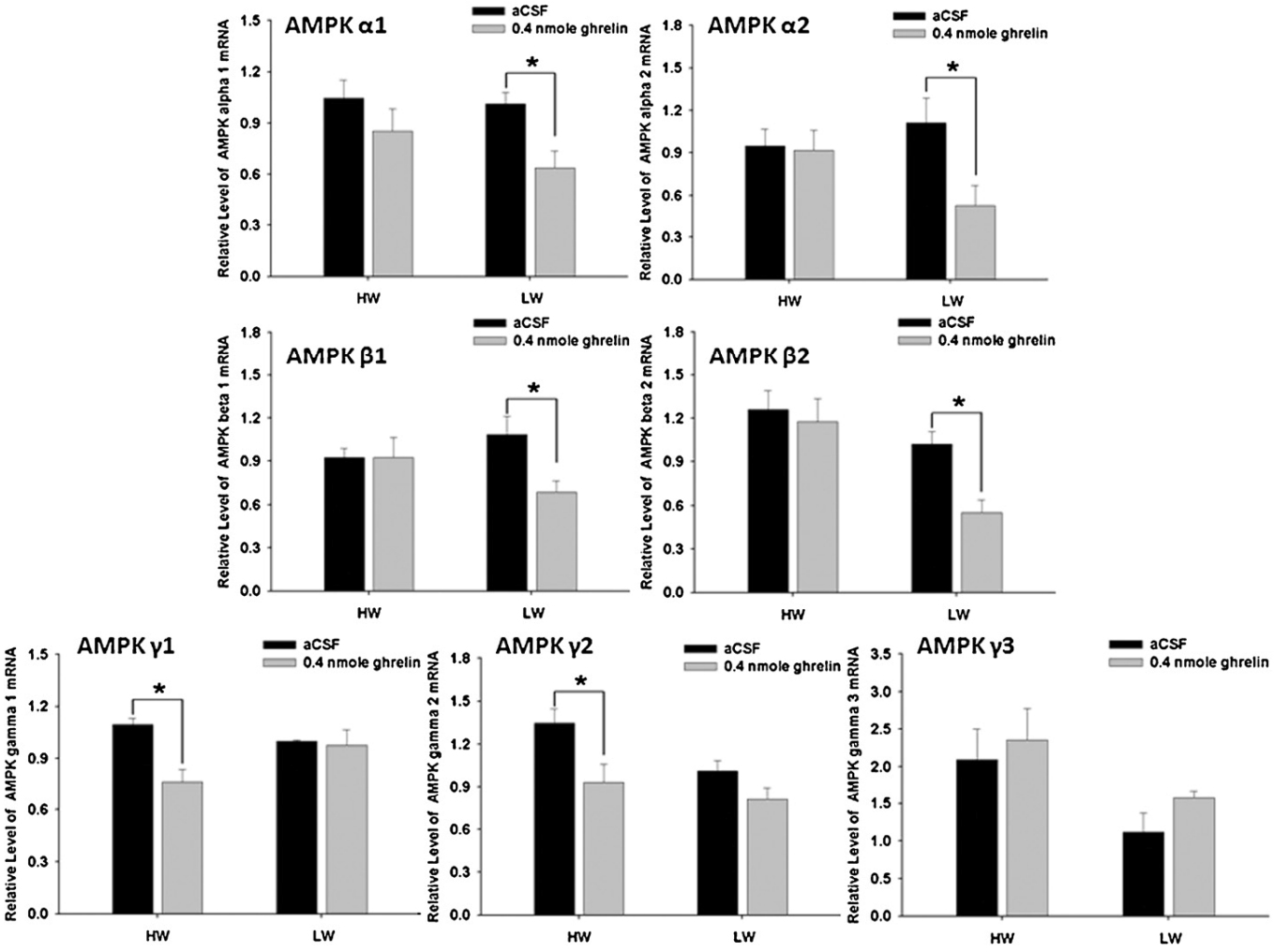

Fig. 2. Gene expression of hypothalamic AMPK subunits in 5-day HWS and LWS lines of chicks following ICV injection of 0.4 nmol ghrelin. The data, which were expressed as fold-change compared to the LWS aCSF group, represent mean ± SEM (n = 6 chicks/group). *P < 0.05 (Experiment 2).

4. Discussion

4.1. Acute effect of ghrelin in HWS and LWS chickens

Chicken ghrelin ICV injection suppresses feeding in chickens regardless of age, sex, and time of injection or dosages [14,15,17,30]. Results from our study were consistent in showing that ICV injections of chicken ghrelin induced a short-term reduction in food intake, as opposed to the orexigenic effect of ghrelin in mammals [31]. Additionally, they provide evidence that HWS chicks, which are hyperphagic, have a higher threshold of response to ICV injection of ghrelin than LWS chicks, which are hypophagic. The differential response of these lines to ICV injection of ghrelin was consistent with those for mature HWS and LWS chickens [32]. In agreement with other reports [14,17,30], ghrelin significantly decreased food intake regardless of injection dosages in LWS chicks, suggesting a hyper-anorexic response in LWS chicks with a low threshold to ghrelin. Conversely, HWS chicks only responded significantly to higher doses, while lower doses of ghrelin had a tendency to inhibit food intake. Thus, long-term selection for high or low body weight from a common base population has altered the brain's response to ghrelin and these comparisons infer genetic differences in the ghrelin downstream pathway facilitating an anorexigenic effect of ghrelin between the two lines.

In mammals, ghrelin's orexigenic effect is mediated by activating hypothalamic AMPK system, which then decreases fatty acid oxidation and increases fatty acid synthesis to stimulate food intake [33–37]. Conversely, in neonatal chicks, ghrelin inhibits food intake by up-regulating FAS mRNA expression and then increasing fatty acid synthesis [38], suggesting the AMPK system and the fatty acid oxidation and synthesis pathway may also be involved in the anorexigenic effect of ghrelin in chickens. Thus, we tested the expression of different genes related to the AMPK system and fatty acid oxidation and synthesis to determine if the anorexigenic effect of ghrelin was mediated by the AMPK system, and if selection for body weight had altered the brains's response to ghrelin by changing the neuronal AMPK system. We found that ICV injection of ghrelin significantly inhibited AMPK α1, α2, β1 and β2 subunits expression in LWS chicks, whereas although ghrelin had a tendency to decrease AMPK α1 expression in HWS chicks, it was not significant. Moreover, ghrelin treated HWS chicks had a lower level of AMPK γ1 and γ2 expression, while ghrelin treated LWS chicks were similar to their controls. The 2 isoforms of the AMPK α subunit, α1 and α2 have a catalytic domain, where AMPK becomes activated when phosphorylation takes place at threonine-172 [39]. The β subunits contain 2 conserved regions that are required to form a functional αβγ complex that is regulated by AMP [40]. The γ subunits include particular cystathionine beta synthase (CBS) domains giving AMPK the ability to sensitively detect shifts in the AMP: ATP

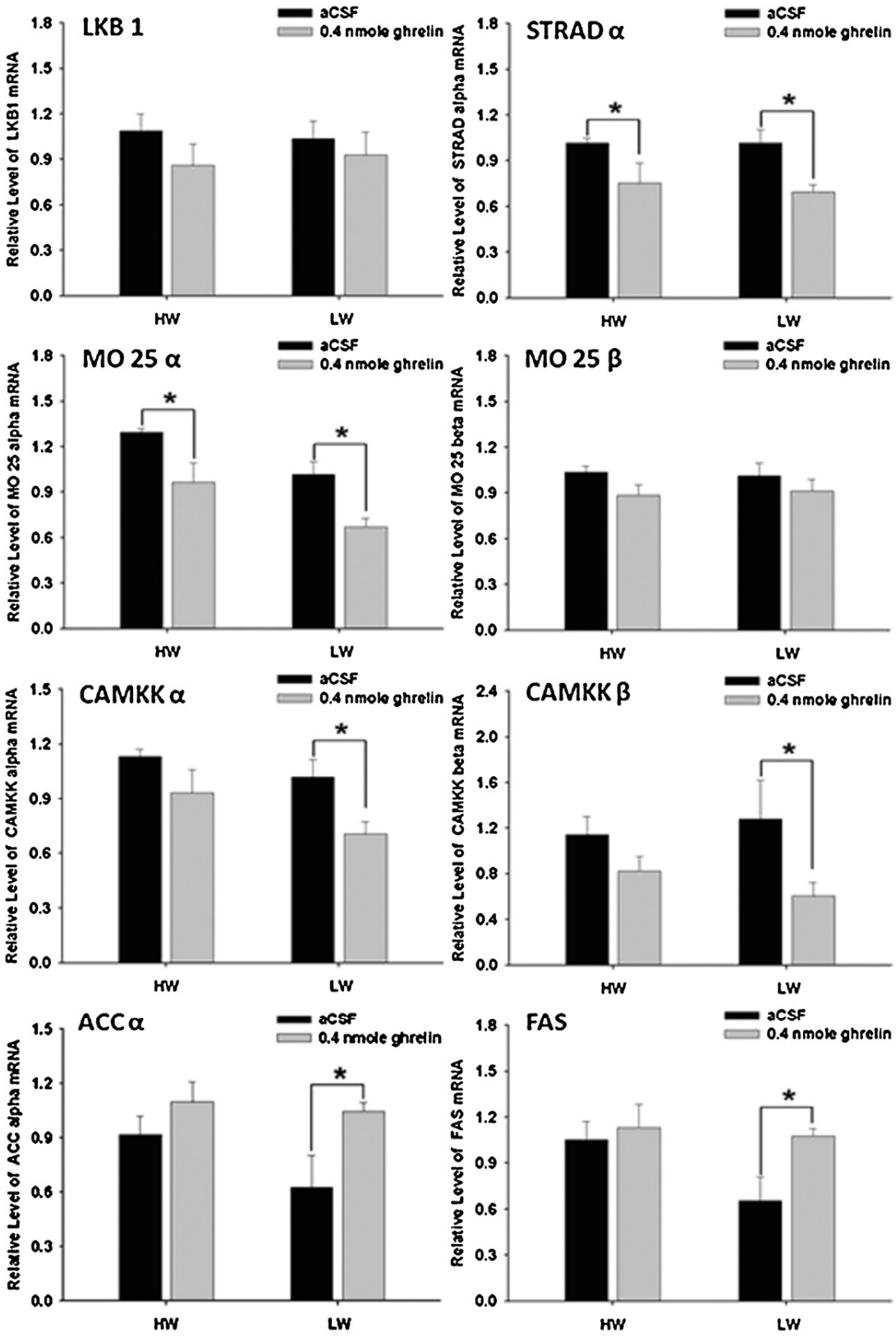

Fig. 3. Gene expression of hypothalamic LKB1/STRAD/MO 25 subunits, CAMKK subunits, ACC α and FAS in 5-day HWS and LWS lines of chicks following ICV injection of 0.4 nmol ghrelin. The data, which were expressed as fold-change compared to the LWS aCSF group, represent mean ± SEM ($n = 6$ chicks/group). *$P < 0.05$ (Experiment 2).

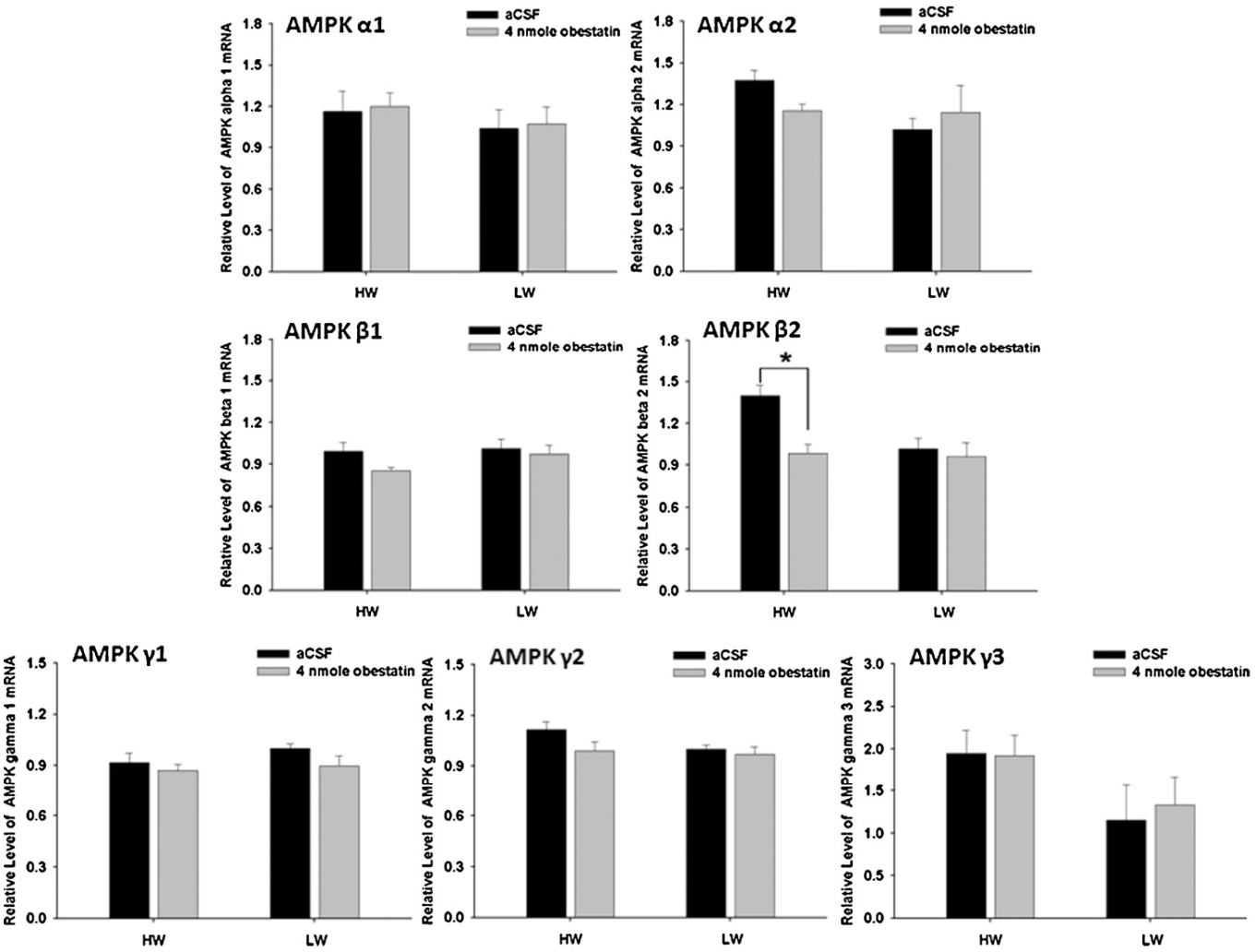

Fig. 4. Gene expression of hypothalamic AMPK subunits in 5-day HWS and LWS lines of chicks following ICV injection of 4 nmol obestatin. The data, which were expressed as fold-change compared to the LWS aCSF group, represent mean ± SEM (n = 6 chicks/group). *P < 0.05 (Experiment 2).

ratio [41]. The cooperation of these different subunits can allosterically activate the enzyme by up to 5-fold [42]. Thus, ICV injection of ghrelin did change mRNA expression of AMPK subunits in both lines, with HWS and LWS chicks having inhibitory effects on different subunit expression. This differential inhibitory effect in AMPK subunit expression may be responsible for the different threshold response of the lines to ghrelin injection observed in Experiment 1.

Although AMPK can be allosterically activated by AMP, the majority of AMPK activity is due to the phosphorylation of a threonine residue by AMPK kinase (AMPKK), which has been identified as a complex between the tumor suppressor protein LKB1 and two accessory subunits, termed STRAD and MO25 [43,44]. LKB1 is allosterically activated by binding to the pseudokinase STRAD and the adaptor protein MO25. The LKB1–STRAD–MO25 complex represents the biologically active unit to phosphorylate and activate AMPK. Thus, the central effects of ghrelin on appetite in chickens may be triggered by down-regulating the LKB1 complex to inhibit AMPK activity. Our results demonstrate that ICV ghrelin administration suppressed hypothalamic STRAD α and MO 25α mRNA levels in both lines. This inhibitory effect of ghrelin on STRAD α and MO 25α may be partially responsible for the down-regulation effect of ghrelin on AMPK subunits in chicks.

Besides the LKB1 complex, another possible upstream regulator responsible for phosphorylation of AMPK is CAMKK, especially the CAMKK β isoform [45–47]. CAMKK was first identified as a protein kinase that activates calmodulin-dependent protein kinase I (CAMK I) and calmodulin-dependent protein kinase IV (CAMK IV) [48], and a purified CAMKK could phosphorylate and activate AMPK in cell-free assays [49]. In our study, ICV injection of ghrelin had an inhibitory effect on CAMKK α and β mRNA expression levels in both lines, with effect significant in LWS chicks. This down-regulation effect of ghrelin on CAMKK α and β provides additional evidence that ghrelin decreases food intake by inhibiting the AMPK system. Furthermore, the difference in the magnitude of CAMKK α and β mRNA suppression between these lines may contribute to their differential responses to ghrelin observed in AMPK subunit expression.

Activation of AMPK can trigger several physiological pathways including the regulation of appetite, metabolism, fatty acid catabolism, coagulation and systemic inflammation [50]. One role of AMPK in metabolic regulation is that of fatty acid oxidation and synthesis. Activation of AMPK reduces expression of ACC and FAS, which are essential bioactive enzymes involved in fatty acid metabolism. The inhibition of ACC, a key regulatory enzyme of fatty acid oxidation, increases the oxidation of fatty acids in mitochondria by suppressing malonyl-CoA expression [51], while the inhibition of FAS, a key lipogenic enzyme, decreases fatty acid synthesis [52]. Considering the inhibitory effect of ghrelin on the AMPK

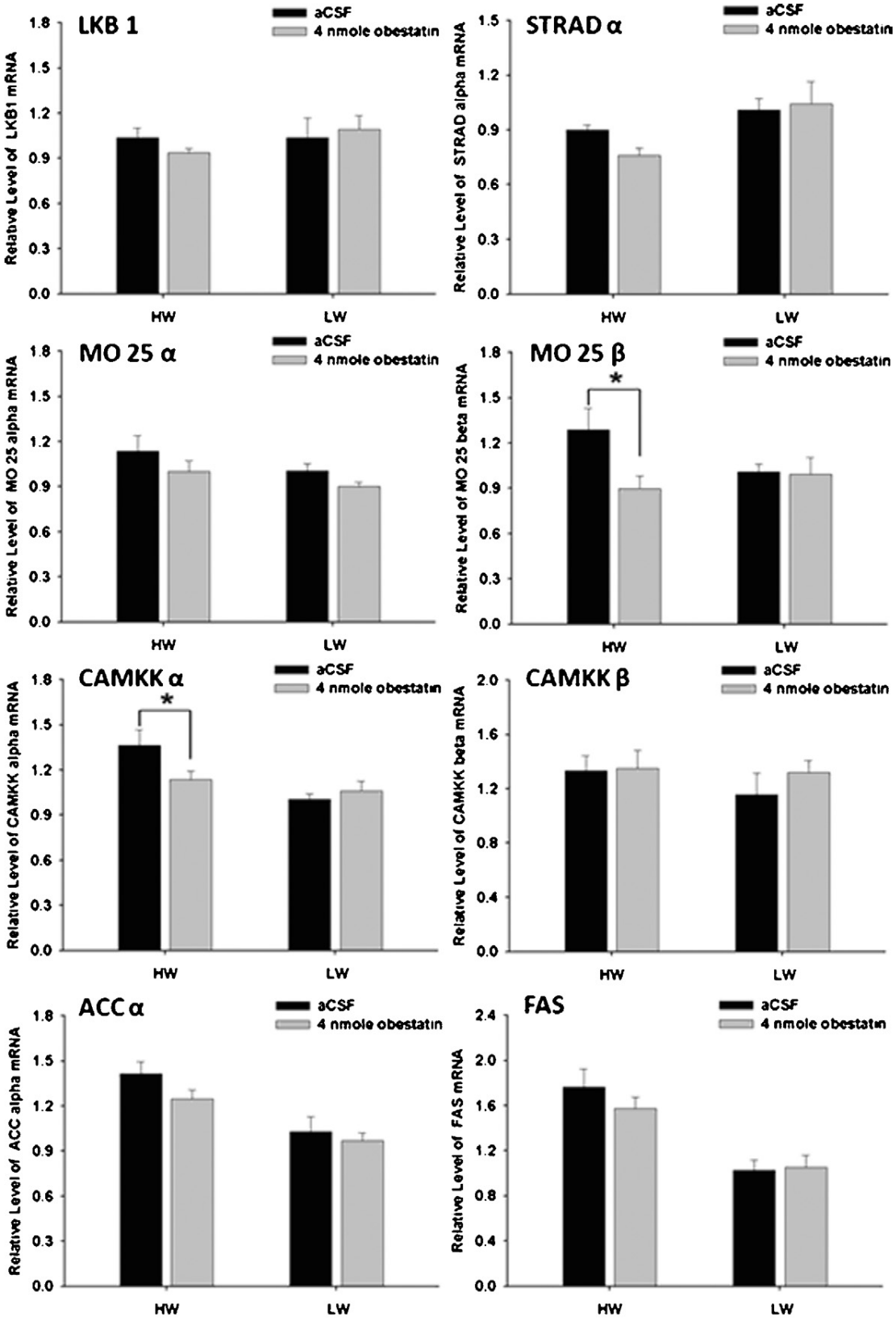

Fig. 5. Gene expression of hypothalamic LKB1/STRAD/MO 25 subunits, CAMKK subunits, ACC α and FAS in 5-day HWS and LWS lines of chicks following ICV injection of 4 nmol obestatin. The data, which were expressed as fold-change compared to the LWS aCSF group, represent mean ± SEM ($n = 6$ chicks/group). *$P < 0.05$ (Experiment 2).

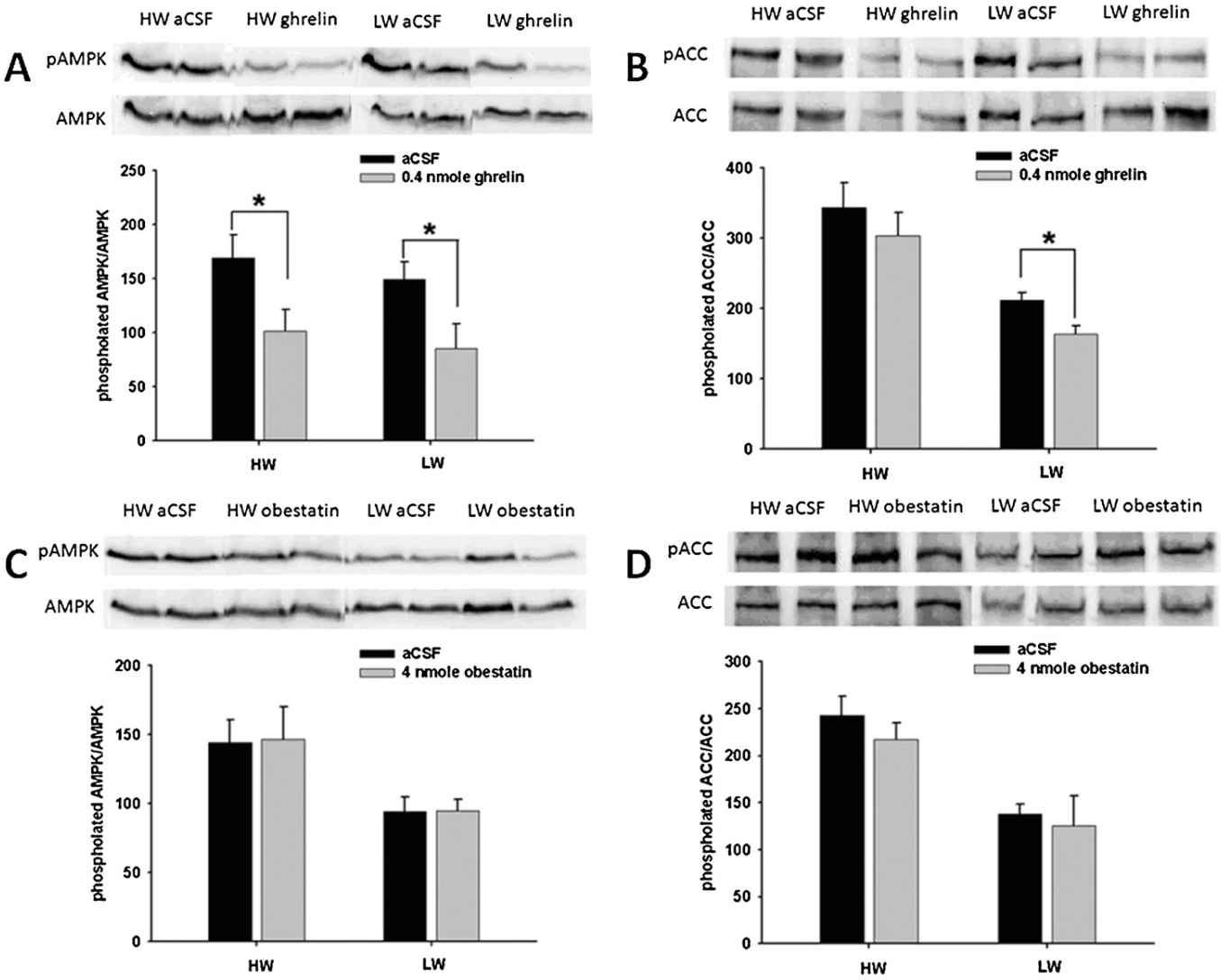

Fig. 6. Effects of ICV injection of ghrelin or obestatin on phosphorylation of hypothalamic AMPK α or ACC in 5-day HWS and LWS lines of chicks. Representative Western blots of phosphorylated AMPK α (pAMPK α) and total AMPK α (AMPK α) or phosphorylated ACC (pACC) and total ACC (ACC) in both lines of chicks are shown. The average levels of pAMPK or pACC in the hypothalamus after a single ICV injection of ghrelin or obestatin were expressed as pAMPK/AMPK or pACC/ACC. (A) Phosphorylation of AMPK α after injection of ghrelin; (B) phosphorylation of ACC after injection of ghrelin; (C) phosphorylation of AMPK α after injection of obestatin; (D) phosphorylation of ACC after injection of obestatin. The data, which were expressed as a percent of the control group, represent the mean ± SEM ($n = 6$ chicks/group). *$P < 0.05$ (Experiment 3).

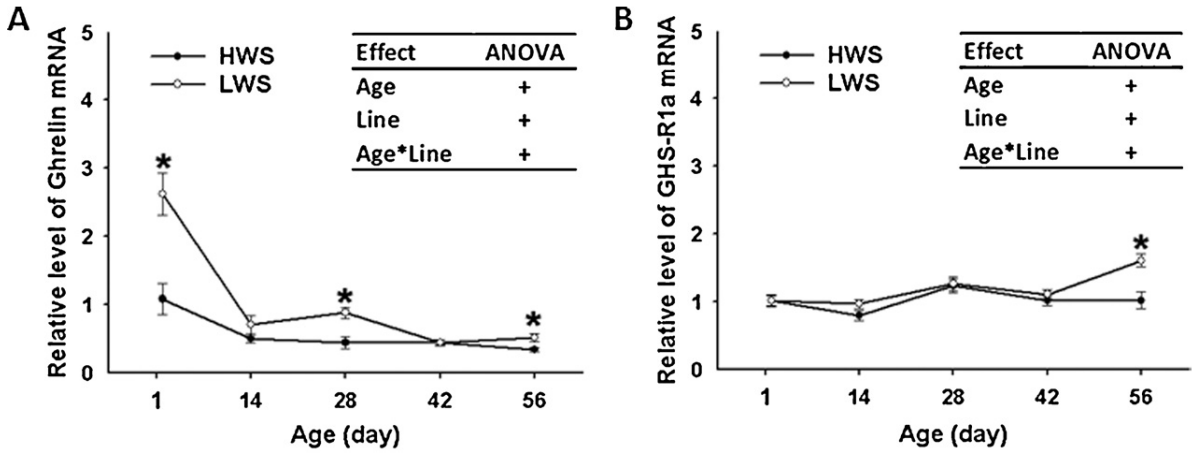

Fig. 7. Gene expression of hypothalamic ghrelin (A) and GHS-R1a (B) in HWS and LWS lines of chickens at 1, 14, 28, 42 and 56 days of age. The data, which were expressed as fold-change compared to the 1-day-old HWS group, represent mean ± SEM ($n = 6$). Asterisk sign (*) denotes significance ($P < 0.05$) in t-test for independent samples and plus sign (+) denotes significance ($P < 0.05$) in ANOVA test.

system of chickens observed in our study, these two downstream factors of the AMPK system may also be involved in the central effects of ghrelin on appetite. Although ghrelin increased ACC α and FAS expression in both lines, it was only significantly in LWS chicks. This stimulatory effect on FAS expression is consistent with previous research in neonatal broiler chicks [38] and opposite to that in rats [36]. The opposite effect on FAS expression between chickens and rodents is consistent with the opposite effect of ghrelin on food intake between avian and mammal species. The up-regulation of ACC α expression provides additional evidence to substantiate the existence of a different AMPK pathway in chick hypothalamus facilitating the anorexigenic effect of ghrelin compared to that of mammals. Furthermore, the difference in the up-regulating magnitude of ACC α and FAS between HWS and LWS lines may contribute to their differential responses to the anorexigenic effect of ghrelin.

To firmly establish the role of AMPK in mediating the anorexigenic effect of ghrelin, we tested the phosphorylation levels of AMPK α and ACC. ACC is a well characterized substrate of AMPK and is often used as an indicator of AMPK activity because AMPK activation decreases ACC activity by phosphorylation [18]. Thus, increased phosphorylated ACC suppresses malonyl-CoA expression, which is considered an inhibitor of feeding. Additionally, the phosphorylation of AMPK α subunits correlates with AMPK activity [52]. We observed that ghrelin significantly decreased hypothalamic phosphorylated AMPK α and ACC in both lines, with the exception of phosphorylated ACC in HWS chicks. These data suggest that hypothalamic AMPK plays an important role in the regulation of food intake in ghrelin-treated chicks. The appetite inhibitory effect of ghrelin in chicks is likely mediated by suppressing AMPK activity. The difference between lines in pACC response to ghrelin's inhibitory effect may also contribute to the different threshold response to ghrelin observed in Experiment 1.

4.2. Acute effect of obestatin in HWS and LWS chickens

Reports on the effects of obestatin on food intake are inconsistent. Some studies reported that obestatin inhibited food intake and suppressed body weight [6,8,53] whereas others reported no effect [54–56]. In one study [10], intraperitoneal obestatin suppressed food intake and body weight gain in rodents with an unusual U-shaped dose–response relationship, which may explain the difficulties in reproducing the effects of obestatin on feeding. Because researches on the effects of obestatin on food intake or body weight have been conducted primarily with rodents, there is need for studies utilizing other species. In our study, obestatin caused a linear dose-dependent increase in food intake in HWS chicks whereas obestatin had no effect on food intake in LWS chicks. This result is in accordance with our previous a report utilizing mature HWS and LWS chickens [32], which showed that ICV injection of low doses of obestatin (0.016, 0.032 and 0.064 nmol) increased food intake in HWS but not LWS chickens. The present study demonstrated that only the high dose of obestatin (4 nmol) stimulated food intake in HWS chicks while lower doses of obestatin (0.016, 0.032 and 0.064 nmol) did not affect food intake in either line (data not shown). These data suggest both a line and an age difference in hypothalamic response to obestatin.

To further clarify if the line difference in hypothalamic response to obestatin was associated with an altered AMPK system, we tested AMPK and related gene expression and phosphorylation of AMPK α and ACC in obestatin treated chicks in both lines. Obestatin did not influence expression of any of these genes or the phosphorylation of AMPK α and ACC, except for a significant inhibitory effect on AMPK β2, CAMKK α and MO 25β subunit expression in HWS chicks. Although the expression of AMPK β2, CAMKK α and MO 25β subunit was inhibited in HWS chicks, the activity and expression level of AMPK did not change with obestatin injection, suggesting that the AMPK system was not altered by obestatin. The orexigenic effect of obestatin in HWS chicks may be mediated by other neuropathways, and thus further study is needed.

4.3. Ontogenic changes of mRNA expression of ghrelin and GHS-R1a

Ghrelin is a natural ligand of the orphan GHS-R1a and is mainly produced in the proventricular region of the stomach, but also expressed in many other tissues such as hypothalamus, liver, intestine, lung and ovary of chickens [12,57,58]. A high level of GHS-R1a expression was detected in the hypothalamus and pituitary, which is consistent with its action of controlling appetite, energy balance and growth hormone (GH) releasing [59]. Unlike in mammals, central ghrelin has an inhibitory effect on food intake by acting through the GHS-R1a receptor in hypothalamus [30], whereas an increase in peripheral circulating ghrelin does not cause the promotion of food intake in chickens [57]. In the present study, we found that LWS chickens had significantly higher levels of ghrelin mRNA in the hypothalamus than HWS chickens. On the first day after hatch, the expression level was 2.6-fold greater in LWS than in HWS chicks. This result is consistent with the known effect of ghrelin on food intake and the fact that LWS chickens are hypophagic relative to the HWS chickens, which are hyperphagic. These different expression levels may also contribute to the different threshold response to ghrelin observed in Experiment 1. The expression of GHS-R1a was greater in LWS than in HWS chickens at 56 days of age. However, the mRNA level of GHS-R1a in the two lines showed no difference from 1 to 42 days of age. Therefore, neonatal HWS and LWS chicks showed a similar mRNA expression pattern of GHS-R1a, and GHS-R1a may not contribute to the different threshold response to ghrelin in these two lines.

5. Conclusions

Acute ICV injection of ghrelin inhibits hypothalamic AMPK related gene expression and phosphorylation of AMPK α and ACC in chicks, suggesting that inhibition of hypothalamic AMPK has an important role in the anorexigenic effect of ghrelin. Comparing chicks from lines developed from a long-term selection from a common founder population, there was a lower threshold for the anorexigenic effect of ICV injected ghrelin in LWS than in HWS chicks. These differential thresholds may be attributed to differences in hypothalamic AMPK signaling. The ICV injection of ghrelin caused a different magnitude of AMPK related factor mRNA expression and phosphorylation inhibition in these two lines. Additionally, the mRNA level of ghrelin in hypothalamus was significantly higher in LWS than HWS, which may contribute to the different threshold response to ghrelin in these two lines. Our results also demonstrate that ICV injection of obestatin had a different appetite regulation effect in these two lines; orexigenic in HWS but not in LWS chicks. This differential response was not caused by the alteration of the AMPK pathway and requires further clarification. These results imply a genetic variation in feeding responses for central ghrelin and obestatin in HWS and LWS lines and may have implications for other species.

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References

[1] Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 1999;402:656–60.

[2] Kaiya H, Miyazato M, Kangawa K, Peter RE, Unniappan S. Ghrelin: a multifunctional hormone in non-mammalian vertebrates. Comp Biochem Physiol A Mol Integr Physiol 2008;149:109–28.
[3] De Vriese C, Delporte C. Ghrelin: a new peptide regulating growth hormone release and food intake. Int J Biochem Cell Biol 2008;40:1420–4.
[4] Kojima M, Kangawa K. Ghrelin: structure and function. Physiol Rev 2005;85:495–522.
[5] Peeters TL. Ghrelin: a new player in the control of gastrointestinal functions. Gut 2005;54:1638–49.
[6] Zhang JV, Ren PG, Avsian-Kretchmer O, Luo CW, Rauch R, Klein C, et al. Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. Science 2005;310:996–9.
[7] Leite-Moreira AF, Soares JB. Physiological, pathological and potential therapeutic roles of ghrelin. Drug Discov Today 2007;12:276–88.
[8] Bresciani E, Rapetti D, Dona F, Bulgarelli I, Tamiazzo L, Locatelli V, et al. Obestatin inhibits feeding but does not modulate GH and corticosterone secretion in the rat. J Endocrinol Invest 2006;29:RC16–8.
[9] Carlini VP, Schioth HB, Debarioglio SR. Obestatin improves memory performance and causes anxiolytic effects in rats. Biochem Biophys Res Commun 2007;352:907–12.
[10] Lagaud GJ, Young A, Acena A, Morton MF, Barrett TD, Shankley NP. Obestatin reduces food intake and suppresses body weight gain in rodents. Biochem Biophys Res Commun 2007;357:264–9.
[11] Kaiya H, Van Der Geyten S, Kojima M, Hosoda H, Kitajima Y, Matsumoto M, et al. Chicken ghrelin: purification, cDNA cloning, and biological activity. Endocrinology 2002;143:3454–63.
[12] Chen LL, Jiang QY, Zhu XT, Shu G, Bin YF, Wang XQ, et al. Ghrelin ligand-receptor mRNA expression in hypothalamus, proventriculus and liver of chicken (*Gallus gallus domesticus*): studies on ontogeny and feeding condition. Comp Biochem Physiol A Mol Integr Physiol 2007;147:893–902.
[13] Baudet ML, Harvey S. Ghrelin-induced GH secretion in domestic fowl in vivo and in vitro. J Endocrinol 2003;179:97–105.
[14] Chen LL, Jiang QY, Kaiya H, Zhu XT, Shu G, Kangawa K, et al. Central administration of chicken ghrelin inhibits food intake in adult chickens. In: 9th International Symposium on Avian Endocrinology. 2008. p. 75.
[15] Saito ES, Kaiya H, Tachibana T, Tomonaga S, Denbow DM, Kangawa K, et al. Inhibitory effect of ghrelin on food intake is mediated by the corticotropin-releasing factor system in neonatal chicks. Regul Pept 2005;125:201–8.
[16] Kaiya H, Furuse M, Miyazato M, Kangawa K. Current knowledge of the roles of ghrelin in regulating food intake and energy balance in birds. Gen Comp Endocrinol 2009;163:33–8.
[17] Furuse M, Tachibana T, Ohgushi A, Ando R, Yoshimatsu T, Denbow DM. Intracerebroventricular injection of ghrelin and growth hormone releasing factor inhibits food intake in neonatal chicks. Neurosci Lett 2001;301:123–6.
[18] Munday MR, Carling D, Hardie DG. Negative interactions between phosphorylation of acetyl-CoA carboxylase by the cyclic AMP-dependent and AMP-activated protein kinases. FEBS Lett 1988;235:144–8.
[19] Kahn BB, Alquier T, Carling D, Hardie DG. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metab 2005;1:15–25.
[20] Kim MS, Lee KU. Role of hypothalamic 5′-AMP-activated protein kinase in the regulation of food intake and energy homeostasis. J Mol Med 2005;83:514–20.
[21] Minokoshi Y, Alquier T, Furukawa N, Kim YB, Lee A, Xue B, et al. AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. Nature 2004;428:569–74.
[22] Kubota N, Yano W, Kubota T, Yamauchi T, Itoh S, Kumagai H, et al. Adiponectin stimulates AMP-activated protein kinase in the hypothalamus and increases food intake. Cell Metab 2007;6:55–68.
[23] Marquez GC, Siegel PB, Lewis RM. Genetic diversity and population structure in lines of chickens divergently selected for high and low eight-week weight. Poult Sci 2010;89:2580–8.
[24] Johansson AM, Pettersson ME, Siegel PB, Carlborg O. Genome wide effects of long-term divergent selection. PLoS Genet 2010;6:e1001188.
[25] Dunnington EA, Siegel PB. Long-term divergent selection for eight-week body weight in white Plymouth rock chickens. Poult Sci 1996;75:1168–79.
[26] Siegel PB, Wolford JH. A review of some results of selection for juvenile body weight in chickens. Poult Sci 2003;40:81–91.
[27] Davis JL, Masuoka DT, Gerbrandt LK, Cherkin A. Autoradiographic distribution of L-proline in chicks after intracerebral injection. Physiol Behav 1979;22:693–5.
[28] Furuse M, Ando R, Bungo T, Ao R, Shimojo M, Masuda Y. Intracerebroventricular injection of orexins does not stimulate food intake in neonatal chicks. Br Poult Sci 1999;40:698–700.
[29] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–8.
[30] Saito ES, Kaiya H, Takagi T, Yamasaki I, Denbow DM, Kangawa K, et al. Chicken ghrelin and growth hormone-releasing peptide-2 inhibit food intake of neonatal chicks. Eur J Pharmacol 2002;453:75–9.
[31] Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, et al. A role for ghrelin in the central regulation of feeding. Nature 2001;409:194–8.
[32] Xu P, Siegel PB, Denbow DM. Ghrelin and obestatin influence on food and water intake in lines of chickens selected for high and low body weight. NC: Poultry Science Raleigh: Abstract 420P; 2009.
[33] Kola B, Hubina E, Tucci SA, Kirkham TC, Garcia EA, Mitchell SE, et al. Cannabinoids and ghrelin have both central and peripheral metabolic and cardiac effects via AMP-activated protein kinase. J Biol Chem 2005;280:25196–201.
[34] Kola B, Farkas I, Christ-Crain M, Wittmann G, Lolli F, Amin F, et al. The orexigenic effect of ghrelin is mediated through central activation of the endogenous cannabinoid system. PLoS One 2008;3:e1797.
[35] Kola B, Korbonits M. Shedding light on the intricate puzzle of ghrelin's effects on appetite regulation. J Endocrinol 2009;202:191–8.
[36] Lopez M, Lage R, Saha AK, Perez-Tilve D, Vazquez MJ, Varela L, et al. Hypothalamic fatty acid metabolism mediates the orexigenic action of ghrelin. Cell Metab 2008;7:389–99.
[37] Sangiao-Alvarellos S, Varela L, Vazquez MJ, Da Boit K, Saha AK, Cordido F, et al. Influence of ghrelin and growth hormone deficiency on AMP-activated protein kinase and hypothalamic lipid metabolism. J Neuroendocrinol 2010;22:543–56.
[38] Buyse J, Janssen S, Geelissen S, Swennen Q, Kaiya H, Darras VM, et al. Ghrelin modulates fatty acid synthase and related transcription factor mRNA levels in a tissue-specific manner in neonatal broiler chicks. Peptides 2009;30:1342–7.
[39] Ojuka EO, Jones TE, Nolte LA, Chen M, Wamhoff BR, Sturek M, et al. Regulation of GLUT4 biogenesis in muscle: evidence for involvement of AMPK and Ca(2+). Am J Physiol Endocrinol Metab 2002;282:E1008–13.
[40] Hudson ER, Pan DA, James J, Lucocq JM, Hawley SA, Green KA, et al. A novel domain in AMP-activated protein kinase causes glycogen storage bodies similar to those seen in hereditary cardiac arrhythmias. Curr Biol 2003;13:861–6.
[41] Adams J, Chen ZP, Van Denderen BJ, Morton CJ, Parker MW, Witters LA, et al. Intrasteric control of AMPK via the gamma1 subunit AMP allosteric regulatory site. Protein Sci 2004;13:155–65.
[42] Carling D, Zammit VA, Hardie DG. A common bicyclic protein kinase cascade inactivates the regulatory enzymes of fatty acid and cholesterol biosynthesis. FEBS Lett 1987;223:217–22.
[43] Hawley SA, Boudeau J, Reid JL, Mustard KJ, Udd L, Makela TP, et al. Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. J Biol 2003;2:28.
[44] Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, et al. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. Curr Biol 2003;13:2004–8.
[45] Carling D, Sanders MJ, Woods A. The regulation of AMP-activated protein kinase by upstream kinases. Int J Obes (Lond) 2008;32Suppl4:S55–9.
[46] Hawley SA, Pan DA, Mustard KJ, Ross L, Bain J, Edelman AM, et al. Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. Cell Metab 2005;2:9–19.
[47] Woods A, Dickerson K, Heath R, Hong SP, Momcilovic M, Johnstone SR, et al. Ca^{2+}/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. Cell Metab 2005;2:21–33.
[48] Tokumitsu H, Brickey DA, Glod J, Hidaka H, Sikela J, Soderling TR. Activation mechanisms for Ca^{2+}/calmodulin-dependent protein kinase. IV. Identification of a brain CaM-kinase IV kinase. J Biol Chem 1994;269:28640–7.
[49] Hawley SA, Selbert MA, Goldstein EG, Edelman AM, Carling D, Hardie DG. 5′-AMP activates the AMP-activated protein kinase cascade, and Ca^{2+}/calmodulin activates the calmodulin-dependent protein kinase I cascade, via three independent mechanisms. J Biol Chem 1995;270:27186–91.
[50] Kola B, Boscaro M, Rutter GA, Grossman AB, Korbonits M. Expanding role of AMPK in endocrinology. Trends Endocrinol Metab 2006;17:205–15.
[51] Winder WW. Energy-sensing and signaling by AMP-activated protein kinase in skeletal muscle. J Appl Physiol 2001;91:1017–28.
[52] Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 2001;108:1167–74.
[53] Green BD, Irwin N, Flatt PR. Direct and indirect effects of obestatin peptides on food intake and the regulation of glucose homeostasis and insulin secretion in mice. Peptides 2007;28:981–7.
[54] Gourcerol G, St-Pierre DH, Tache Y. Lack of obestatin effects on food intake: should obestatin be renamed ghrelin-associated peptide (GAP)? Regul Pept 2007;141:1–7.
[55] Seoane LM, Al-Massadi O, Pazos Y, Pagotto U, Casanueva FF. Central obestatin administration does not modify either spontaneous or ghrelin-induced food intake in rats. J Endocrinol Invest 2006;29:RC13–5.
[56] Sibilia V, Bresciani E, Lattuada N, Rapetti D, Locatelli V, De Luca V, et al. Intracerebroventricular acute and chronic administration of obestatin minimally affect food intake but not weight gain in the rat. J Endocrinol Invest 2006;29:RC31–4.
[57] Kaiya H, Saito ES, Tachibana T, Furuse M, Kangawa K. Changes in ghrelin levels of plasma and proventriculus and ghrelin mRNA of proventriculus in fasted and refed layer chicks. Domest Anim Endocrinol 2007;32:247–59.
[58] Richards MP, Poch SM, McMurtry JP. Characterization of turkey and chicken ghrelin genes, and regulation of ghrelin and ghrelin receptor mRNA levels in broiler chickens. Gen Comp Endocrinol 2006;145:298–310.
[59] Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, et al. A receptor in pituitary and hypothalamus that functions in growth hormone release. Science 1996;273:974–7.

CHAPTER 6

Genetic Selection for Body Weight in Chickens Altered Hypothalamic Feeding Response to Ghrelin by Corticotrophin Releasing Hormone, and to Obestatin by Leptin Receptor

ABSTRACT This study was designed to determine feeding responses to ghrelin and obestatin in two lines of chickens that had undergone 52 generations of selection for high (HWS) or low (LWS) 56-day body weight. If different, were they attributed to the alteration of the neuronal corticotrophin-releasing hormone (CRH) pathway or obesity and appetite related factors. Intracerebroventricular (ICV) injection of 0.4 of nmole ghrelin strongly suppressed food intake in both lines. Inhibited in LWS but not HWS chicks were hypothalamic CRH mRNA and protein and mRNA of glucocorticoid receptor (GR), 20-hydroxysteroid dehydrogenase (20HSD), carnitine palmitoyltransferase I (CPT1) and fat mass and obesity associated (FTO). Not affected in either line were GR protein and the mRNA of thyrotropin-releasing hormone (TRH), leptin receptor (LEPR), neuropeptide Y (NPY), pro-opiomelanocortin (POMC) and orexin. An ICV injection of 4 nmole of obestatin increased food intake in HWS but not LWS chicks. Although obestatin decreased GR mRNA in HWS chicks, neither the mRNA nor protein of CRH and 20HSD was altered by obestatin. Obestatin decreased the mRNA of LEPR and FTO in HWS but not LWS chicks. Not affected by obestatin were mRNA of CPT1, NPY, POMC and orexin, while the mRNA of TRH was inhibited by obestatin in both lines. These results show that selection for body weight has altered

hypothalamic response to ghrelin by CRH pathway, CPT1 and FTO, and to obestatin by LEPR and FTO.

Key words: obesity, chicken, ghrelin, obestatin, body weight

6.1. INTRODUCTION

Ghrelin, a 28-amino acid peptide produced mainly by the stomach, is involved in the regulation of body weight and food intake in both mammals (Kojima et al., 1999; Cummings et al., 2003) and avians (Furuse et al., 2001; Chen et al., 2008). The effect of ghrelin on food intake in avian and mammalian species, however, is a polar opposite. That is while intracerebroventricular (ICV), intravenous (IC) or subcutaneous (SC) administration of ghrelin can potently stimulate food intake in mammals (Kamegai et al., 2001; Nakazato et al., 2001; Healy et al., 2011). In avians, it inhibits feeding regardless of age, sex, and time of injection or dosages (Furuse et al., 2001; Saito et al., 2002, 2005; Xu et al., 2011). Obestatin, a 23 amino acid peptide encoded by the same gene as ghrelin, reduces food intake and body weight gain in rats (Carlini et al., 2007; Lagaud et al., 2007), whereas it increases food intake in 5-day-old high body weight (HWS) chicks (Xu et al., 2011). These two highly related appetite regulatory peptides constitute a dynamic network with interacting effector elements.

In mammals, the orexigenic effect of ghrelin is mediated by activating arcuate nucleus (ARC) neuropeptides, such as neuropeptide Y (NPY) and orexin (Kamegai et al., 2001; Toshinai et al., 2003; Chen et al., 2004), and hypothalamic AMP-activated protein kinase (AMPK), which then stimulate fatty acid oxidation and inhibit fatty acid synthesis (Kola

et al., 2005; Lopez et al., 2008). In chickens, however, the anorexigenic effect of ghrelin is independent of the NPY pathway and mediated by corticotropin-regulating factor (CRF) (Saito et al., 2005). The suppression of AMPK and its downstream fatty acid metabolism related pathway also plays a role in the inhibitory effect of ghrelin on food intake in chickens (Xu et al., 2011). These results suggest that the appetite regulatory effects of ghrelin in mammals and avians involve different cell signaling pathways or molecular mechanisms.

The lines of chickens used in the present study had undergone 52 generations of divergent selection for high (HWS) or low (LWS) body weight at 56 days of age from a common founder White Rock population (Marquez et al., 2010). These lines of chickens differ in body weight at selection age by more than 10-fold (Siegel et al., 2003). They also exhibit hypophagic (LWS) and hyperphagic (HWS) characteristics (Johansson et al., 2010). Our objectives were to evaluate whether long-term divergent selection for body weight has altered central feeding response to ghrelin and obestatin by modifying the neuronal CRH pathway or obesity and appetite related factors.

6.2. MATERIALS AND METHODS

6.2.1. Animals

Eggs from same-age parents from each line were incubated in the same machine for each experiment. After hatch, chicks were placed as flocks in electric heated batteries with raised wire floors for 2 days, and then transferred to individual cages in a room at 32±2°C. A mash diet (20% crude protein, 2684 kcal ME/kg) and water were provided *ad*

libitum. The individual cages were designed to allow visual and auditory but not physical contact among chicks. All trials were conducted at 5 days post-hatching, which allowed time for chicks to adjust to this environment and for absorption of yolk. Data were collected from both lines simultaneously and injections followed the sequence: HWS, LWS, HWS, and so forth. Experimental procedures were approved by the Virginia Tech Animal Care and Use Committee.

6.2.2. Peptides and ICV Injection

Chicken ghrelin and obestatin were synthesized by the United States Department of Agriculture (Washington, DC, USA) according to the peptide sequence of chicken ghrelin and obestatin. The peptides were dissolved in artificial cerebrospinal fluid (aCSF) with 0.1% Evans Blue dye to facilitate the localization of the injection site. The ICV injection of 5 μL of each substance was made using a microsyringe according to the method of Davis et al (1979). The procedure does not induce physiological stress (Furuse et al., 1999).

6.2.3. Experiment 1: Quantification of Hypothalamic mRNA Expression by Real-time RT-PCR

Twelve male chicks from each line were fasted 180 min, then weighed and randomly assigned to receive either 0 or 0.4 nmole ghrelin by ICV injection. A separate trial followed the same procedure with chicks given 0 or 4 nmole obestatin by ICV injection. The ICV injection concentration used was based on a previous experiment (Xu et al., 2011). After injection, chicks were provided free access to both food and water. Food

intake data were analyzed by ANOVA using GLM (SAS Institute Inc, Cary, NC, USA). The model included line, treatment (trt) and the interactions among them. Treatment effect was also analyzed in each line. All data were expressed as mean ± S.E.M. and the level of significance was set a priori at $P<0.05$. All food intakes were expressed as percentage of food consumption on a body weight basis. One hour after injection, chicks were decapitated and the preoptic-hypothalamic area was dissected from each brain using the landmarks of the optic chiasm rostrally, and the mammillary bodies caudally. Dissected hypothalamic tissues were quickly frozen in liquid nitrogen, and stored at -80 °C for later analysis.

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

Real-time RT-PCR was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems, Inc., Carlsbad, CA, USA). One μL of 4-fold diluted RT product was used for PCR in a final volume of 20 μL containing 10 μL 2×Fast SYBR Green Master Mix (Applied Biosystems, Inc.) and 0.5 μM primer mix (Table 6.1). Tested genes include 20-hydroxysteroid dehydrogenase (20HSD), corticotrophin-releasing hormone (CRH), glucocorticoid receptor (GR), thyrotropin-releasing hormone (TRH), carnitine palmitoyltransferase I (CPT1), leptin receptor (LEPR), fat mass and obesity associated (FTO), NPY, pro-opiomelanocortin (POMC) and orexin. Chicken glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as a reference gene for normalization purposes. The expression of GAPDH mRNA was similar for both lines. Thermal cycling conditions were: initial denaturation (20 s at 95°C), then a two-step amplification program (3 s at 95°C, 30 s at 60°C) repeated 40 times. Real-time RT-PCR data were analyzed by the relative quantification ($\Delta\Delta C_T$) method according to instructions from Applied Biosystems, Inc. (Carlsbad, CA, USA). The statistical model used was the same as that in food intake analysis. All mRNA expression levels were expressed as fold-change compared to the HWS aCSF group.

Table 6. 1. Nucleotide sequences of specific primers.

[1]Target gene	GenBank accession	PCR products (bp)	Primer sequences
20HSD	DQ079061	88	F: 5'-CGGCTCCAACAAAGGGATT-3'
			R: 5'-AGGGTCTCGGGCAGTAAGGT-3'
CRH	NM_001123031	68	F: 5'-TTAGGACCAAATCTGATCACAAGTTC-3'
			R: 5'-CGGCGTCCTTCCTCTGC-3'
GR	DQ227738	102	F: 5'-CTTCCATCCGCCCTTCA -3'
			R: 5'-TCGCATCTGTTTCACCC-3'
TRH	NM_001030383	76	F: 5'-TTGCAGAAAATCACAATGCCA-3'
			R: 5'-TGAGGCAAACACCAGACAAGG-3'
CPT1	AY675193	110	F: 5'-ACTATCAACGAGTCAGACACCACA-3'
			R: 5'-AAACACCGTAACCATCATCAGC-3'

LEPR	NM_204323	87	F: 5'-GCATCTCTGCATCTCAGGAAAGA-3'
			R: 5'-GCAGGCTACAAACTAACAGATCCA-3'
FTO	XM_414084	124	F: 5'-TGAAGGTAGCGTGGGACATAGA-3'
			R: 5'-GGTGAAAAGCCAGCCAGAAC-3'
NPY	M87294	83	F: 5'-ATGAGGCTGTGGGTGTCGGT-3'
			R: 5'-TTGGAGGGGTACGCTTCTGC-3'
POMC	NM_001031098	123	F: 5'-GGAGGAAAAGAAGGATGGAGG-3'
			R: 5'-AGAGTCATCAGCGGGGTCTG-3'
Orexin	NM_204185	86	F: 5'-GCTCCTGCCGTATCTACGACCT-3'
			R: 5'-CGGGATGCTCTTCCTCTTGC-3'
GAPDH	NM_204305	141	F: 5'-TGGCATCCAAGGAGTGAGC-3'
			R: 5'-GGGGAGACAGAAGGGAACAG-3'

[1]20HSD (20-hydroxysteroid dehydrogenase), CRH (corticotrophin-releasing hormone), GR (glucocorticoid receptor), TRH (thyrotropin-releasing hormone), CPT1 (carnitine palmitoyltransferase I), LEPR (leptin receptor), FTO (fat mass and obesity associated), NPY (neuropeptide Y), POMC (pro-opiomelanocortin), and GAPDH (glyceraldehyde 3-phosphate dehydrogenase).

6.2.5. Experiment 2: Quantification of Hypothalamic CRH and GR Protein by Western Blotting

Twelve male chicks from each line received the same treatment as described in Experiment 1. One hour after injection, they were decapitated and the preoptic-hypothalamic area dissected from each brain. Proteins were extracted with SDS lysis buffer (25 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol and 5% β-mercaptoethanol). Proteins extracts were separated on a 12.5% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Blots were blocked in 5% bovine serum albumin (BSA) dissolved in Tris-buffered saline with Tween-20 (TBST, 20 mmole Tris, pH 7.4, 150 mmole NaCl, and 0.1% Tween-20) for 180 min at 4°C and then briefly washed twice for 5 min each in TBST. The blots were incubated overnight at 4°C with primary antibodies against CRH (1:3000; Bachem Americas, Inc., Torrance, CA, USA), GR (1:1000; AbD Serotec, MorphoSys U.S. Inc., Raleigh, NC, USA) and β-actin (1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA) in TBST with 1% BSA. Each blot was washed twice for 5 min, three times for 10 min and then incubated with IgG horseradish

peroxidase-conjugated antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at room temperature for 60 min. Finally, the blots were washed and detected by enhanced chemiluminescence (ECL). A chemiluminescent substrate, SuperSignal West Pico (Pierce, Rockford, IL, USA), was applied according to instructions of the manufacturer and the signal was detected using a Gel Doc XR System (Bio-Rad, Hercules, CA, USA). Densitometry analyses were performed using Quantity One (Bio-Rad, Hercules, CA, USA) image analysis software. CRH and GR protein expression levels were expressed as a density ratio (CRH/β-actin, GR/β-actin). The statistical model used was the same as that in Experiment 1.

6.3. RESULTS

6.3.1. Food Intake after ICV Injection

The effects of 0.4 nmole ghrelin and 4 nmole obestatin ICV injections on food intake of HWS and LWS chicks are shown in Fig. 1. At 30 and 60 min post-injection, ghrelin significantly decreased food intake in both lines (Fig. 6.1A and 6.1B), whereas obestatin significantly increased food intake of HWS (Fig. 6.1C) but not LWS chickens (Fig. 6.1D).

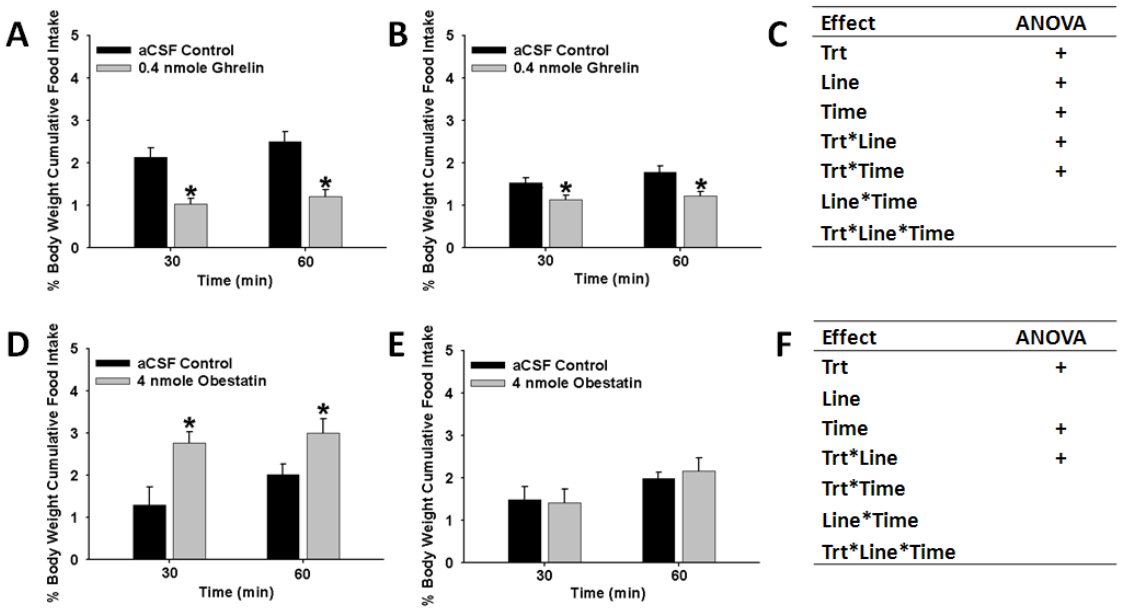

Fig. 6. 1. Cumulative food intake expressed as percent body weight of 5-day HWS and LWS lines of chicks following ICV injection of 0.4 nmole ghrelin or 4 nmole obestatin (n=12 chicks/group). (A) HWS chicks injected with ghrelin; (B) LWS chicks injected with ghrelin; (C) ANOVA test for HWS and LWS chicks injected with ghrelin; (D) HWS chicks injected with obestatin; (E) LWS chicks injected with obestatin; (F) ANOVA test for HWS and LWS chicks injected with obestatin. Asterisk sign (*) and plus sign (+) denotes significance (P<0.05).

6.*3.2. mRNA Expression of 20HSD, CRH, GR and TRH in Preoptic-hypothalamus*

The effects of ICV injection of 0.4 nmole ghrelin on mRNA expression of 20HSD, CRH, GR and TRH are illustrated in Fig. 6.2. There was a significant trt effect for 20HSD. Ghrelin significantly decreased preoptic-hypothalamic 20HSD expression in LWS but not HWS chicks. For CRH, the trt, line and trt×line interaction effects were significant. The interaction was a result of ghrelin significantly decreasing preoptic-hypothalamic

CRH expression in LWS but not HWS chicks. The mRNA expressions of GR and TRH were not affected by ghrelin.

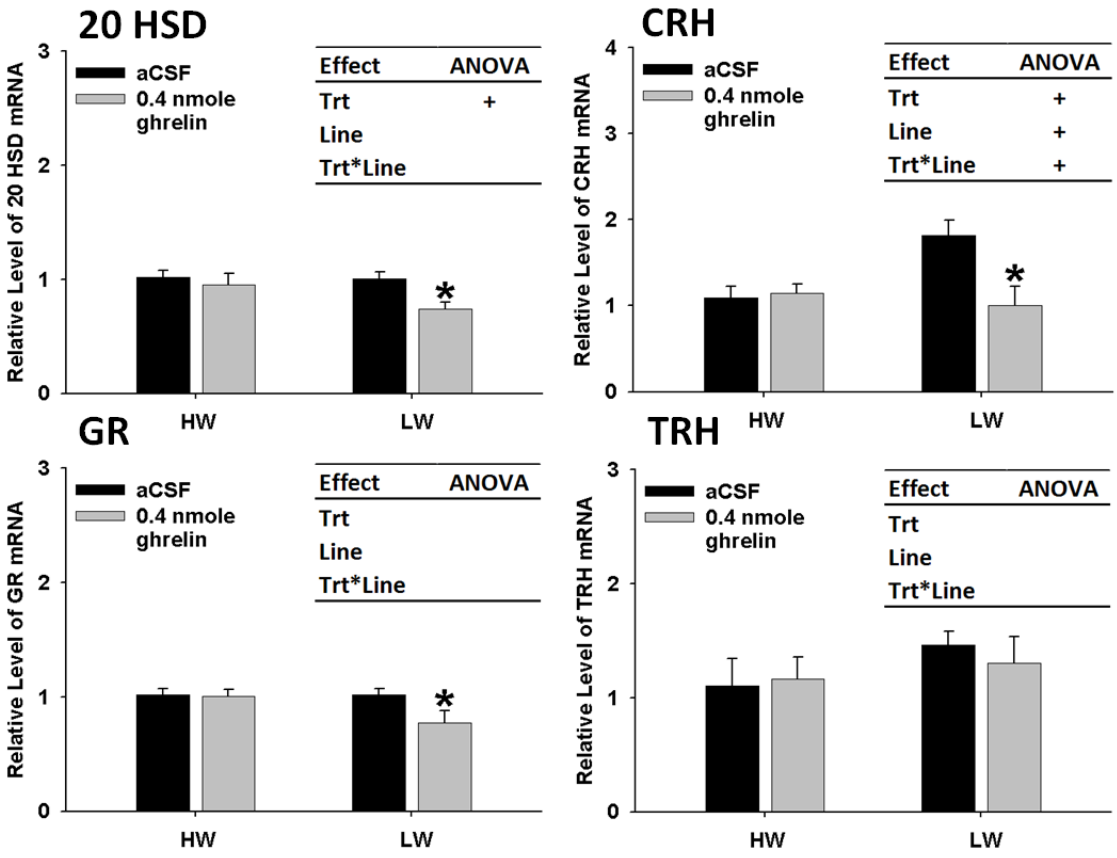

Fig. 6. 2. Gene expression of hypothalamic 20 HSD, CRH, GR and TRH in 5-day HWS and LWS lines of chicks following ICV injection of 0.4 nmole ghrelin. The data expressed as fold-change compared to the HWS aCSF group, represent mean ± SEM (n=6 chicks/group). Asterisk sign (*) and plus sign (+) denotes significance (P<0.05).

Although obestatin did not affect mRNA expressions of 20HSD and CRH, there was an acute effect of obestatin on the expression of GR and TRH in HWS and LWS chicks (Fig. 6.3). For GR, there were significant trt and line effects and obestatin caused a

significant reduction in HWS but not LWS chicks, while for TRH, the trt effect was significant and obestatin significantly deceased TRH expression in both lines.

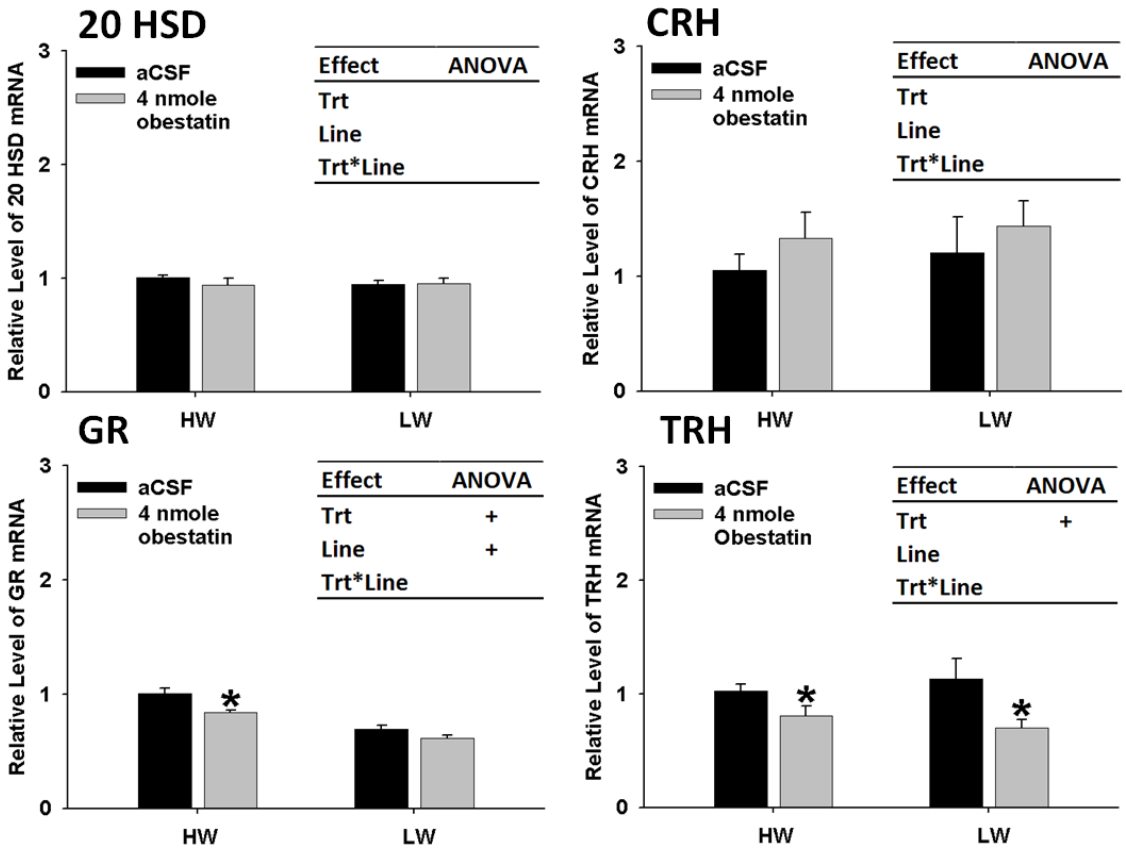

Fig. 6. 3. Gene expression of hypothalamic 20 HSD, CRH, GR and TRH in 5-day HWS and LWS lines of chicks following ICV injection of 4 nmole obestatin. The data expressed as fold-change compared to the HWS aCSF group, represent mean ± SEM (n=6 chicks/group). Asterisk sign (*) and plus sign (+) denotes significance (P<0.05).

6.3.3. mRNA Expression of Obesity and Appetite Related Factors in Preoptic-hypothalamus

The effect of ICV injection of 0.4 nmole ghrelin on the mRNA expression of obesity related factors including CPT1, LEPR and FTO, and appetite regulatory neuropeptides including NPY, POMC and orexin is shown in Fig. 6.4. There was a significant trt effect for CPT1 and ghrelin significantly decreased its expression in LWS but not HWS chicks. For FTO, the trt and trt×line interaction effects were significant. ICV injection of ghrelin resulted in significantly lower levels of FTO in the preoptic-hypothalamus compared to the aCSF control chicks. Ghrelin had no effect on LEPR, NPY, POMC or orexin mRNA expression in either line.

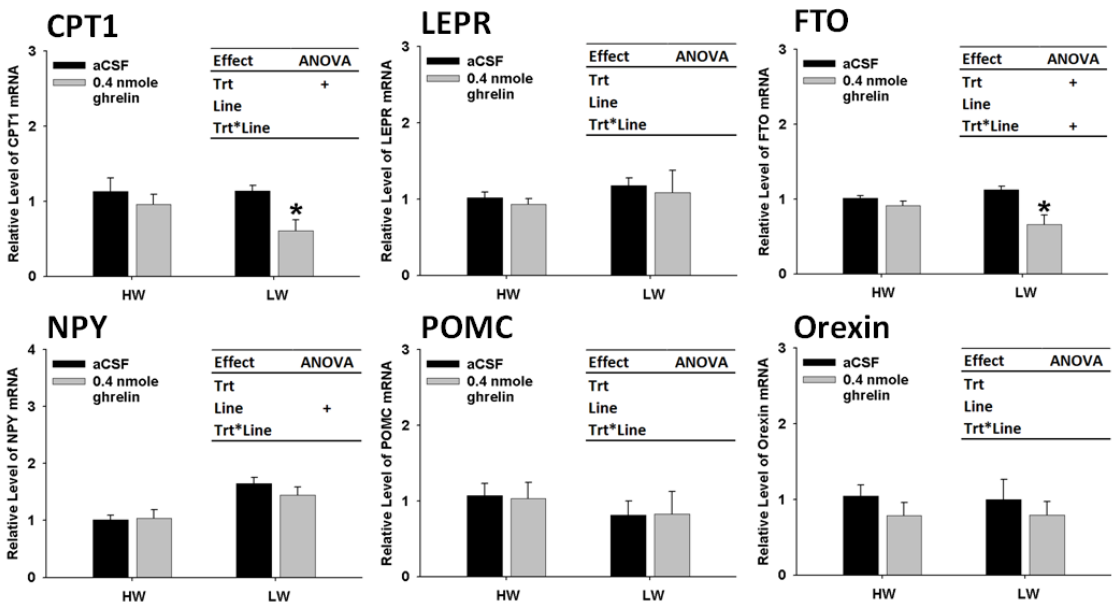

Fig. 6. 4. Gene expression of hypothalamic CPT1, LEPR, FTO, NPY, POMC and orexin in 5-day HWS and LWS lines of chicks following ICV injection of 0.4 nmole ghrelin. The data expressed as fold-change compared to the HWS aCSF group, represent mean ± SEM (n=6 chicks/group). Asterisk sign (*) and plus sign (+) denotes significance (P<0.05).

Obestatin did not influence hypothalamic expression of CPT1, NPY, POMC or orexin in either line (Fig. 6.5). For LEPR, there were significant trt, line and trt×line effects, while for FTO, there were line and trt×line interaction not trt effects. ICV injection of obestatin effectively inhibited LEPR and FTO expression in HWS but not LWS chicks.

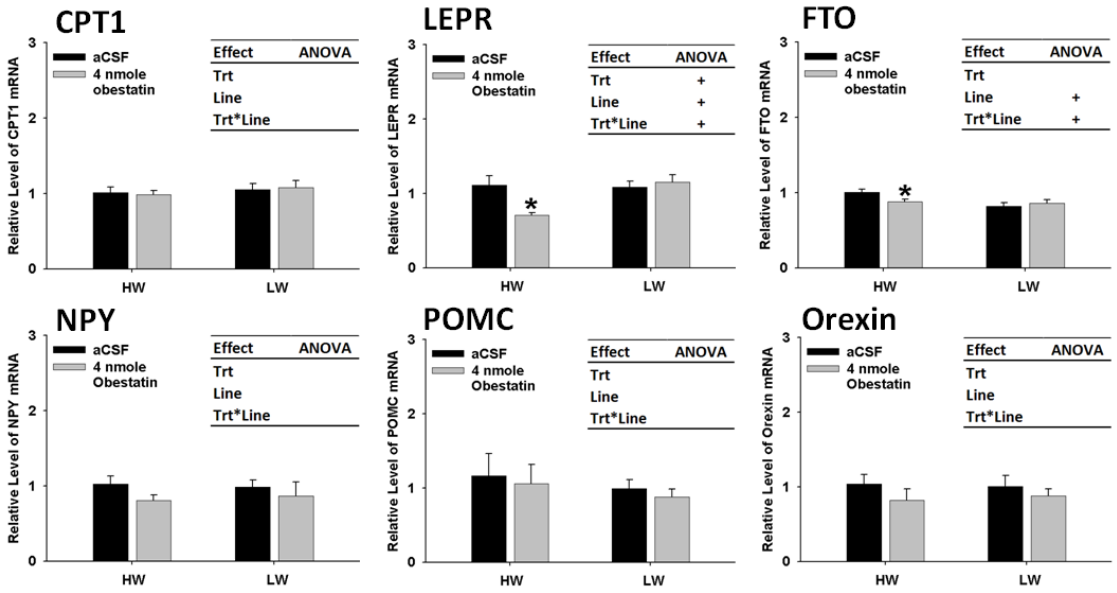

Fig. 6. 5. Gene expression of hypothalamic CPT1, LEPR, FTO, NPY, POMC and orexin in 5-day HWS and LWS lines of chicks following ICV injection of 4 nmole obestatin. The data expressed as fold-change compared to the HWS aCSF group, represent mean ± SEM (n=6 chicks/group). Asterisk sign (*) and plus sign (+) denotes significance (P<0.05).

6.3.4. CRH and GR Protein Expression in Preoptic-hypothalamus

By western blot, the effects of central ghrelin or obestatin administration on preoptic-hypothalamic CRH and GR protein levels were measured in 5-day-old HWS and LWS chicks (Fig. 6.6). Ghrelin significantly downregulated CRH in LWS but not HWS chicks

and the effects of trt and line were significant (Fig. 6.6A). The protein level of GR was not affected by ghrelin (Fig. 6.6B). There was a line effect for the preoptic-hypothalamic GR (HWS > LWS), but the trt and trt×line effects were not significant. Obestatin did not alter CRH (Fig. 6.6C) or GR (Fig. 6.6D) protein levels in either HWS or LWS chicks.

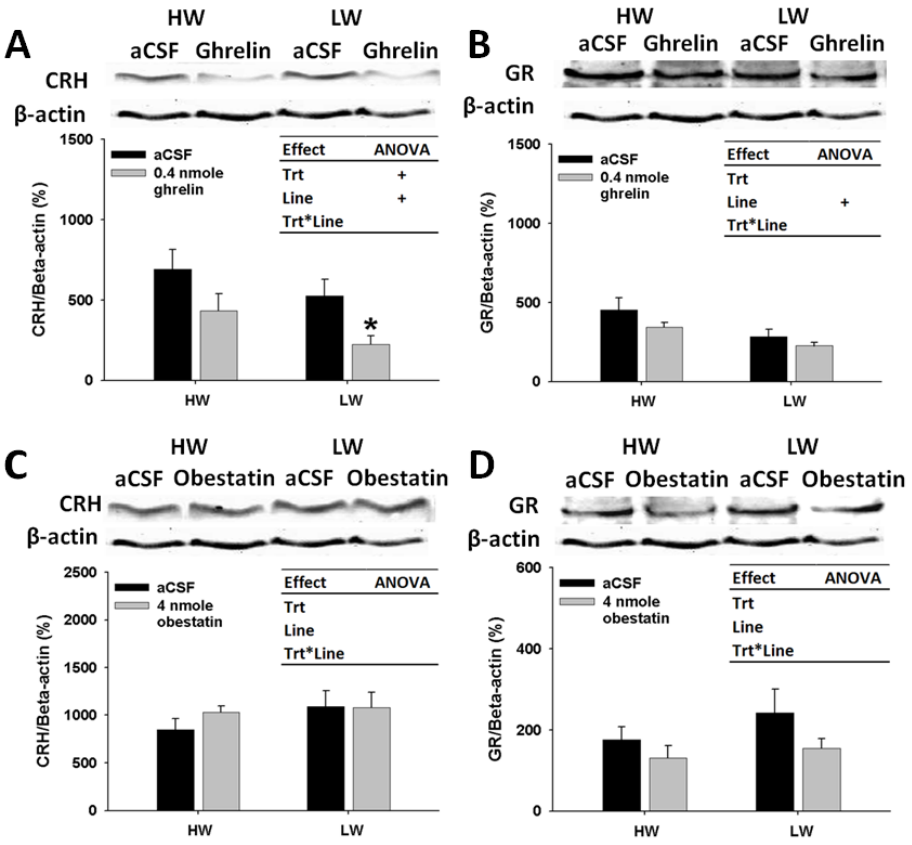

Fig. 6. 6. Effects of ICV injection of ghrelin or obestatin on preoptic-hypothalamic CRH and GR protein expression in 5-day HWS and LWS lines of chicks. Representative Western blots of CRH and GR in both lines of chicks are shown. The average levels of CRH and GR in the preoptic-hypothalamus after a single ICV injection of ghrelin or obestatin were expressed as CRH/β-actin and GR/β-actin. (A) CRH expression after injection of ghrelin; (B) GR expression after injection of ghrelin; (C) CRH expression

after injection of obestatin; (D) GR expression after injection of obestatin. The data expressed as a percent of the control group, represent the mean ± SEM (n=6 chicks/group). Asterisk sign (*) and plus sign (+) denotes significance (P<0.05).

6.4. DISCUSSION

6.4.1. Ghrelin

As opposed to the orexigenic effect of ghrelin in mammals (Cummings et al., 2003), ICV injection of chicken ghrelin inhibited food intake in chickens regardless of line, age, or sex (Furuse et al., 2001; Saito et al., 2002, 2005; Chen et al., 2008; Xu et al., 2011). In our previous study (Xu et al., 2011), hyperphagic HWS chicks had a higher threshold of feeding response to ICV injection of ghrelin than hypophagic LWS chicks. According to the differential dosage effect of ghrelin in these two lines, the most effective dose (0.4 nmole) of ghrelin was used in the present study. The results were consistent in showing that at this dose, ghrelin strongly suppressed food intake in both lines.

In chickens, hypothalamic ghrelin closely interacts with CRH and subsequently the hypothalamic-pituitary-adrenal (HPA) axis (Lu et al., 2008), with the anorexigenic effect of ghrelin mediated by the hypothalamic CRF pathway (Saito et al., 2005). A previous study with HWS and LWS chicks (Cline et al., 2009), although ICV injections of CRF inhibited food intake in both lines, the threshold dose was lower in LWS than HWS chicks, suggesting selection for body weight has altered the central CRF anorexigenic system. It has been reported that ICV injection of ghrelin increased plasma corticosterone levels (Saito et al., 2005), our results, however, demonstrated that ICV injection of

ghrelin inhibited the mRNA and protein expression of hypothalamic CRH in LWS chicks. This inconsistency may be explained by the negative feedback system of the HPA axis. Naively, high levels of plasma corticosterone caused by ICV injection of ghrelin may affect the hypothalamus in a negative feedback cycle to decrease hypothalamic CRH expression. Additionally, the difference between lines in hypothalamic CRH response to ghrelin's inhibitory effect may contribute to their differential responses to the anorexigenic effect of ghrelin.

Corticosterone induced repression of CRH is mediated through ligand-dependent interactions with GR, which involves direct binding of GR to the promoter of CRH (Dahlman-Wright et al., 1992) or indirect protein-protein interactions (Wissink et al., 1997). The full-length cDNA for chicken GR has been cloned and its tissue-specific expression was characterized (Kwok et al., 2007). As in mammals (Lu et al., 2006), corticosterone or aldosterone induces chicken GR transcriptional activity and its translocation into the nucleus is dose-dependent (Proszkowiec-Weglarz et al., 2010). The higher content of hypothalamic GR mediates the augmented negative feedback signal from corticosterone, which then contributes to the repression of CRH (Yuan et al., 2009). Our results that mRNA expression of hypothalamic GR is inhibited by ICV injection of ghrelin in LWS but not HWS chicks is consistent with the lower level of CRH in ghrelin-treated LWS chicks. The downregulation of GR may blunt the negative feedback of corticosterone and compensate corticosterone-induced suppression of CRH. The line-specific hypothalamic GR response to central ghrelin we observed may account, at least in part, for differences between lines in the hypothalamic response to ghrelin. However, GR appears to be regulated differently at the transcriptional and translational levels. That

is while ghrelin had a tendency to decrease hypothalamic GR protein levels in both lines, it suppressed mRNA but not protein expression in LWS chicks. This inconsistency may be attributed to the latency of alteration of translation compared to the transcription by blood corticosterone feedback.

Chicken 20HSD, an abundantly and ubiquitously expressed steroid dehydrogenase, plays an important role in the biosynthesis and inactivation of steroid hormones (Bryndova et al., 2006). As in mammalian 20βHSDs, chicken 20HSD reduces and inactivates corticosterone to 20-dihydrocorticosterone (Kucka et al., 2006). We observed that mRNA expression of 20HSD was downregulated by ICV injection of ghrelin in LWS but not HWS chicks. Considering the inactivation effect of 20HSD on corticosterone, the downregulation of 20HSD in hypothalamus may increase the content availability of corticosterone and its negative feedback regulatory effect on CRH. This suggests that the hypothalamic glucocorticoid receptor as well as glucocorticoid metabolism is involved in ghrelin's regulatory effect on HPA function. This differential inhibitory effect of ghrelin on 20HSD expression in these two lines may account, to some extent, for the line differences in hypothalamic response to ghrelin.

Hypothalamic TRH has an essential role in the regulation of the hypothalamic-pituitary-thyroid (HPT) axis (Nillni and Sevarino, 1999). Elevated thyroid hormone caused by hypothalamic TRH, which is associated with hyperthyroidism, decreases adipose tissue mass and increases food intake in mammals via enhanced hypothalamic AMP-activated kinase (AMPK) activity (Dhillo, 2007; Ishii et al., 2008). In chickens, TRH has both thyrotropic and somatotropic effects. Thus its function is closely related to the

corticotropic axis (Kuhn et al., 2005). Intraperitoneal injection of triiodothyronine to broiler chicks increased growth, feed conversion, and visceral organ mass (Chang et al., 2003). Considering the corticotropic effect of ghrelin and the close relation between corticotropic and thyrotropic axes, the anorexigenic effect of ghrelin may also involve TRH. When the mRNA effect of TRH after ICV injection of ghrelin was tested, we found that ghrelin did not affect TRH expression in either line. Thus, TRH may be not involved in the anorexigenic effect of ghrelin.

The anorexigenic effect of ghrelin in chickens involves AMPK-mediated stimulation of fatty acid synthase (FAS) and inhibition of phosphorylation of acetyl-CoA carboxylase (ACC) (Xu et al., 2011). It is likely that except for these two enzymes, other factors related to fatty acid metabolism or obesity are involved in the appetite regulation effect of ghrelin in chickens. Our results demonstrate that ghrelin decreases the mRNA expression of CPT1 and FTO in LWS chicks, while mRNA expression of these factors had a tendency to decrease, but not significantly, in HWS chicks. LEPR expression was not affected by ghrelin. CPT1 is a mitochondrial enzyme mediating the transport of fatty acids across the membrane of mitochondria for fatty acid oxidation (Bonnefont et al., 2004). Ghrelin caused inhibition of CPT1 and resulted in the decrease of fatty acid oxidation, which is consistent with previous results (Xu et al., 2011). FTO is a well conserved gene associated with body mass index and obesity, and its expression was significantly up-regualted in the hypothalamus of rats after fasting (Fredriksson et al., 2008). Hypothalamic expression of FTO had different patterns in broiler and layer chickens, suggesting FTO may participate in the central control of energy homeostasis of chickens, as in rats (Yuan et al., 2009). Here, we demonstrated that ghrelin had an

inhibitory effect on hypothalamic expression of FTO, which is consistent with FTO's correlation with obesity and body weight gain. To our knowledge, this is the first time the relationship between ghrelin and FTO expression has been demonstrated in chickens. Differences in the magnitude of the inhibitory effect of ghrelin on CPT1 and FTO expression in the two lines of chickens may also play a role in the different feeding response to ghrelin in these two lines.

In mammals, there is a close relationship between ghrelin and various appetite regulatory neuropeptides such as NPY and AgRP secreted from ARC and orexin expressed in the lateral hypothalamus area (LHA) (Kamegai et al., 2001; Toshinai et al., 2003; Chen et al., 2004). The orexigenic effect of ghrelin is primarily mediated via the hypothalamic NPY/AgRP and orexin pathway (Kalra and Kalra, 2003; Toshinai et al., 2003). In contrast, in chickens, the anorexigenic effect of ghrelin is independent from the NPY pathway (Saito et al., 2005). Consistently, in our study, the mRNA expressions of appetite regulatory neuropeptides including NPY, POMC and orexin were not altered by ICV injection of ghrelin, suggesting not only NPY but also POMC and orexin are not involved in the anorexigenic effect of ghrelin in chickens.

6.4.2. Obestatin

Effects of obestatin on food intake in mammals are either inhibition (Zhang et al., 2005; Bresciani et al., 2006; Green et al., 2007) or nil (Seoane et al., 2006; Sibilia et al., 2006; Gourcerol et al., 2007), while in chickens, obestatin had a line-specific orexigenic effect (Xu et al., 2009; Xu et al., 2011). Here, we show that obestatin has an inhibitory effect on food intake of HWS but not LWS chicks. This differential feeding response to central

obestatin clearly implies an alteration of appetite related downstream pathway for obestatin by selection for body weight, which may contribute to the extreme phenotypic difference in body weight of these two lines.

Although obestatin caused a reduction in mRNA expression of GR in HWS chicks, neither the mRNA nor protein expression of CRH and 20HSD was altered by ICV injection of obestatin. Thus, the orexigenic effect of obestatin may be not mediated by the CRH axis. However, the mRNA expression of an important HPT axis related factor TRH was inhibited by obestatin in both lines, suggesting a possible role for TRH in the appetite regulatory effect of obestatin in chickens. The possibility that other signal components of the HPT axis are involved in the orexigenic effect of obestatin should be examined by measuring TRH targets such as pituitary thyroid stimulating hormone (TSH) and plasma triiodothyronine.

In rodents, there were no differences in hypothalamic mRNA expression levels of NPY, AgRP, POMC, or CART after chronic ICV obestatin treatment (Nogueiras et al., 2007). Similarly, in our study, the mRNA expression of appetite regulatory neuropeptides including NPY, POMC and orexin was not changed by ICV injection of obestatin, indicating that the appetite regulatory effect of obestatin may not involve ARC appetite regulatory neuropeptites in either mammals or chickens. Additionally, the mRNA expression of fatty acid oxidation stimulatory enzyme CPT1 was not altered by central obestatin, which is consistent with an absence of AMPK related fatty acid metabolism regulation in obestatin treated chicks (Xu et al., 2011).

Leptin inhibits food intake and increases energy expenditure via interaction with hypothalamic LEPR (Meister et al., 2000). Absence of functionally active LEPR in rats increases food intake and body weight gain, resulting in obesity (Phillips et al., 1996). As in mammals, chicken LEPR expressed in different tissues is capable of binding endogenous ligand as well as exogenous mammalian leptin (Adachi et al., 2008). In 3-week-old broiler chickens, the activation of LEPR by leptin reduced food intake by decreasing the expression of NPY, orexin, orexin receptor, melanocortin receptors 4/5 (MCR-4/5) and FAS (Dridi et al., 2005). In the present study, obestatin decreased expression of hypothalamic LEPR in HWS but not LWS chicks, which is consistent with the line-specific orexigenic effects of obestatin. However, the appetite related downstream pathway factors for leptin including NPY, orexin and FAS in these lines (Xu et al., 2011) were not altered by obestatin, which indicates other downstream pathways of leptin may be involved in the food regulatory effect of obestatin.

As with ghrelin, obestatin suppressed expression of obesity related factor FTO with the inhibition observed in HWS and not LWS chicks. This line specific inhibition on FTO may have a role in the differential feeding response to obestatin in these two lines. However, why obestatin and ghrelin have the same inhibitory effect on FTO expression despite their opposite food intake regulation effects needs further study.

6.5. CONCLUSION

The experiments reported here provide evidence for differences in hypothalamic CRH response to ghrelin's inhibitory effect in lines HWS and LWS. It may contribute to the different dose threshold for the anorexigenic effect of ghrelin in these two lines. The

expression of CRH, GR and 20HSD were downregulated by ghrelin in LWS but not in HWS chicks, suggesting that the differential response of hypothalamic glucocorticoid receptor and glucocorticoid metabolism may be involved in line-specific CRH responses to ghrelin. Similarly, the inhibitory effect of ghrelin on CPT1 and FTO expression observed in LWS but not HWS chicks may also play a role in the line differential feeding response to ghrelin. Obestatin had an orexigenic effect in HWS, but not LWS chicks. Unlike ghrelin, this differential feeding response was not caused by an alteration of the CRH pathway; alternatively, different LEPR and FTO responses to obestatin between lines may contribute to the line-specifc orexigenic effect. Additionally, our results also demonstrate that TRH expression was effectively inhibited by obestatin in both lines, suggesting a possible role for TRH in the appetite regulatory effect of obestatin in chickens.

6.6. REFERENCES

Adachi, H., Y. Takemoto, T. Bungo, and T. Ohkubo. 2008. Chicken leptin receptor is functional in activating JAK-STATpathway in vitro. J. Endocrinol. 197:335-342.

Bonnefont, J. P., F. Djouadi, C. Prip-Buus, S. Gobin, A. Munnich, and J. Bastin. 2004. Carnitine palmitoyltransferases 1 and 2: biochemical, molecular and medical aspects. Mol. Aspects Med. 25:495-520.

Bresciani, E., D. Rapetti, F. Dona, I. Bulgarelli, L. Tamiazzo, V. Locatelli, and A. Torsello. 2006. Obestatin inhibits feeding but does not modulate GH and corticosterone secretion in the rat. J. Endocrinol. Invest. 29:RC16-18.

Bryndova, J., P. Klusonova, M. Kucka, K. Mazancova-Vagnerova, I. Miksik, and J. Pacha. 2006. Cloning and expression of chicken 20-hydroxysteroid dehydrogenase. J. Mol. Endocrinol. 37:453-462.

Carlini, V. P., H. B. Schioth, and S. R. Debarioglio. 2007. Obestatin improves memory performance and causes anxiolytic effects in rats. Biochem. Biophys. Res. Commun. 352:907-912.

Chang, S. C., M. J. Lin, J. Croom, and Y. K. Fan. 2003. Administration of triiodothyronine and dopamine to broiler chicks increases growth, feed conversion and visceral organ mass. Poult. Sci. 82:285-293.

Chen, H. Y., M. E. Trumbauer, A. S. Chen, D. T. Weingarth, J. R. Adams, E. G. Frazier, Z. Shen, D. J. Marsh, S. D. Feighner, X. M. Guan, Z. Ye, R. P. Nargund, R. G. Smith, L. H. Van der Ploeg, A. D. Howard, D. J. MacNeil, and S. Qian. 2004. Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein. Endocrinology 145:2607-2612.

Chen, L. L., Q. Y. Jiang, H. Kaiya, X. T. Zhu, G. Shu, K. Kangawa, P. Gao, and Y. L. Zhang. 2008. Central administration of chicken ghrelin inhibits food intake in adult chickens. Pages 75 in 9th International Symposium on Avian Endocrinology.

Cline, M. A., A. Y. Kuo, M. L. Smith, W. Nandar, B. C. Prall, P. B. Siegel, and D. M. Denbow. 2009. Differential feed intake responses to central corticotrophin releasing factor in lines of chickens divergently selected for low or high body weight. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 152:130-134.

Cummings, D. E., and M. H. Shannon. 2003. Roles for ghrelin in the regulation of appetite and body weight. Arch. Surg. 138:389-396.

Dahlman-Wright, K., A. Wright, J. Carlstedt-Duke, and J. A. Gustafsson. 1992. DNA-binding by the glucocorticoid receptor: a structural and functional analysis. J. Steroid Biochem. Mol. Biol. 41:249-272.

Davis, J. L., D. T. Masuoka, L. K. Gerbrandt, and A. Cherkin. 1979. Autoradiographic distribution of L-proline in chicks after intracerebral injection. Physiol. Behav. 22:693-695.

Dhillo, W. S. 2007. Appetite regulation: an overview. Thyroid 17:433-445.

Dridi, S., Q. Swennen, E. Decuypere, and J. Buyse. 2005. Mode of leptin action in chicken hypothalamus. Brain Res. 1047:214-223.

Fredriksson, R., M. Hagglund, P. K. Olszewski, O. Stephansson, J. A. Jacobsson, A. M. Olszewska, A. S. Levine, J. Lindblom, and H. B. Schioth. 2008. The obesity gene, FTO, is of ancient origin, up-regulated during food deprivation and expressed in neurons of feeding-related nuclei of the brain. Endocrinology 149:2062-2071.

Furuse, M., R. Ando, T. Bungo, R. Ao, M. Shimojo, and Y. Masuda. 1999. Intracerebroventricular injection of orexins does not stimulate food intake in neonatal chicks. Br. Poult. Sci. 40:698-700.

Furuse, M., T. Tachibana, A. Ohgushi, R. Ando, T. Yoshimatsu, and D. M. Denbow. 2001. Intracerebroventricular injection of ghrelin and growth hormone releasing factor inhibits food intake in neonatal chicks. Neurosci. Lett. 301:123-126.

Gourcerol, G., D. H. St-Pierre, and Y. Tache. 2007. Lack of obestatin effects on food intake: should obestatin be renamed ghrelin-associated peptide (GAP)? Regul. Pept. 141:1-7.

Green, B. D., N. Irwin, and P. R. Flatt. 2007. Direct and indirect effects of obestatin peptides on food intake and the regulation of glucose homeostasis and insulin secretion in mice. Peptides 28:981-987.

Healy, J. E., J. L. Bateman, C. E. Ostrom, and G. L. Florant. 2011. Peripheral ghrelin stimulates feeding behavior and positive energy balance in a sciurid hibernator. Horm. Behav.

Ishii, S., J. Kamegai, H. Tamura, T. Shimizu, H. Sugihara, and S. Oikawa. 2008. Triiodothyronine (T3) stimulates food intake via enhanced hypothalamic AMP-activated kinase activity. Regul. Pept. 151:164-169.

Johansson, A. M., M. E. Pettersson, P. B. Siegel, and O. Carlborg. 2010. Genome-wide effects of long-term divergent selection. PLoS Genet. 6:e1001188.

Kalra, S. P., and P. S. Kalra. 2003. Neuropeptide Y: a physiological orexigen modulated by the feedback action of ghrelin and leptin. Endocrine 22:49-56.

Kamegai, J., H. Tamura, T. Shimizu, S. Ishii, H. Sugihara, and I. Wakabayashi. 2001. Chronic central infusion of ghrelin increases hypothalamic neuropeptide Y and Agouti-related protein mRNA levels and body weight in rats. Diabetes 50:2438-2443.

Kojima, M., H. Hosoda, Y. Date, M. Nakazato, H. Matsuo, and K. Kangawa. 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402:656-660.

Kola, B., E. Hubina, S. A. Tucci, T. C. Kirkham, E. A. Garcia, S. E. Mitchell, L. M. Williams, S. A. Hawley, D. G. Hardie, A. B. Grossman, and M. Korbonits. 2005. Cannabinoids and ghrelin have both central and peripheral metabolic and cardiac effects via AMP-activated protein kinase. J. Biol. Chem. 280:25196-25201.

Kucka, M., K. Vagnerova, P. Klusonova, I. Miksik, and J. Pacha. 2006. Corticosterone metabolism in chicken tissues: evidence for tissue-specific distribution of steroid dehydrogenases. Gen. Comp. Endocrinol. 147:377-383.

Kuhn, E. R., S. M. Geelissen, S. Van der Geyten, and V. M. Darras. 2005. The release of growth hormone (GH): relation to the thyrotropic- and corticotropic axis in the chicken. Domest. Anim. Endocrinol. 29:43-51.

Kwok, A. H., Y. Wang, C. Y. Wang, and F. C. Leung. 2007. Cloning of chicken glucocorticoid receptor (GR) and characterization of its expression in pituitary and extrapituitary tissues. Poult. Sci. 86:423-430.

Lagaud, G. J., A. Young, A. Acena, M. F. Morton, T. D. Barrett, and N. P. Shankley. 2007. Obestatin reduces food intake and suppresses body weight gain in rodents. Biochem. Biophys. Res. Commun. 357:264-269.

Lopez, M., R. Lage, A. K. Saha, D. Perez-Tilve, M. J. Vazquez, L. Varela, S. Sangiao-Alvarellos, S. Tovar, K. Raghay, S. Rodriguez-Cuenca, R. M. Deoliveira, T. Castaneda, R. Datta, J. Z. Dong, M. Culler, M. W. Sleeman, C. V. Alvarez, R. Gallego, C. J. Lelliott, D. Carling, M. H. Tschop, C. Dieguez, and A. Vidal-Puig. 2008. Hypothalamic fatty acid metabolism mediates the orexigenic action of ghrelin. Cell Metab. 7:389-399.

Lu, F. Z., X. X. Wang, Q. X. Pan, R. H. Huang, and H. L. Liu. 2008. Expression of genes involved in the somatotropic, thyrotropic, and corticotropic axes during development of Langshan and Arbor Acres chickens. Poult. Sci. 87:2087-2097.

Lu, N. Z., S. E. Wardell, K. L. Burnstein, D. Defranco, P. J. Fuller, V. Giguere, R. B. Hochberg, L. McKay, J. M. Renoir, N. L. Weigel, E. M. Wilson, D. P. McDonnell, and J. A. Cidlowski. 2006. International Union of Pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily: glucocorticoid, mineralocorticoid, progesterone, and androgen receptors. Pharmacol. Rev. 58:782-797.

Marquez, G. C., P. B. Siegel, and R. M. Lewis. 2010. Genetic diversity and population structure in lines of chickens divergently selected for high and low 8-week body weight. Poult. Sci. 89:2580-2588.

Meister, B. 2000. Control of food intake via leptin receptors in the hypothalamus. Vitam. Horm. 59:265-304.

Nakazato, M., N. Murakami, Y. Date, M. Kojima, H. Matsuo, K. Kangawa, and S. Matsukura. 2001. A role for ghrelin in the central regulation of feeding. Nature 409:194-198.

Nillni, E. A., and K. A. Sevarino. 1999. The biology of pro-thyrotropin-releasing hormone-derived peptides. Endocr. Rev. 20:599-648.

Nogueiras, R., P. Pfluger, S. Tovar, M. Arnold, S. Mitchell, A. Morris, D. Perez-Tilve, M. J. Vazquez, P. Wiedmer, T. R. Castaneda, R. DiMarchi, M. Tschop, A. Schurmann, H. G. Joost, L. M. Williams, W. Langhans, and C. Dieguez. 2007.

Effects of obestatin on energy balance and growth hormone secretion in rodents. Endocrinology 148:21-26.

Phillips, M. S., Q. Liu, H. A. Hammond, V. Dugan, P. J. Hey, C. J. Caskey, and J. F. Hess. 1996. Leptin receptor missense mutation in the fatty Zucker rat. Nat. Genet. 13:18-19.

Proszkowiec-Weglarz, M., and T. E. Porter. 2010. Functional characterization of chicken glucocorticoid and mineralocorticoid receptors. Am. J. Physiol. Regul. Integr. Comp. Physiol. 298:R1257-1268.

Saito, E. S., H. Kaiya, T. Tachibana, S. Tomonaga, D. M. Denbow, K. Kangawa, and M. Furuse. 2005. Inhibitory effect of ghrelin on food intake is mediated by the corticotropin-releasing factor system in neonatal chicks. Regul. Pept. 125:201-208.

Saito, E. S., H. Kaiya, T. Takagi, I. Yamasaki, D. M. Denbow, K. Kangawa, and M. Furuse. 2002. Chicken ghrelin and growth hormone-releasing peptide-2 inhibit food intake of neonatal chicks. Eur. J. Pharmacol. 453:75-79.

Seoane, L. M., O. Al-Massadi, Y. Pazos, U. Pagotto, and F. F. Casanueva. 2006. Central obestatin administration does not modify either spontaneous or ghrelin-induced food intake in rats. J. Endocrinol. Invest. 29:RC13-15.

Sibilia, V., E. Bresciani, N. Lattuada, D. Rapetti, V. Locatelli, V. De Luca, F. Dona, C. Netti, A. Torsello, and F. Guidobono. 2006. Intracerebroventricular acute and chronic administration of obestatin minimally affect food intake but not weight gain in the rat. J. Endocrinol. Invest. 29:RC31-34.

Siegel, P. B., and J. H. Wolford. 2003. A review of some results of selection for juvenile body weight in chickens. Poult. Sci. 40:81-91.

Toshinai, K., Y. Date, N. Murakami, M. Shimada, M. S. Mondal, T. Shimbara, J. L. Guan, Q. P. Wang, H. Funahashi, T. Sakurai, S. Shioda, S. Matsukura, K. Kangawa, and M. Nakazato. 2003. Ghrelin-induced food intake is mediated via the orexin pathway. Endocrinology 144:1506-1512.

Wissink, S., E. C. van Heerde, M. L. Schmitz, E. Kalkhoven, B. van der Burg, P. A. Baeuerle, and P. T. van der Saag. 1997. Distinct domains of the RelA NF-kappaB subunit are required for negative cross-talk and direct interaction with the glucocorticoid receptor. J. Biol. Chem. 272:22278-22284.

Xu, P., P. B. Siegel, and D. M. Denbow. 2009. Ghrelin and obestatin influence on food and water intake in lines of chickens selected for high and low body weight in NC: Poultry Science Abstract 420P, Raleigh.

Xu, P., P. B. Siegel, and D. M. Denbow. 2011. Genetic selection for body weight in chickens has altered responses of the brain's AMPK system to food intake regulation effect of ghrelin, but not obestatin. Behav. Brain Res. 221: 216-226.

Yuan, L., Y. Ni, S. Barth, Y. Wang, R. Grossmann, and R. Zhao. 2009. Layer and broiler chicks exhibit similar hypothalamic expression of orexigenic neuropeptides but distinct expression of genes related to energy homeostasis and obesity. Brain Res. 1273:18-28.

Zhang, J. V., P. G. Ren, O. Avsian-Kretchmer, C. W. Luo, R. Rauch, C. Klein, and A. J. Hsueh. 2005. Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. Science 310:996-999.

CHAPTER 7

SUMMARY AND CONCLUSION

This study was to determine if long-term genetic selection for high (HWS) or low (LWS) 56-day body weight in chickens has altered the hypothalamic AMP-activated protein kinase (AMPK) system and if this alteration contributes to the different feeding response to various appetite modulators between HWS and LWS lines.

Our results demonstrated that the mRNA expression of genes related to energy homeostasis and obesity, including acetyl-CoA carboxylase (ACC), carnitine palmitoyltransferase I (CPT1), fatty acid synthase (FAS), leptin receptor (LEPR) and fat mass and obesity associated gene (FTO), and feeding-related neuropeptides, including neuropeptide Y (NPY), pro-opiomelanocortin (POMC) and orexin, have been altered by the long-term divergent selection for body weight. Compared to HWS chickens, LWS chickens had a higher level of AMPK phosphorylation, which may be caused by upregulated upstream factor calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK β). The greater activity of the AMPK complex in LWS chickens could trigger a greater rate of fatty acid oxidation by inhibiting ACC to activate CPT1 and a decreased rate of fatty acid synthesis by inactivating FAS. For obesity related factors, LEPR was upregulated while FTO was downregulated in LWS chickens, which is consistent with the hypophagia and low lipogenic characteristic of this genotype. HWS chickens had decreased NPY and increased POMC and orexin expressions, suggesting the existence of a counter-regulatory mechanism, triggered by downregulated AMPK activity, to maintain energy balance and limit obesity development.

To investigate if selection for body has altered central appetite control, the feeding responses to various food intake modulators were measured in HWS and LWS chickens. Intracerebroventricular (ICV) injections of chicken ghrelin strongly suppressed food intake in both lines. LWS chickens, which are hypophagic, were more sensitive to central ghrelin than HWS chickens, which are hyperphagic. Obestatin caused a linear dose-dependent increase in food intake in HWS chickens while having no effect in LWS chickens. For glucagon-like peptide-1 (GLP-1), LWS chickens responded to a lower dose with decreased food intake than did HWS chickens. All doses of chicken glucagon-like peptide-2 (GLP-2) decreased food intake at a similar magnitude in HWS chickens, while only the highest dose (1.7 nmole) inhibited food intake in LWS chickens. NPY caused a linear dose-dependent increase of food intake in LWS but not HWS chickens. For peptide YY (PYY), HWS chickens responded at a similar magnitude of food intake stimulation to all doses, while LWS chickens had an increased dose threshold of response (5 μg vs. 2.5 μg) than HWS chickens. High dose (48 μg) of α-lipoic acid stimulated food intake in HWS but not LWS chickens. AICAR i.e. 5-amino-4-imidazole carboxamide riboside caused a linear dose-dependent decrease in food intake in LWS but not HWS chickens. Compared to AICAR, 4-Amino-5-imidazolecarboxamide hydrochloride (AICA) had a more potent anorexigenic effect in both lines with differing dose thresholds (3 nmole in HWS vs. 6 nmole in LWS). For 6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine (Compound C), all doses tested resulted in a sustained increase in food intake in line LWS with the greatest effect being 12 μg. In line HWS, responses differed among doses with only the lowest dose (3 μg) being significantly higher than the control. Gonadotropin-inhibitory hormone (GnIH) and 4-methylene-2-

octyl-5-oxotetrahydrofuran-3-carboxylic acid (C75) did not affect food intake in either HWS or LWS chickens. Astressin had a transient (30 to 45 min) inhibitory effect on food intake in HWS but not LWS chickens.

To determine if selection for body weight altered the brain's feeding response to AMPK stimulator AICAR and AMPK inhibitor Compound C by changing AMPK pathway, the effects of ICV injection of AICAR and Compound C on AMPK and its related kinase expression were measured. AICAR caused a quadratic dose-dependent decrease in food intake in LWS but not HWS chicks. Compound C caused a quadratic dose-dependent increase in food intake in HWS but not in LWS chicks. Key aspects of the AMPK pathway, including upstream kinases mRNA expression, AMPK subunit α mRNA expression and phosphorylation, and a downstream target ACC phosphorylation were not affected by either AICAR or Compound C in either line. The exception was a significant inhibitory effect of AICAR on ACC phosphorylation ratio due to increased total ACC protein content without changing phosphorylated ACC protein levels. Thus, the anorexigenic effect of AICAR in LWS chicks and orexigenic effect of Compound C in HWS chicks resulted from activation or inhibition of other kinase pathways separate from AMPK.

To determine if the effects of ghrelin and obestatin on food intake in chickens are mediated by the AMPK system and if selection for body weight alters the brain's response to ghrelin and obestatin by changing the neuronal AMPK system, we investigated central effects of ghrelin and obestatin in HWS and LWS chickens. Although ICV injection of ghrelin decreased food intake in both lines, the threshold for

the anorexigenic effect of central ghrelin was lower in LWS than HWS chicks, which is consistent with the results in adolescent HWS and LWS chickens. Obestatin caused a linear dose-dependent increase in food intake in HWS but not LWS chicks. ICV injection of 0.4 nmole ghrelin inhibited hypothalamic AMPK related gene expression and phosphorylation of AMPK α and ACC with the magnitude of inhibition different in the two lines. In contrast, ICV injection of 4 nmole obestatin did not affect mRNA expression of AMPK system or phosphorylation of AMPK and ACC in either line. These data support the premise of a lower threshold for the anorexigenic effect of central ghrelin in LWS than HWS chicks, and this difference may be associated with differential hypothalamic AMPK signaling. Additionally, the mRNA level of ghrelin in the hypothalamus was significantly higher in LWS than HWS, which may also contribute to the different threshold response to ghrelin in these two lines. Thus, selection for body weight has resulted in differences in the central ghrelin and obestatin system, and an altered brain AMPK system may contribute to the different neuronal response to ghrelin, but not obestatin.

To determine if other neuronal factors or pathways are involved in the differential feeding response to ghrelin and obestatin in HWS and LWS chickens, the effects of ICV injection of ghrelin and obestatin on the expression of corticotrophin-releasing hormone (CRH) or obesity and appetite related factors were investigated. ICV injection of 0.4 nmole ghrelin strongly suppressed food intake in both lines. At this dose, ghrelin inhibited hypothalamic CRH mRNA and protein and mRNA of glucocorticoid receptor (GR), 20-hydroxysteroid dehydrogenase (20HSD), CPT1 and FTO in LWS but not HWS chicks. However, GR protein and the mRNA of thyrotropin-releasing hormone (TRH),

LEPR, NPY, POMC and orexin were not affected by ghrelin in either line. ICV injection of 4 nmole obestatin increased food intake in HWS but not LWS chicks. Although obestatin decreased GR mRNA in HWS chicks, neither the mRNA nor protein of CRH and 20HSD was altered by obestatin. Obestatin decreased the mRNA of LEPR and FTO in HWS but not LWS chicks. The mRNA of CPT1, NPY, POMC and orexin was not affected by obestatin. Additionally, the mRNA of TRH was inhibited by obestatin in both lines. Thus, selection for body weight may alter hypothalamic response to ghrelin by CRH pathway, CPT1 and FTO, and to obestatin by LEPR and FTO.

Taken together, our data suggest long-term selection for body weight affects hypothalamic regulation of energy balance and food intake. Alterations of AMPK activity could contribute to these changes (Fig. 7.1). There is genetic variation in the feeding response to central AICAR, Compound C, ghrelin, and obestatin in these two lines. Selection for body weight alters hypothalamic responses to AICAR, Compound C and obestatin independent from AMPK. Altered AMPK system and CRH pathway may be involved in the differential response to ghrelin (Fig. 7.2), while different LEPR and FTO may contribute to the line-specific feeding response to obestatin. In short, LWS line has greater hypothalamic AMPK activity than does HWS line, which may contribute to the differential appetite regulation by ghrelin and body weight in these two lines.

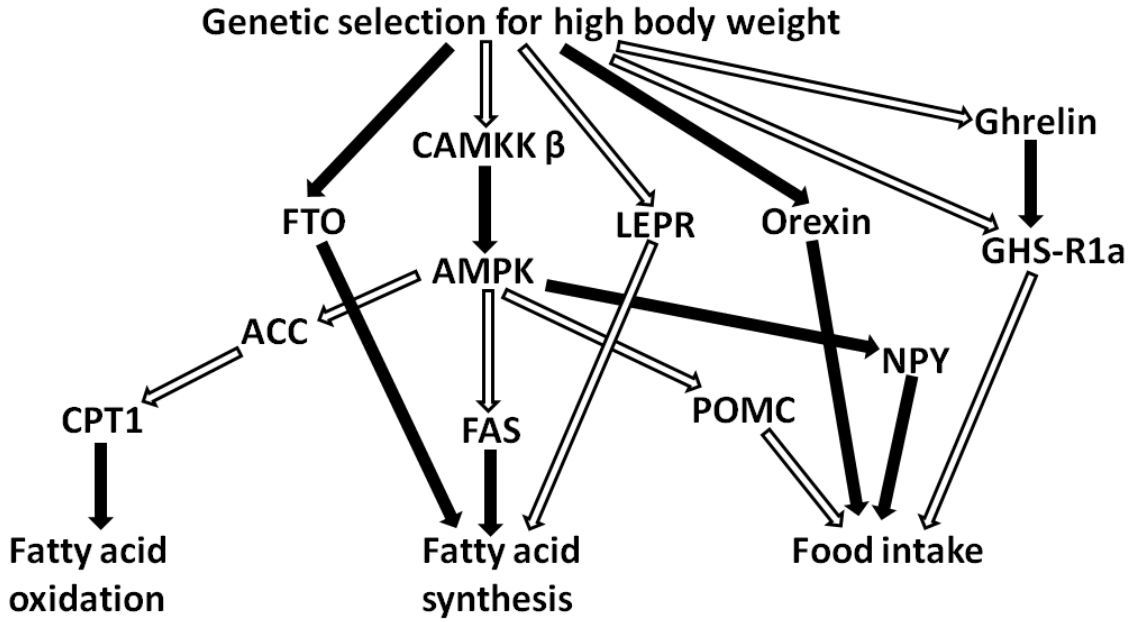

Fig. 7.1. Genetic selection for high body weight in chickens altered hypothalamic regulation of fatty acid metabolism and food intake. Black arrows represent stimulation and block arrows represent inhibition.

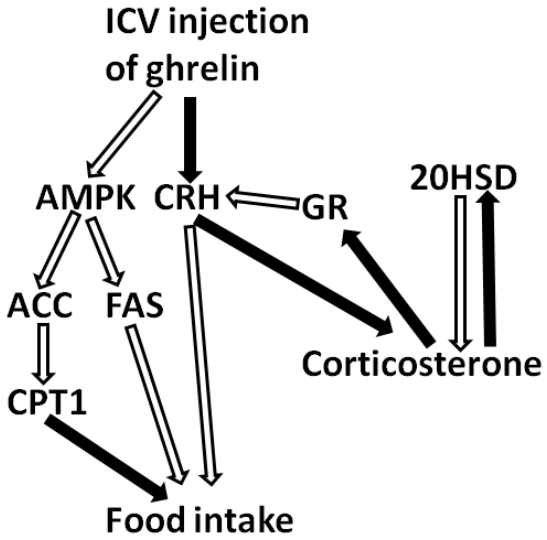

Fig. 7.2. Mechanism of appetite regulation by ghrelin in chickens. Black arrows represent stimulation and block arrows represent inhibition.