Physiological and biochemical studies on the central and peripheral regulation of food intake in chicks

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Physiological and biochemical studies on the central and peripheral regulation of food intake in chicks

（ニワトリヒナの中枢および末梢の摂食調節に関する生理生化学的研究）

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Contents

Chapter1

General Introduction

Over the last 80 years, chicken breeds have undergone intensive selection to optimize their productive performance. Rapid growth in meat-type chickens requires more feed consumption to cover the nutrient requirements (Collins et al., 2014). As a result, the intensive genetic selection in modern meat-type chickens has led to the development of hyperphagia (Richards, 2003; Bornelöv et al., 2018). The rise in food consumption in broiler chickens has led to increased fat accretion (Zuidhof et al., 2014), metabolic and health complications such as leg problems, and fatty liver syndrome (Julian, 2005; Hartcher and Lum, 2020). Feed restriction programs in broiler breeders has raised concerns regarding animal welfare due to the excessive feeling of hunger in birds (Jong et al., 2003; Hartcher and Lum, 2020). Therefore, identifying the regulatory mechanism of food intake in chickens is essential to finding solutions for health problems and improving animal welfare in the poultry industry.

Although the key genes involved in mammalian energy homeostasis were cloned and found to be conserved not only across mammals but also across all vertebrates, the anatomical and functional data on these genes varies among species from comparable to variable (Michel, 2018). For example, the adiposity signals leptin and insulin, which transmit the body fat levels to the brain, suppress food intake in mammals (Clemmensen et al., 2017), whereas there is evidence that leptin and insulin may not function as adiposity hormones in chickens; the expression level of leptin was very low in adipose tissue (Seroussi et al., 2016), and there was no significant correlation between the plasma insulin levels and abdominal fat accumulation (Honda et al., 2015a). The hunger signal ghrelin stimulates food intake in mammals but not in chickens (Kaiya et al., 2013). These findings clearly demonstrated that some aspects of the mechanisms of appetite regulation in chickens may differ from mammals.

Appetite-regulating neurons in the brain sense nutrient changes through satiety signals from peripheral hormones such as intestinal peptide YY (Ueno et al., 2008) and cholecystokinin (CCK) (Woods, 2009) in mammals. Recent findings suggest that additional satiety signals, such as hepatic insulin-like growth factor-1 and pancreatic PYY, function in chickens. For example, central and peripheral administration of IGF-1 suppressed food intake in chicks (Fujita et al., 2017). *PYY* mRNA levels were markedly higher in the pancreas than in the intestines, suggesting that the pancreas is the primary site of PYY production (Reid et al., 2017). In addition, intestinal receptors of gut hormones may also be involved in appetite regulation in chickens. CCK activates *CCK* receptor A (CCKAR) on gastrointestinal vagal afferents, causing suppression of the food intake in mammals (Woods, 2009). In chickens, Dunn et al. (2013) showed that the *CCKAR* expression levels in high growth haplotype chickens were lower than those in slow growth haplotype chickens, indicating an altered response to the CCK satiety signal in high growth haplotype chickens. These findings suggest that IGF-1, PYY, CCK, and their receptors are involved in the satiation of chickens.

The nucleus solitaries (NTS) in the brain stem receive satiety signals and convey these signals to the hypothalamus, the center of appetite regulation in mammals (Murphy and Bloom, 2006). Glucagon-like peptide *(GLP)-1* and 2, and *CCK* expressed in the NTS function as appetite suppressive neurotransmitters in mammals (Guan, 2014; van Bloemendaal et al., 2014; D'Agostino et al., 2016). The central administration of GLP-1 and 2 strongly suppressed food intake in chickens (Honda et al., 2015a). The proglucagon mRNA levels in the chicken medulla oblongata were reduced by fasting (Honda et al., 2015c). These findings raise the hypothesis that GLPs convey satiety signals to the hypothalamus in chickens.

In the present study, I investigated possible roles of satiety signals, such as pancreatic PYY, intestinal CCK, PYY, and their receptors, and hepatic IGF-related

proteins. I also examined whether IGF-2 functions as a satiety signal in chicks like IGF-1. Finally, I investigated how GLP-1 and 2 influence appetite-regulating factors and signaling pathways in the hypothalamus in chicks. Our findings add new pieces, such as pancreatic PYY and IGF-related proteins in the circulation and GLPs in the brain, to the complex puzzle of the avian appetite-regulating system. This study gives a better understanding of avian-species-specific food intake regulation, which may provide potential targets for manipulating appetite regulation and solutions for metabolic-related problems in birds.

Chapter2

Effects of fasting and re-feeding on the expression of CCK, PYY, hypothalamic neuropeptides, and IGF-related genes in layer and broiler chicks

1. Introduction

The appetite regulation system in birds is a sophisticated signal system that involves both central and peripheral regulation (Richards and Proszkowiec-Weglarz, 2007; Boswell and Dunn, 2017) as in mammals (Woods, 2009; Williams and Elmquist, 2012). Cholecystokinin (CCK) and peptide YY (PYY) have been investigated as gut hormones that send satiation signals to the brain in mammals (Woods, 2009) and possibly in chickens. For example, peripheral administration of CCK and PYY reduced food intake in mammals (Scott et al., 2005a; Sayegh et al., 2014) and chicks (Tachibana et al., 2012; Aoki et al., 2017). Plasma concentrations of CCK and PYY were elevated postprandially in mammals (Woods, 2009). There is evidence that the serum CCK concentration was elevated by food intake in chicks (Melo-Duran et al., 2019). PYY expression in the jejunum of chicks was down-regulated under fasting conditions (Aoki et al., 2017). The production areas of CCK and PYY are different between species. CCK is secreted from the duodenum, the upper part of the small intestine in mammals (Côté et al., 2014), whereas *CCK* is primarily expressed in the ileum, the lower part of the small intestine in chicks (Honda et al., 2017; Reid and Dunn, 2018). Intestinal PYY is secreted by L cells in the intestinal mucosa and mainly expressed in the colon and cecum of mammals (Zhou et al., 2006), whereas in chicks, the major gastrointestinal site for expression of PYY is the jejunum (Aoki et al., 2017; Reid et al., 2017). Recent findings clearly

demonstrated that chicken *PYY* mRNA expression was the highest in the pancreas compared to other tissues (Gao et al., 2017; Reid et al., 2017). These facts suggest that CCK and PYY act as anorexigenic hormones in both mammals and chicks and raise the hypothesis that different production areas may be involved in the species-specific mechanism of appetite regulation.

In mammals, the anorexigenic effects of CCK and PYY are mediated by the CCK-A receptor (CCKAR) (Beglinger et al., 2001; Dockray, 2012) and neuropeptide Y2 receptor (Y2R), respectively, (Abbott et al., 2005; Scott et al., 2005b; Reidelberger et al., 2013). In chickens, high growth haplotype chickens showed decreased expression of CCKAR and resistance to the anorectic effect of exogenously administered CCK when compared to low growth haplotype chickens (Dunn et al., 2013). Chicken PYY showed high affinity binding to Y2R *in vitro* (Salaneck et al., 2000). However, the physiological changes in intestinal Y2R and CCKAR in response to food intake have not yet been examined in chickens.

Recently, we reported that intracerebroventricular administration of insulin-like growth factor (IGF)-1 significantly suppressed food intake in broiler and layer chicks (Fujita et al., 2017; 2019). Six hours of fasting significantly reduced the mRNA levels of *IGF-1* in the liver, and this change was reversed by 6 h of re-feeding in broiler chicks (Fujita et al., 2017). In contrast, 6 h of fasting significantly increased the mRNA levels of Insulin-like growth factor-binding protein *(IGFBP)-1* and *-2* in the liver, and these changes were reversed by 6 h of re-feeding in broiler chicks (Fujita et al., 2018). IGFBPs were thought to be carrier proteins in the bloodstream, but the availability of blood IGF-1 to the receptor of target cells is limited by binding to *IGFBPs* (Allard and Duan, 2018). It is therefore likely that IGF-1, IGFBP-1, and -2 are involved in the regulation of food intake in chicks.

The hypothalamus is known as the central site for integrating satiety signals

in mammals (Hussain and Bloom, 2013). Both the mammalian and avian hypothalamic arcuate nuclei contain anorexigenic pro-opiomelanocortin (POMC) and orexigenic neuropeptide Y (NPY)/agouti-related protein (AgRP) neurons that play important roles in the central regulation of food intake (Morton et al., 2006; Boswell and Dunn, 2017). Central administration of NPY and AgRP stimulates food intake, whereas central administration of α-melanocyte-stimulating hormone (α-MSH, a neuropeptide derived from POMC) suppresses it in mammals (Rossi et al., 1998; Edwards et al., 1999; Tung et al., 2006) and chicks (Tachibana et al., 2001; Saneyasu et al., 2011; Honda et al., 2012). Food deprivation induces *NPY* and *AgRP* expression and suppresses *POMC* expression in the hypothalami of mammals (Bertile et al., 2003) and chicks (Fang et al., 2014). Therefore, the mRNA levels of hypothalamic *NPY*, *AgRP*, and *POMC* can be used as indicators for appetite.

In the present study, to clarify the physiological importance of CCK, PYY, and IGF-related proteins in the appetite regulatory system of avian species, we focused on the mRNA levels of intestinal *CCK*, *CCKAR*, *PYY*, and *Y2R*, pancreatic *PYY*, hepatic *IGF*-related proteins, and hypothalamic *NPY*, *AgRP*, and *POMC* in response to fasting and re-feeding in chicks. We also used two different types of chicks, layer and broiler chicks, because broiler chicks show hyperphagia that may be caused by weakened satiation signals.

2. Materials and Methods

2.1. Animals and diet

This study was approved by the Institutional Animal Care and Use Committee and was performed according to the Kobe University Animal Experimentation

Regulations (25-08-01). One day old male layer (White leghorn) and broiler (Ross 308) chicks were purchased from local hatcheries (Japan Layer K. K., Gifu, Japan and Yamamoto Co., Ltd., Kyoto, Japan, respectively). The commercial chick starter diet met the nutritional requirements for both broiler and layer chicks. They were given free access to water and a commercial chick starter diet (NICHIWA SANGYO Co., Ltd., Kobe, Japan) under a 23-h/1-h light - dark cycle, a light schedule commonly used in the poultry industry.

We previously showed that 12 h of fasting significantly decreased *PYY* mRNA levels in the ileum in chicks (Aoki et al., 2017). Reid et al. (2017) reported that 11 h of fasting significantly decreased *PYY* mRNA levels in the pancreas in broiler-layer hybrid chicks. However, 7.5 h of fasting did not influence *CCK* mRNA levels in the ileum in NOVO gen brown birds (Reid et al., 2017). Thus, in order to compare the physiological importance of CCK and PYY, we selected 12 h of fasting, which downregulates *PYY* expression in both ileum and pancreas. A total of 24 twenty-one-day-old male layer and broiler chicks were weighed and allocated to three cages based on body weight (eight birds in each group). Chicks were reared in electrically heated battery cages. The temperature was kept at 31 ± 2℃ during the first 7 days, and then reduced gradually according to age until reaching 25 ± 2℃ at 21 d. Chicks in the feeding group were euthanized by skilled person by decapitation after 0 h of fasting. Chicks in the fasting group were euthanized by decapitation after 12 h of fasting. After 12 h of fasting, chicks in the re-feeding group were refed for 12 h and euthanized by decapitation. Blood was collected from the carotid artery. Plasma was separated immediately by centrifugation at 1,910 g for 10 min at 4 ℃, and the plasma concentrations of glucose and triglycerides (TG) were measured using commercial kits (Lab Assay™ Glucose and Lab Assay™ Triglyceride, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan, respectively). The liver, pancreas, and a central section of the jejunum and ileum were accurately

excised, washed with saline, and frozen immediately in liquid nitrogen for real-time PCR analysis. The diencephalon was collected and preserved in RNAlater® tissue storage reagent (Sigma-Aldrich CO., St. Louis, Mo, USA), and the hypothalami were excised with reference to the stereotaxic atlas drawn by Kuenzel and Masson (1988). The septopallio-mesencephalic tract, third cranial nerves, 1.5 mm from the midline, and the dorsal section from the anterior commissure to 1.0 mm ventral to the posterior commissure were used as landmarks.

2.2.Real-time PCR

Total RNA was extracted from the tissues using Sepazol-RNA I Super G (Nacalai Tesque, Inc., Kyoto, Japan). First-strand cDNA was synthesized from total RNA using Rever Tra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo Co.Ltd, Osaka, Japan). The levels of mRNA were quantified for each primer using TB Green Premix Ex Taq II (Tli RNaseH Plus; Takara Bio Inc., Otsu, Japan) according to the supplier's recommendations, in an Applied Biosystems 7300 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Complementary DNA of *CCK* (GenBank accession no. NM_001001741) and *CCKAR* (GenBank accession no. NM_001081501) was amplified using the following primers: *CCK* sense, 5' -GCG CTG CTG GCO AAG TA-3'; *CCK* antisense, 5' -GAC AGA GAA CCT CCC AGT GGA A-3' ; *CCKAR* sense, 5' -TGG TTG CGT ATG GCC TCA TT-3'; *CCKAR* antisense, 5' -GGC GAT GCT GGT ACT TCC TT-3'. Complementary DNA of ribosomal protein S17 (internal standard) (Saneyasu et al., 2019b), *NPY*, *POMC* and *AgRP* (Fujita et al., 2019), *PYY*, and *Y2R* (Aoki et al., 2017), and *IGF*-related proteins (Fujita et al., 2017; 2018) were amplified using primers as described previously.

2.3. Data analysis

Data were analyzed by one-way analysis of variance. If a significant difference ($P < 0.05$) was detected, Fisher's PLSD test was performed for multiple comparisons. All statistical analyses were performed using a commercial software package (StatView version 5, SAS Institute, Cary, NC, USA,1998).

3. Results

In layer chicks, the mRNA levels of intestinal *CCK*, *PYY*, *Y2R*, and pancreatic *PYY* were significantly reduced after 12 h of fasting, and these changes were significantly reversed by 12 h of re-feeding (Fig. 1A). Hypothalamic *NPY* mRNA levels were significantly increased after 12 h of fasting, and this change was reversed by 12 h of re-feeding (Fig. 1B). Plasma glucose and TG were significantly reduced after12 h hours of fasting, and these changes were reversed by 12h of re-feeding (Fig. 1C).

In broiler chicks, the mRNA levels of intestinal *PYY* and *Y2R* were reduced after 12 h of fasting, and these changes were significantly reversed by 12 h of re-feeding (Fig. 2A). Hypothalamic *NPY* and *AgRP* mRNA levels increased after 12h of fasting, whereas 12 h of re-feeding significantly reversed the mRNA levels of hypothalamic *NPY*, but not *AgRP* (Fig. 2B). Plasma glucose and TG were reduced significantly after 12 h of fasting and these changes were reversed by 12 h of re-feeding (Fig. 2C).

Effects of fasting and re-feeding on the mRNA levels of *IGF*-related proteins in the liver are shown in Fig. 3. Fasting and re-feeding did not influence hepatic *IGF-1* mRNA levels in layer chicks (Fig. 3A). On the other hand, hepatic *IGF-1* mRNA levels were significantly reduced after 12 h of fasting, and this change was not reversed by 12 h of re-feeding in broiler chicks (Fig. 3B). The mRNA levels of *IGFBP-1* and -2 were markedly increased by fasting and reduced by re-feeding in both types of chicks (Fig. 3A

and B). Hepatic *IGFBP-3* mRNA levels were significantly increased by fasting only in layer chicks, and this change was reversed by re-feeding (Fig. 3A). In broiler chicks, re-feeding significantly reduced hepatic *IGFBP-3* mRNA levels, although fasting did not influence them (Fig. 3B). Re-feeding significantly reduced hepatic *IGFBP-4* mRNA levels only in layer chicks, although fasting did not influence them (Fig. 3A).

4. Discussion

Broiler chicks have undergone intensive selection for growth for more than 50 years. As a result, they do not adequately regulate voluntary food intake commensurate with their energy needs (Richards, 2003; Bornelöv et al., 2018). In the present study, we proposed that the gut hormones CCK and PYY, intestinal Y2R, and pancreatic PYY are involved in the post prandial changes in appetite in layer chicks. However, several changes were not observed in broiler chicks. All these findings suggest that the physiological roles of appetite regulation-related genes may be different between broiler and layer chicks.

In the present study, we found that pancreatic *PYY* mRNA levels were significantly changed in response to feeding only in layer chicks. Gao et al. (2017) and Reid et al. (2017) clearly demonstrated that chicken *PYY* is highly expressed in the pancreas when compared to other tissues, including the intestines. Reid et al. (2017) also demonstrated that the mRNA levels of chicken *PYY* were significantly reduced by 11 h of fasting in the pancreas in two-week-old broiler-layer hybrid chicks. We previously reported that intravascular administration of chicken PYY significantly suppressed food intake in broiler chicks (Aoki et al., 2017). However, pancreatic *PYY* mRNA levels were not changed by fasting and re-feeding in broiler chicks in the present experimental condition. It is therefore possible that only jejunal PYY functions as a satiety signal in

broiler chicks.

The mRNA levels of pancreatic *PYY* were reported in the frogs (Sundström et al., 2012) and fish (Cheung et al., 1991; Al-Mahrouki and Youson, 1998; Chen et al., 2015), but the levels were not higher than intestinal *PYY*. In mammals, PYY functions as a neuropeptide in the central nervous system and gut hormone in the intestines (Ueno et al., 2008). However, pancreatic PYY may exhibit species-specific roles in chickens. Ding et al. (1997) reported that PYY-positive endocrine cells were scattered in the exocrine region in chickens and turtles when compared to eels, bullfrogs, mice, rats, guinea pigs, dogs, and humans. Tatemoto (1982) reported that PYY suppresses pancreatic exocrine excretion in cats. In the present study, re-feeding significantly increased pancreatic *PYY* expression in layer chicks. It is therefore possible that pancreatic PYY suppresses the excretion of pancreatic juice in layer chicks in response to food intake.

The effects of gut hormones on food intake in chickens have been investigated in recent decades (Denbow, 1994; Honda et al., 2017). However, the interactions of endogenous gut hormones and their receptors in the appetite regulation of chickens have not been fully elucidated. In the present study, our findings clearly demonstrated that both fasting and re-feeding affect the mRNA levels of not only *PYY* but also *Y2R* in the jejunum of layer and broiler chicks. This is the first report suggesting that expression of intestinal *Y2R* may be involved in the peripheral regulation of food intake in chicks. There is evidence that Y2R is expressed in the myenteric neurons of the intestines in mammals (Wang et al., 2010). The enteric nervous system is involved in the brain-gut axis in mammals (Bauer et al., 2016; Bliss and Whiteside, 2018). It is therefore possible that intestinal PYY functions as a satiety signal via the enteric nervous system in chicks. However, distribution of Y2R in the intestines in chicks has not been investigated. Further study is needed to identify the Y2R-expressing cells in chicken intestines.

In the present study, intestinal *CCK* mRNA levels were significantly reduced

by 12 h of fasting, and restored after 12 h of re-feeding in layer chicks. However, in broiler chicks, no significant change was observed in the *CCK* mRNA levels. Reid and Dunn (2018) showed that neither short-term (6 h) fasting nor re-feeding influenced the mRNA levels of *CCK* in the ileum of 17-day-old brown chicks. Therefore, it is likely that *CCK* expression in the ileum is more responsive to long-term fasting or the re-feeding state or shows different responses in different chicken strains.

In the present study, hepatic *IGFBP-3* mRNA levels were significantly increased by fasting only in layer chicks. IGFBPs binds IGF-1 in plasma and blocks the binding to the receptor (Allard and Duan, 2018). In chickens, only 6% of serum IGF-1 exists in free form, suggesting that the functions of plasma IGF-1 are significantly influenced by IGFBPs (McMurtry et al., 1997). It is therefore possible that the plasma concentration of free form IGF-1 is decreased by increasing IGFBP-3 under the fasting condition in layer chicks. Interestingly, a reduction in free IGF-1 after fasting has been reported in humans (Frystyk et al., 2002) and rats (Frystyk et al., 1999), but serum IGFBP-3 was reduced by fasting in rats (Frystyk et al., 1999). It seems likely that the physiological roles of IGFBP-3 are completely different between rats and layer chicks. Further studies will provide insights into the different roles of IGFBP-3 not only between in layer and broiler chicks, but also in mammals and birds.

In mammals, the hypothalamus plays an important role in directly sensing nutrients and hormones such as glucose, insulin, and leptin, and the area postrema in the caudal brainstem receives circulating metabolic signals including gut hormones (Clemmensen et al., 2017). Thus, the regulation of food intake is coordinated by a complex neurocircuitry involving multiple brain regions. Although, the role of CCK and PYY in the appetite regulatory pathway in chick brains has not yet been identified, we have reported that the hypothalamus may be involved in the IGF-1-induced anorexigenic pathway in chicks (Fujita et al., 2019). It is therefore possible that CCK, PYY, and IGF-

1 affect the expression of hypothalamic neuropeptides via different pathways. Further studies are needed to clarify the target region of CCK and PYY in the brain and the interaction of the effects of gut hormones and IGF-1 in chicks.

Shurlock and Forbes (1981) reported that infusion of glucose into the hepatic portal vain depressed food intake in 28-week-old layer chickens. Lacy et al. (1986) reported that lipid infusion intrahepatically depressed food intake in 11-week-old layer chickens. In the present study, re-feeding significantly increased not only the mRNA levels of *PYY* but also plasma glucose and TG levels in layer and broiler chicks. These findings suggest that PYY and plasma metabolites may coordinately induce satiety in both types of chickens.

Hypothalamic *NPY* expression was upregulated by 12 h of fasting and these changes were reversed by 12 h of re-feeding in layer and broiler chicks, suggesting that changes of feeding conditions influence appetite in both types of chicks. On the other hand, significant upregulation of hypothalamic *AgRP* was found only in broiler chicks, and this change was not reversed by 12 h of re-feeding. It is therefore likely that 12 h of re-feeding is not enough to suppress appetite in broiler chicks. However, Tachibana et al. (2001) reported that central administration of AgRP significantly suppressed food intake in layer chicks but not in broiler chicks. Therefore, further study is needed to evaluate the physiological importance of AgRP in the central regulation of food intake in broiler chicks.

The effects of fasting on the hypothalamic POMC expression in chicks were different in previous reports. For example, 24 h of fasting in 14-day-old yellow-feathered broiler chicks (Fang et al., 2014) and 3 h of fasting in 5-day-old low or high body weight line chicks (Yi et al., 2015) significantly reduced hypothalamic *POMC* mRNA levels. On the other hand, Song et al. (2012) reported that 48 h of fasting did not influence the mRNA levels of hypothalamic *POMC* in 7-day-old broiler chicks. In the present study, 12 h of

fasting did not influence the mRNA levels of hypothalamic *POMC* in either layer or broiler chicks. It is therefore likely that the regulatory mechanism underlying fasting-induced hypothalamic POMC expression is different depending on age, strain, or experimental conditions.

In the present study, plasma TG levels were significantly higher under the re-feeding condition in both layer and broiler chicks. The reason for significant increases in plasma TG is not clear. However, Leveille (1969) reported that refeeding for 3 days following a 3-day fast significantly increased liver fat upon refeeding chicks when compared to control chicks. Leveille et al. (1975) also demonstrated that hepatic synthesis of fatty acids was markedly depressed by fasting, but increased rapidly upon refeeding, "overshooting" the control rate of fatty acid synthesis in chicks. Thus, one possible explanation is that 12 h of re-feeding following 12 h of fasting produced an "overshoot" in lipogenesis to levels surpassing that of the *ad libitum* feeding group.

5. Conclusion

We examined the effects of fasting and re-feeding on the appetite regulation-related genes in broiler and layer chicks and found that several genes showed different responses in the two types of chicks. Our findings suggest that pancreatic PYY and ileum CCK may not play important roles in the regulation of food intake in broiler chicks.

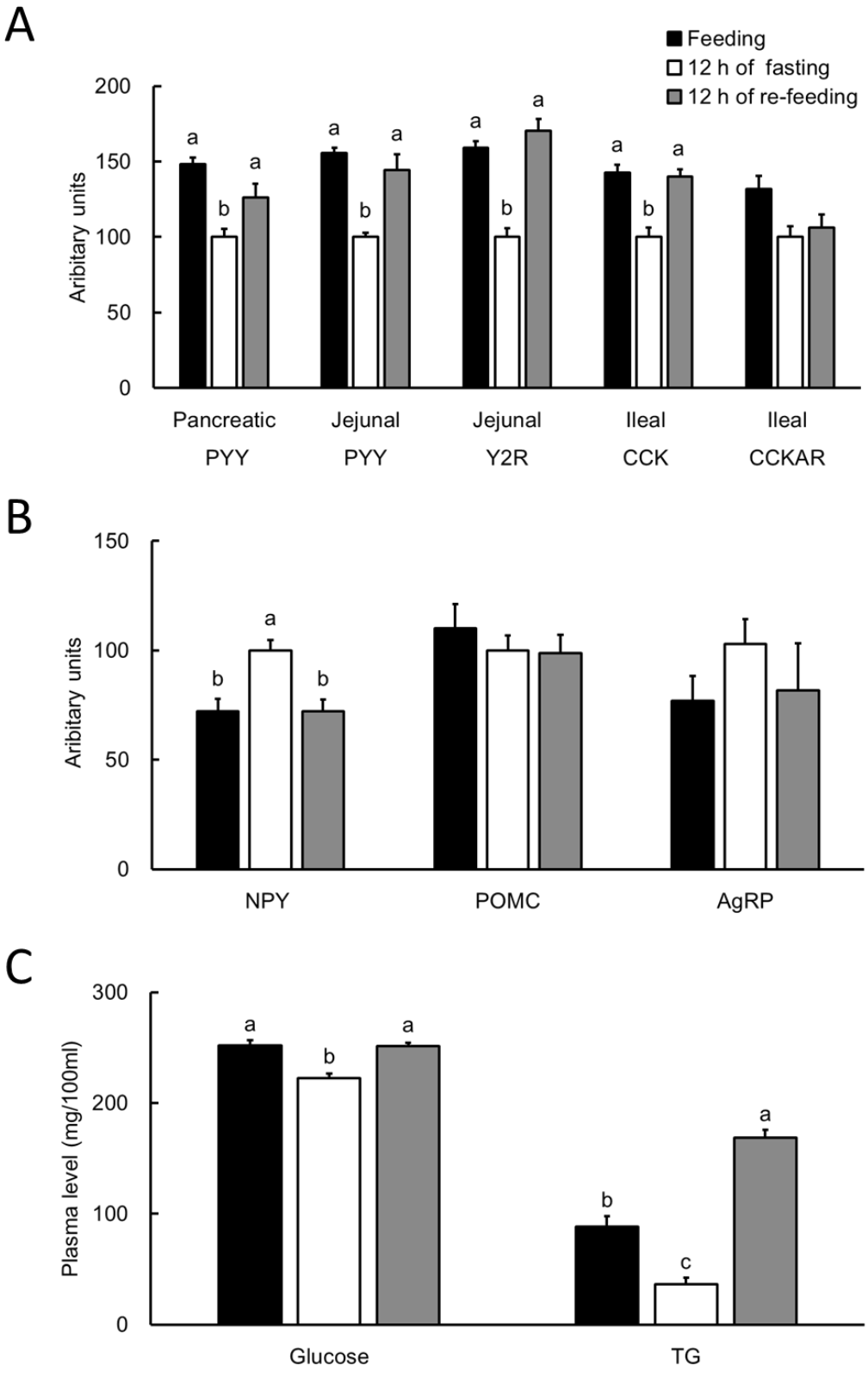
Figure Captions

Fig. 1. Effects of fasting and re-feeding on the mRNA levels of (A) intestinal CCK, Jejunal PYY, and their receptors, and pancreatic PYY, (B) hypothalamic NPY, POMC, and AgRP, and (C) plasma metabolites in layer chicks. Data are the means ± S.E.M. of eight chicks in each group and are expressed as a percentage of the mean in the 12 h fasting group. Groups with different letters for each gene are significantly different ($P < 0.05$).

Fig. 2. Effects of fasting and re-feeding on the mRNA levels of (A) intestinal CCK, Jejunal PYY, and their receptors, and pancreatic PYY, (B) hypothalamic NPY, POMC, and AgRP, and (C) plasma metabolites in broiler chicks. Data are the means ± S.E.M. of eight chicks in each group and are expressed as a percentage of the mean in the 12 h fasting group. Groups with different letters for each gene are significantly different ($P < 0.05$).

Fig. 3. Effects of fasting and re-feeding on the mRNA levels of hepatic IGF-1 and IGFBPs in layer (A) and broiler (B) chicks. Data are the means ± S.E.M. of eight chicks in each group and are expressed as a percentage of the mean in the 12 h fasting group. Groups with different letters for each gene are significantly different ($P < 0.05$).

Fig. 1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12 h of re-feeding
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b
a
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Pancreatic PYY
Jejunal PYY
Jejunal Y2R
Ileal CCK
Ileal CCKAR
B
Aribitary units
150
100
50
0
b
a
b
NPY
POMC
AgRP
C
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a
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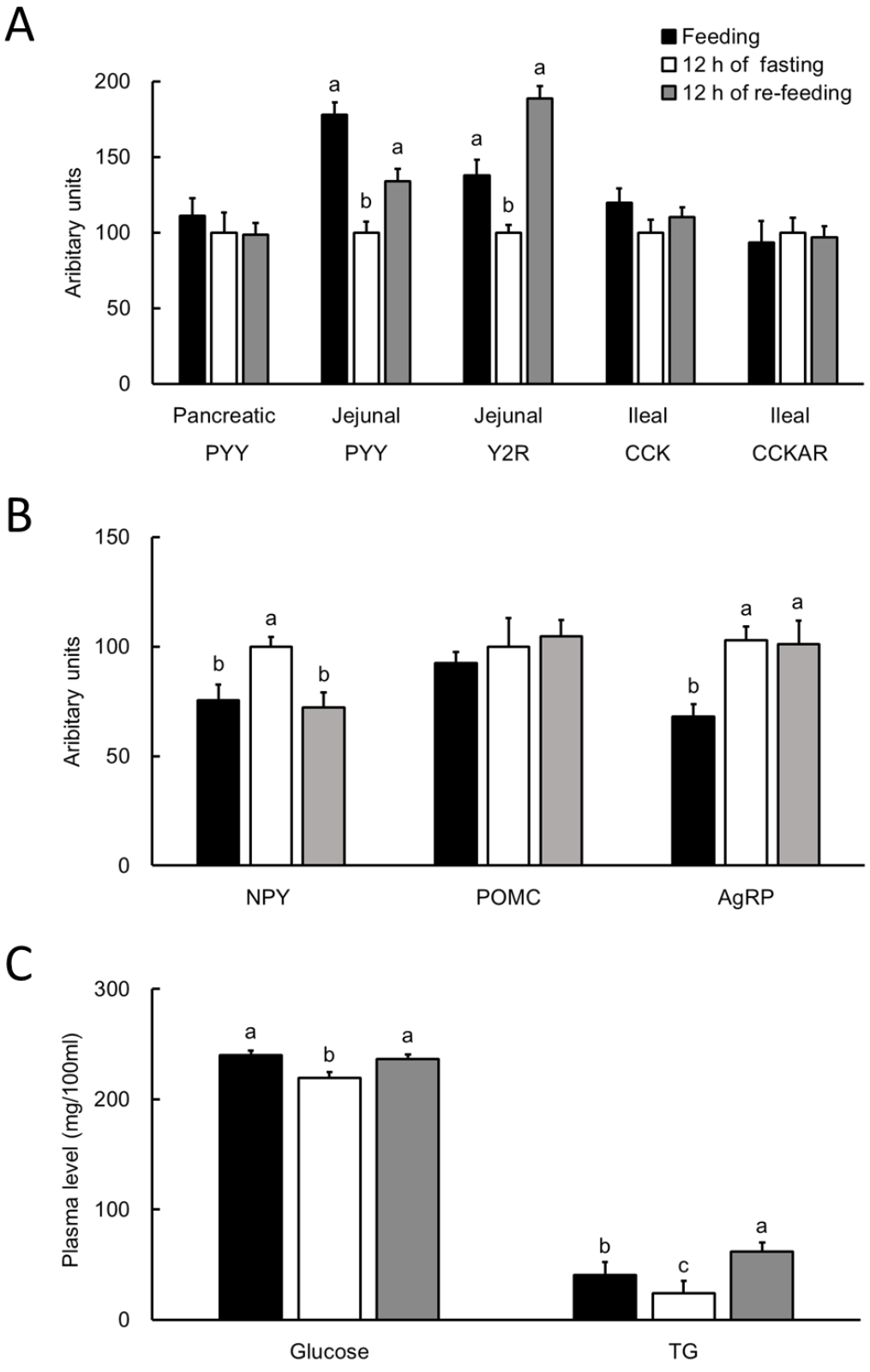
c
a
Glucose
TG

Fig. 2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Feeding
12 h of fasting
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Jejunal PYY
Jejunal Y2R
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Ileal CCKAR
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NPY
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Glucose
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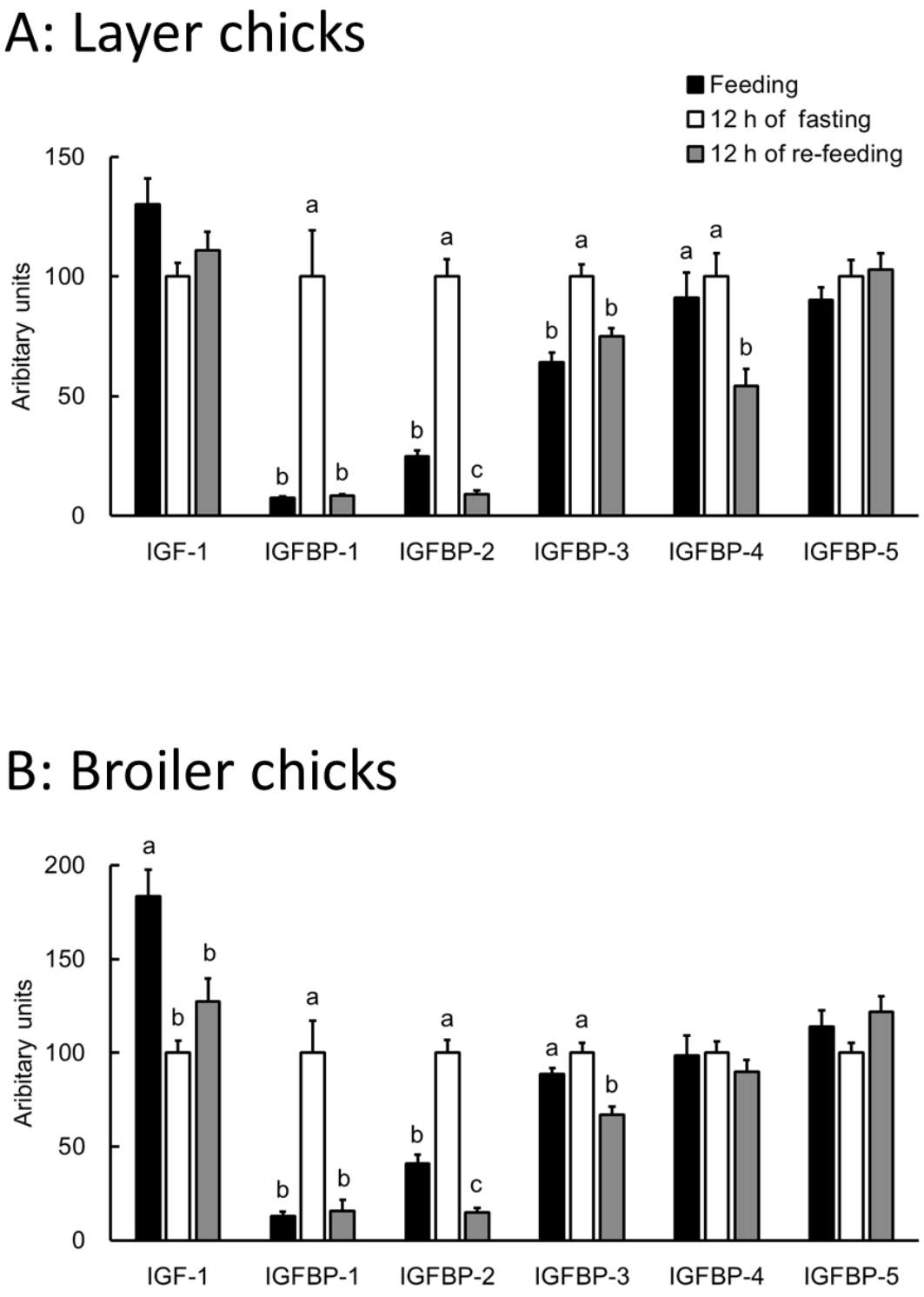
Fig. 3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12 h of re-feeding
Aribitary units
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IGF-1
IGFBP-1
IGFBP-2
IGFBP-3
IGFBP-4
IGFBP-5
B: Broiler chicks
Aribitary units
200
150
100
50
0
IGF-1
IGFBP-1
IGFBP-2
IGFBP-3
IGFBP-4
IGFBP-5

Chapter 3

Central administration of insulin-like growth factor-2 suppresses food intake in chicks

1. Introduction

Insulin-like growth factor (IGF)-1 plays important roles in the muscle development and growth of chickens (Duclos et al., 1999; Duclos, 2005). We recently found that central administration of IGF-1 suppresses food intake in chicks (Fujita et al., 2017; Fujita et al., 2019). The mRNA levels of proopiomelanocortin, an anorexigenic in the hypothalamus, are increased by central administration of IGF-1 (Fujita et al., 2019). There is evidence that the plasma concentration of insulin and IGF-1 were postprandially elevated in chickens (Kita, 1998). It is therefore likely that IGF-1 functions as a postprandial satiety hormone in chickens.

IGF-2 is a multifunctional hormone with structural and functional similarity to IGF-1 in mammals and chickens (McMurtry et al., 1997). Central administration of 100 ng (~13.3 pmol) of IGF-2 suppressed food intake in rats (Lauterio et al., 1987). Chicken IGF-2 can bind chicken *IGF-1* receptor (IGF-1R) and express the function (Duclos and Goddard, 1990). In addition, the chicken mannose 6-phosphate receptor (MPR300), which is known to be an IGF-2 receptor (IGF-2R) in mammals, can bind chicken IGF-2 *in vitro* (Koduru et al., 2006) and was expressed in brains of chickens (Matzner et al., 1996). Feed restriction reduced the plasma IGF-2 concentration in chickens (Leili et al., 1997). The mRNA levels of *IGF-2* in the liver were increased by a high energy diet in growing chicks (Saxena et al., 2020). These findings raise the hypothesis that IGF-2 may function as a satiety hormone in chickens.

IGF binding proteins (IGFBPs) bind IGF-1 and -2 in plasma and block their

binding to the receptors, indicating that IGFBPs influence the physiological roles of IGFs. For example, addition of either IGFBP-1 or IGFBP-2 to sera reduced free IGF-1 *in vitro* (Frystyk et al., 2002). An increase in IGFBP-1 and reduction in free IGF-1 are accompanied by an increase in IGFBP-1 complexed IGF-1 after fasting in humans (Frystyk et al., 2001). In chickens, only 5% of serum IGF-2 exists in free form, suggesting that the function of almost all plasma IGF-2 is suppressed by IGFBPs (McMurtry et al., 1997). Interestingly, recent evidence demonstrated that locally expressed IGFBPs increased IGFs availability for binding to the receptors, and that IGF exhibited an independent action in mammals and fishes (Shimizu and Dickhoff, 2017; Allard and Duan, 2018).

In the present study, we investigated the possible involvement of IGF-2 in the mechanism of food intake regulation in chicks. We also examined the effects of fasting on the mRNA levels of *IGFBPs* in the liver and hypothalamus, the production area of *IGFBPs* and the target site of *IGF-2*, respectively. Our findings suggest that hepatic IGFBP-1, -2, and -3 production may suppress the anorexigenic function of IGF-2 in chicks under *ad libitum* feeding conditions.

2. **Materials and methods**

2.1.Animals and peptides

This study was approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation Regulations (25-08-01 and 27-07-01). Day-old male chicks (White leghorn) were purchased from a local hatchery (Japan Layer K.K., Gifu, Japan). They were given free access to water and a commercial chick starter diet (Nippon Formula Feed Mfg. Co., Ltd., Kanagawa, Japan) in an electrically heated cages (1725 mm x 425 mm x 320 mm) maintained at 28±2°C in a room with an automatically controlled 23 h light/1h dark cycle (23:00-24:00 dark).

Room temperature was 22±2℃.

Amino acid sequencing of IGF-2 showed the presence of 12 amino acid substitutions compared with humans (McMurtry et al., 1997). However, Upton et al. (1995) reported that recombinant chicken IGF-2 was equipotent with human IGF-2 in both biological and receptor binding studies in chick embryo fibroblasts. Purified chicken MPR 300 binds both chicken and human IGF-2 (Koduru et al., 2006). The metabolic clearance of chicken IGF-2 and human IGF-2 was similar when administered intravascularly in 7-week-old chickens (McMurtry et al., 1996). These findings suggest that human IGF-2 and chicken IGF-2 show similar effects in chicks. Therefore, in the present study, we used human IGF-2 (Novus Biologicals, LLC, Co, USA) instead of chicken IGF-2.

2.2.Experiment 1: Effects of central administration of IGF-2 on food intake in chicks

Forty-eight 8-day-old chicks were weighed and allocated to four groups based on body weight (12 birds in each group). IGF-2 was dissolved in 0.85% (w/v) saline solution containing 0.1% (w/v) Evans Blue. The peptide was intracerebroventricularly administered according to the method of Davis et al. [4] at a volume of 10 μl after three hours of fasting. Chicks were administered with IGF-2 (0, 30, 100, or 300 pmol). Food intake was measured at 60 and 120 min after administration of IGF-2 in each individual cage (260 mm x 185 mm x 148 mm). Feed and water were supplied in plastic boxes (78 mm x 58 mm x 48 mm and 62 mm x 50 mm x 40 mm, respectively). Each feeder filled with food was pre-weighed. A paper was put under the feeder. At in each time point, any spilled food on the paper was collected and food consumption was weighted using an electric digital balance (Readability: 10 mg). Food intake was calculated as follows:
Food intake = (The amount of food decrease in the feeder) - (The amount of spilled food).
At the end of the experiment, the chicks were euthanized by decapitation. Verification of

injection was made by observation of the presence of Evans Blue dye in the lateral ventricle. Data from chicks without Evans Blue dye in the lateral ventricle were omitted. Effects of central administration of IGF-2 on food intake were also measured under an *ad libitum* feeding condition.

2.3.Experiment 2: Effects of six hours of fasting on IGF-related genes' mRNA levels in the liver and hypothalamus of chicks

Twelve 7-day-old chicks were weighed, allocated based on body weight, and euthanized by decapitation after 0 or 6 hours of fasting. The liver and diencephalon were collected, and preserved in RNAlater tissue storage reagent (Sigma-Aldrich, St. Louis, Mo, USA). The hypothalamus was excised based on reference to a stereotaxic atlas drawn by Kuenzel and Masson as described previously (Honda et al., 2015a). Total RNA extraction and cDNA synthesis were performed as described previously (Fujita et al., 2019). Complementary DNA of *IGFBPs* were amplified using primers as described previously (Fujita et al., 2018). Complementary DNA of *IGF-1* and *IGF-1R* were amplified using primers as described previously (Fujita et al., 2017). Complementary DNA of *IGF-2*, *IGF-2R* and ribosomal protein S17 (*RPS17*: an internal standard) were amplified using primers as follows, *IGF-2* sense, 5'-CCT GGC TCT GCT GGA AAC C-3' ; antisense, 5'-GAG AGG TCA CGC TCT GAC TTG A-3'; *IGF-2R* sense, 5'-AAC ATC GGG TGT TTC CTA CAA ATA C-3'; antisense, 5'-TGA TTT GGT GCT GCA ATT TCC-3'; *RPS17* sense, 5'- GCG GGT GAT CAT CGA GAA GT-3'; antisense, 5'-GCG CTT GTT GGT GTG GAA GT-3'. Messenger RNA levels were quantified in duplicate using the Thermo Scientific PikoReal Real time PCR System (Thermo Fisher Scientific Oy, Vantaa, Finland) and SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara Bio Inc., Shiga, Japan) according to the manufacturer's recommendations.

2.4.Experiment 3: Effects of a peroxisome proliferator-activated receptor alpha (PPARα) agonist on IGF-related genes' mRNA levels in chicken hepatoma cells

There is evidence that a PPARα agonist significantly upregulates hepatic *IGFBP-1* (Degenhardt et al., 2006) and *IGFBP-2* (Kang et al., 2015) expression in mammalian hepatoma cells. Therefore, we examined the effects of the PPARα agonist WY14643 on *IGFBPs* expression in chicken hepatoma (LMH) cells. Degenhardt et al. (2006) demonstrated the direct effect of WY14643 on gene expression in a human hepatoma cell line (HepG2) at two hours after addition of WY14643. We previously showed that 50 μM of WY14643 significantly upregulated the target gene *carnitine palmitoyltransferase 1A* (*CPT1A*) in LMH cells (Honda et al., 2015a). According to these findings, we confirmed the effect of a two-hour incubation with 50 μM of WY14643 on LMH cells. Cell culture, RNA extraction, and cDNA synthesis were performed as described previously (Honda et al., 2015a). Complementary DNA of *CPT1A* was amplified using primers as described previously (Honda et al., 2015a).

2.5.Data analysis

Data in Experiment 1 were analyzed by one-way analysis of variance. If a significant difference ($P < 0.05$) was detected, Tukey-Kramer test was performed for multiple comparisons. Data in Experiment 2 were analyzed by Student's t-test. All statistical analyses were performed using the commercial package (StatView version 5, SAS Institute, Cary, NC, USA, 1998).

3. **Results**

In the present study, we firstly examined the effect of central administration of IGF-2 on food intake in chicks. Intracerebroventricular administration of 300 pmol of IGF-2 significantly suppressed food intake under both three hours of fasting and *ad*

libitum feeding conditions (Fig. 4A and B, respectively).

The hypothalamus plays important roles in the central regulation of food intake in mammals (Woods, 2009) and birds (Richards and Proszkowiec-Weglarz, 2007). Hence, in order to evaluate the possible role of central *IGF-2* and related proteins in the central regulation of food intake in chicks, we next examined the effects of fasting on the mRNA levels of *IGF-2*, *IGF-1R*, *IGF-2R*, and *IGFBPs* in the hypothalamus. None of these mRNA levels in the hypothalamus of chicks were affected by six hours of fasting (Table 1).

In order to evaluate the possible role of hepatic IGF-2 and IGFBPs production in response to feeding status in chicks, we next examined the effects of fasting on their mRNA levels in the liver. Six hours of fasting did not influence the mRNA levels of *IGF-2* but markedly increased the mRNA levels of *IGFBP-1* and *-2* (Fig. 5). The mRNA levels of *IGFBP-3* were also significantly increased.

We finally examined the effects of WY14643 on *IGFBPs* expression in LMH cells and found that WY14643 significantly increased the mRNA levels of *IGFBP-1*, as well as those of a *PPARα* target gene *CPT1A* (Fig. 6).

Thus, our findings suggest that increased hepatic production of IGFBP-1, -2, and -3 may decrease free IGF-2 in the circulation under *ad libitum* feeding conditions, which in turn suppresses the anorexigenic action of IGF-2 in chicks. Our findings also suggest that the upregulation of hepatic *IGFBP-1* expression may be involved in the activation of PPARα in chicken liver.

4. **Discussion**

We showed that central administration of IGF-1 (Fujita et al., 2017) and IGF-2 (present study) significantly suppressed food intake in chicks. In rats, both IGF-1 and -2 probably cross the blood-brain barrier via IGFs receptors (Reinhardt and Bondy, 1994).

As described in the Introduction section, IGFBPs bind IGF-1 and -2 in plasma and block their binding to the receptors. In the present study hepatic *IGFBP-1*, *-2*, and *-3* mRNA levels showed significantly lower levels in the *ad libitum* feeding condition as compared to the fasting condition. All these findings raise the hypothesis that down regulation of IGFBP-1, -2, and -3 production in the liver elevates plasma free IGFs, which in turn facilitates IGFs crossing to the brain and suppressing food intake in chicks.

There are three major IGFBPs, IGFBP-28 (28kDa), -34 (34kDa), and -40 (40kDa) in the plasma of chickens. Plasma IGFBP-28 and -34 were increased after 48 hours of fasting, whereas IGFBP-40 did not show any significant change (Beccavin et al., 1999). IGFBP-28, -34, and -40 are suggested to be IGFBP-1, -2, and -3, respectively (Duclos et al., 1999). In the present study, mRNA levels of *IGFBP-1* and *-2* in the liver under six hours of fasting condition were 5.47- and 6.95-fold higher than that under the *ad libitum* feeding condition. On the other hand, mRNA levels of *IGFBP-3* under the fasting condition were only 1.36-fold higher than that under the feeding condition. The effects of feeding on the plasma concentration of free IGFs in chickens have not been investigated. However, these findings suggest that hepatic IGFBP-1 and -2 production may play important roles in the diet-induced changes in plasma free IGFs levels in chickens.

Forty-eight hours of fasting significantly decreased the plasma total IGF-2 concentration in 9-week-old fat chickens, but not in lean chickens (Beccavin et al., 1999). Sixteen hours of fasting significantly decreased plasma total IGF-2 concentration in 16-week-old lean chickens (Beccavin et al., 2001). In the present study, six hours of fasting did not influence *IGF-2* mRNA levels in the liver in 7-day-old layer chicks. It is therefore possible that six hours of fasting is not enough to elevate the plasma total IGF-2 concentration in layer chicks, although the plasma free IGF-2 concentration may be changed. Further study is needed to clarify the effects of fasting on the plasma free IGF-

2 concentration in chicks.

We previously showed that four hours of fasting significantly elevated plasma non-esterified fatty acid (NEFA) and increased the mRNA levels of hepatic *PPARα* in chickens (Saneyasu et al., 2013). PPARα is activated by fatty acids (Nakamura et al., 2014). In mammalian hepatoma cells, hepatic *IGFBP-1* (Degenhardt et al., 2006) and *IGFBP-2* (Kang et al., 2015) expression are upregulated by a peroxisome proliferator-activated receptor α (PPAR α). Therefore, we finally examined the effects of the PPARα agonist WY14643 on the mRNA levels of *IGFBPs* and a *PPARα* target gene, *CPT1A*, in chicken hepatoma cells and found that the PPARα agonist significantly increased the mRNA levels of *IGFBP-1*. It is therefore possible that fasting-elevated plasma NEFA upregulates hepatic IGFBP-1 via PPARα in chickens, which in turn inhibits the anabolic effects of IGFs. Further study is required to clarify the mechanism underlying the fasting-induced upregulation of *IGFBP-2* and *-3* expression in chicken liver.

In the present study, six hours of fasting increased hepatic *IGFBP-3* expression in 8-day-old layer chicks. On the other hand, we previously showed that six hours of fasting did not influence hepatic *IGFBP-3* expression in 8-day-old broiler chicks. These findings raise the hypothesis that fasting induced IGFBP-3 production may suppress the anorexigenic action of circulating IGFs in layer chicks but not in broiler chicks. However, it is well known that broiler chicks eat more food than layer chicks (Mahagna and Nir, 1996). Therefore, IGFBP-3 may play a minor role in the regulation of food intake in chicks as compared to IGFBP-1 and -2.

Tachibana et al. (2003) reported that central administration of 477 pmol of cocaine- and amphetamine-regulated transcript (CART) significantly suppressed food intake under an *ad libitum* feeding condition, but the same dose of CART did not suppress food intake in chicks fasted for three hours. Shiraishi et al. (2009) reported that central administration of 2 ng of porcine insulin significantly suppressed food intake under an *ad*

libitum feeding condition, whereas central administration of 10 ng of porcine insulin did not suppress food intake in chicks fasted for three hours. These findings suggest that the feeding condition can influence the sensitivity to appetite regulatory peptide in chicks. However, in the present study, central administration of 300 pmol of IGF-2 significantly suppressed food intake under both three hours of fasting and *ad libitum* feeding conditions. Six hours of fasting did not influence the mRNA levels of *IGF-1R*, *IGF-2R*, and *IGFBPs* in the hypothalamus of chicks. It is therefore likely that short term fasting does not influence the anorexigenic effect of IGF-2 in chicks.

IGF-2 plays important roles in chick embryonic development (McMurtry et al., 1997). Liu et al. (2016) reported that *IGF-2* mRNA levels in the liver and skeletal muscles increased during embryonic growth and showed higher levels in the later stages (embryonic days 17-19). A high concentration (60-80 ng/mL) of plasma IGF-2 during embryonic days 13-21 fell to 40-50 ng/mL after hatching (Lu et al., 2007). McMurtry (1998) also showed that plasma IGF-2 concentration increased before hatching and decreased after hatching. Holzenberger and Lapointe (2000) reported that *IGF-2* expression in the chicken brain is downregulated shortly after hatching. These findings raise the hypothesis that IGF-2 suppresses appetite before hatching. Thus, it will be interesting to clarify whether downregulation of IGF-2 production after hatching functions as the trigger for appetite induction in neonatal chicks.

Interestingly, the anorexigenic action of IGF-1 has been observed in diabetic rats (Lu et al., 2001), but not in non-diabetic mammals (Lauterio et al., 1987; Foster et al., 1991). These findings raise the hypothesis that the appetite regulatory role of IGF-1 is changed by the physiological condition or type of animal. In the present study, we showed that IGF-2 suppresses food intake in chicks. The anorexigenic action of IGF-2 has been observed in non-diabetic rats (Lu et al., 2001). It seems likely that the anorexigenic function of IGF-2 may have been well conserved between mammals and birds. However,

we also found that central administration of 300 pmol of IGF-2 did not influence food intake in meat type chicks (unpublished data). Higher doses of IGF-2 may suppress food intake in broiler chicks. Further studies are needed to compare the anorexigenic effect of IGF between different types of chicks in a wider range of doses (i.e. 0-3,000pmol).

IGF-2 actions are possibly mediated by both IGF-1R and IGF-2R. Our previous observations suggest that hypothalamic POMC and AKT may be involved in the IGF-1-induced anorexigenic pathway in chicks. However, Versteyhe et al. (2013) investigated the gene expression regulated via IGF-1 receptor and found significant difference in responses between equipotent concentrations of IGF-1 and -2 in mice fibroblasts. It is therefore likely that IGF-1 and -2 shows have different actions even through the same receptor. Further study is needed to examine the effects of central administration of IGF-2 on the phosphorylation of signaling molecules and expression of appetite regulating neuropeptides in the hypothalamus of chickens.

5. **Conclusion**

In the present study, we found that central administration of IGF-2 suppressed food intake in chicks. We also showed that hepatic *IGFBP-1*, *-2*, and *-3* mRNA levels were markedly increased in response to fasting. These findings suggest that IGF-2, IGFBP-1, -2, and -3 may be involved in the regulation of food intake in chickens.

Figure captions

Fig. 4. Effects of central administration of insulin-like growth factor-2 on food intake in chicks. A: IGF-2 was administered after three hours of fasting. B: IGF-2 was administered under an *ad libitum* feeding condition. Data represent means ± S.E.M. The number of chicks used is shown in parentheses. * indicates significant difference with respect to the 0 pmol group ($P < 0.05$).

Fig. 5. Effects of six hours of fasting on the mRNA levels of insulin-like growth factor-related genes in the livers of chicks. Data were normalized to the respective average of each feeding group. Data represent means ± S.E.M. of six chicks. ** indicates significant difference with respect to the feeding group ($P < 0.01$).

Fig. 6. Effects of WY14643 on the mRNA levels of insulin-like growth factor-related genes in chicken hepatoma cells. Data represent means ± S.E.M. of four wells in each group. * indicates significant difference with respect to the control group ($P < 0.05$).

Fig. 4

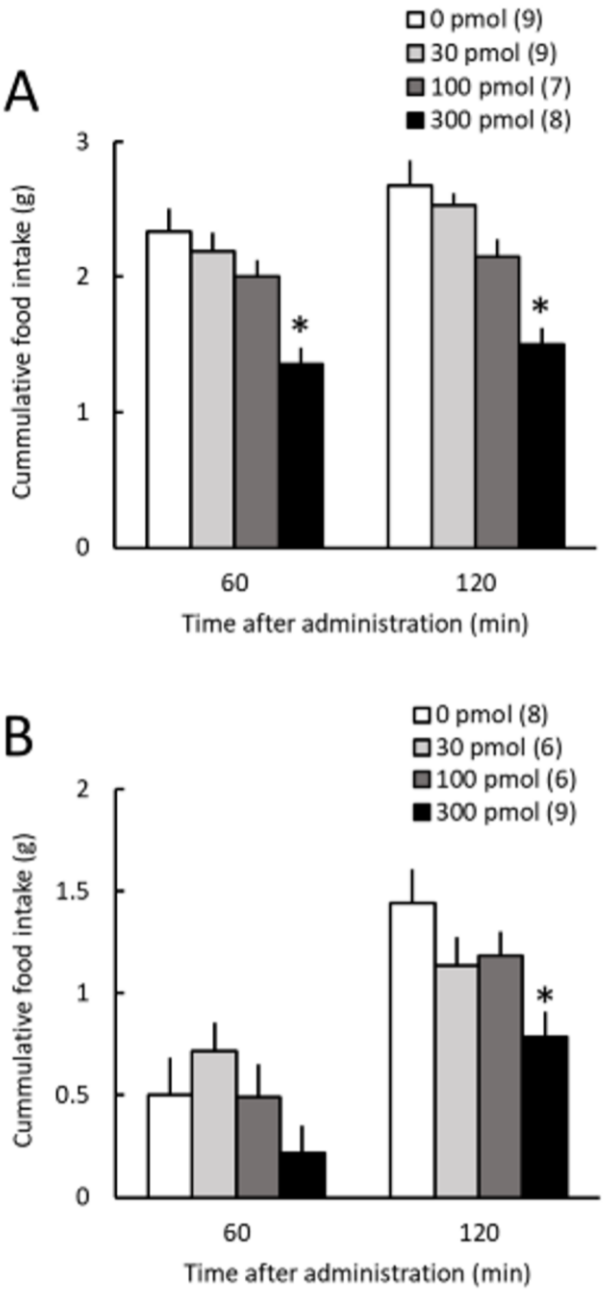

A
0 pmol (9)
30 pmol (9)
100 pmol (7)
300 pmol (8)
Cummulative food intake (g)
3
2
1
0
*
*
60
120
Time after administration (min)
B
0 pmol (8)
30 pmol (6)
100 pmol (6)
300 pmol (9)
Cummulative food intake (g)
2
1.5
1
0.5
0
*
60
120
Time after administration (min)

Table 1. Effects of six hours of fasting on the mRNA levels of insulin-like glowth factor-related genes in the hypothalamus of chicks

	Feeding	Fasting
IGF-2	1.00 ± 0.08	0.85± 0.10
IGF-1R	1.00 ± 0.06	0.87± 0.05
IGF-2R	1.00 ± 0.03	1.01 ± 0.07
IGFBP-1	1.00 ± 0.02	1.01 ± 0.04
IGFBP-2	1.00 ± 0.08	0.82 ± 0.16
IGFBP-3	1.00 ± 0.03	0.89 ± 0.04
IGFBP-4	1.00 ± 0.11	0.75 ± 0.06
IGFBP-5	1.00 ± 0.01	0.99 ± 0.06

Data were normalized to respective average of each feeding group. Data represent means ± S.E.M. of six chicks.

Fig. 5

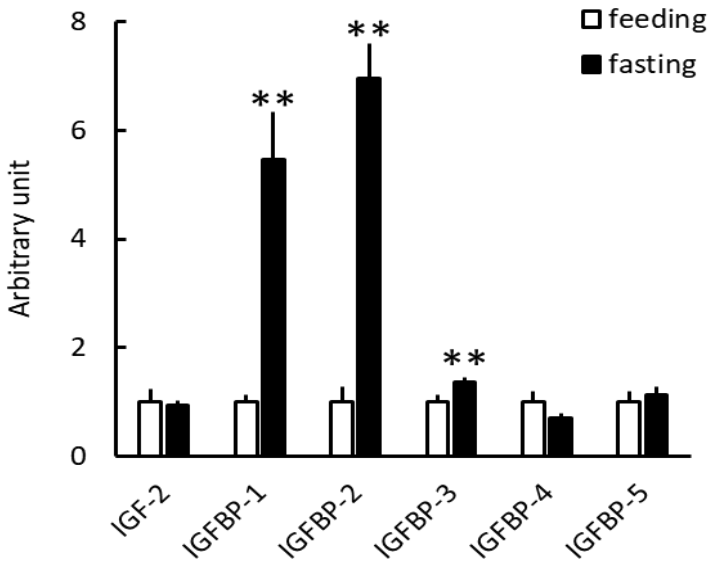
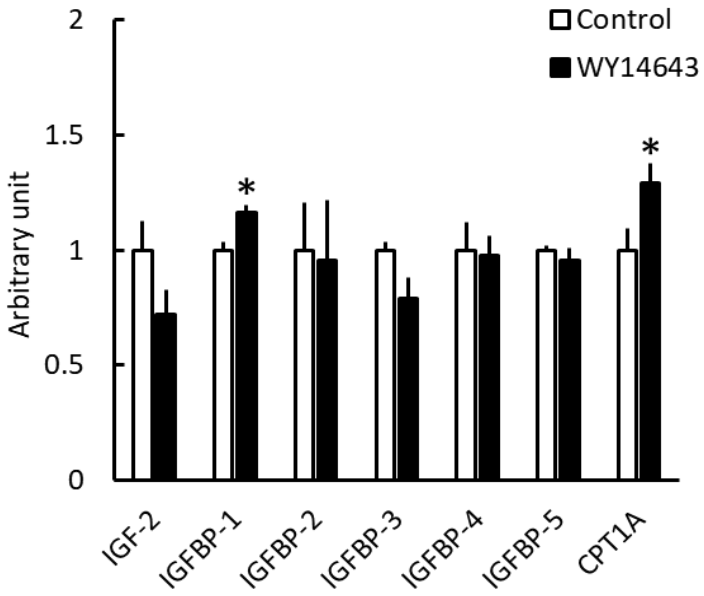

feeding
fasting
Arbitrary unit
8
6
4
2
0
**
**
**
IGF-2
IGFBP-1
IGFBP-2
IGFBP-3
IGFBP-4
IGFBP-5

Fig. 6


Control
WY14643
Arbitrary unit
2
1.5
1
0.5
0
*
*
IGF-2
IGFBP-1
IGFBP-2
IGFBP-3
IGFBP-4
IGFBP-5
CPT1A

Chapter 4

Comparison of the effects of intracerebroventricular administration of glucagon-like peptides 1 and 2 on hypothalamic appetite regulating factors and sleep-like behavior in chicks.

Introduction

Glucagon-like peptide (GLP)-1 and GLP-2, proglucagon-derived brain-gut peptides, function as anorexigenic neuropeptides in mammals and chickens. For example, central administration of GLP-1 and GLP-2 suppressed food intake in rats (Turton et al., 1996; Tang-Christensen et al., 2000) and chicks (Honda et al., 2015b). GLP-1 and GLP-2-producing neurons in the nucleus of the solitary tract (NTS) in the medulla oblongata project to several food-regulating areas in the hypothalamus (Guan et al., 2012; van Bloemendaal et al., 2014). We previously showed that proglucagon mRNA levels in the chicken medulla oblongata were reduced by fasting (Honda et al., 2015c). mRNA levels of the *GLP-1* receptor (*GLP1R*) (Huang et al., 2012) and *GLP-2* receptor (*GLP2R*) (Mo et al., 2014) were distributed in different brain regions in adult chickens. GLP-1 and GLP-2 specifically activate *GLP1R* and *GLP2R*, respectively, in chickens (Honda, 2016). These findings raise the hypothesis that both GLP-1 and GLP-2 function as anorexigenic peptides in the chicken brain but the mechanisms underlying the anorexigenic effects are different between them.

In mammals, AMP-kinase (AMPK) regulates feeding behavior by modulating orexigenic neuropeptide Y (NPY)/agouti-related protein (AgRP) neurons and anorexigenic proopiomelanocortin (POMC) neurons (Yang et al., 2011). AMPK activity was reduced by re-feeding in the mouse hypothalamus (Minokoshi et al., 2004). Central GLP1R activation suppresses food intake via inhibition of hypothalamic AMPK

(Burmeister et al., 2013). Hypothalamic POMC neurons are involved in anorexigenic pathways induced by GLP-1 (van Bloemendaal et al., 2014) and GLP-2 (Guan et al., 2012). It is therefore likely that AMPK and POMC are involved in the GLPs-induced anorexigenic pathway in the hypothalamus in mammals.

In chickens, fasting induced phosphorylation of AMPKα in the hypothalamus (Song et al., 2012). POMC neurons play important roles in the central regulation of food intake (Bungo et al., 2011). Tachibana et al. (2006) suggested that the anorexigenic effects of GLP-1 are mediated by CRF in chicks. Fang et al. (2014) proposed that hypothalamic pyruvate dehydrogenase kinase 4 (PDK4) is the key factor in appetite regulation in chicks and possibly influences the gene expression of *NPY*, *AgRP*, and *POMC*. It is therefore possible that AMPK, POMC, CRF, and PDK4 in the hypothalamus are involved in the anorexigenic pathway of GLPs in chicks.

Bungo et al. (1999) reported that ICV administration of GLP-1 induced sleep-like behavior in chicks. They also showed that ICV administration of fusaric acid, an inhibitor of a noradrenaline synthesis rate limiting enzyme, attenuated the anorexigenic effects of GLP-1 in chicks (Bungo et al., 2011). In addition, noradrenaline induces sleep-like behavior and suppresses food intake in chicks (Steinman et al., 1987). All these findings suggest interactive relationships between GLP-1-induced sleep-like behavior and anorexia in chicks. However, the effects of GLP-2 on sleep-like behavior have not yet been examined.

In the present study, we compared the distribution of the *GLP1R* and *GLP2R* in the chicken brain and the effects of GLP-1 and GLP-2 on hypothalamic appetite regulating factors and sleep-like behavior in chicks. Our findings suggest that GLP-1 and GLP-2 are produced in the same region in the chicken brain, but the mechanism underlying GLP-1 and GLP-2-induced behavioral changes may differ between them.

2. Materials and methods

2.1 Animals and diet

This study was approved by the Institutional Animal Care and Use Committee and was performed according to the Kobe University Animal Experimentation Regulations (24-03-06, 26-09-07). One day old male layers (White leghorn, Julia strain) were purchased from local hatcheries (Japan Layer K. K., Gifu, Japan). Fifty chicks were reared in electrically heated battery cages (1,725 mm×850 mm×320 mm). They were given free access to water in four waterers (ϕ 170 mm×140 mm) and a commercial chick starter diet (NICHIWA SANGYO Co., Ltd., Kobe, Japan) in four feeders (75 mm×407 mm×49 mm) under a 23-h/1-h light - dark cycle (23:00-24:00 dark). The temperature was kept at 31 ± 2℃. We previously investigated the effects of ICV administration of GLPs on food intake in 8-day-old chicks, but GLP-1-induced sleep-like behavior has been reported only in 3-day-old chicks (Bungo et al., 1999). Therefore, distribution of GLPs-related genes in the brain and the effects of GLPs on hypothalamic appetite-regulating factor were examined at 7 and 8 days of age, while the effects of GLPs on sleep-like behavior were examined at a younger age (4 days of age).

2.2 Experiment 1: Distribution of GLP1R, GLP2R, and proglucagon mRNA in the brains of chicks.

Four 7-day-old chicks were euthanized by decapitation by a skilled person at 13:00. The whole brains were divided into six regions: telencephalon, optic lobe, cerebellum, rostral part of the brainstem (diencephalon), middle part of the brainstem, and caudal part of the brainstem, as described previously (Aoki et al., 2017). Total RNA

was extracted from the tissues using Sepazol-RNA-I Super G (Nakalai Tesque, Inc., Kyoto, Japan). Real-time PCR analysis was performed as described previously (Kewan et al., 2021). Complementary DNA of *GLP1R* (GenBank accession no. EU770586.1), *GLP2R* (GenBank accession no. FJ899744.1), and proglucagon (GenBank accession no. NM_205260.3) were amplified using the following primers: *GLP1R* sense, 5'-CCC CGC CAG GCG TAGT-3'; *GLP1R* antisense, 5'-GTA CTC CTT CCA CTT CTG CAC AAC-3'; *GLP2R* sense, 5'-TCT CGT CTG CGG GCA AGT-3'; *GLP2R* antisense, 5'-GAT CTT TTG AAA TAC TGT GGC TGT TG-3'; proglucagon sense, 5'-GCA CTA AAA GAA ATG GCC AAC AAG-3'; proglucagon antisense, 5'-GCT GAT CCG GGA ATT TGT CA-3'. Complementary DNA of ribosomal protein S17 (internal standard) was amplified using primers as described previously (Saneyasu et al., 2019a).

2.3 Experiment 2: Effects of central administration of GLP-1 and GLP-2 on the mRNA levels of hypothalamic appetite-regulating factors in chicks

We have already reported that ICV administration of either 30 pmol of GLP-1 or GLP-2 in chicks strongly suppressed food intake for 120 min after administration (Honda et al., 2015b). Therefore, we used a dose of 30 pmol in the present study. Forty-two 8-day-old chicks were weighed and allocated to three groups based on body weight (14 birds in each group). Chicken GLP-1 and GLP-2 were dissolved in 0.85% (w/v) saline solution containing 0.1% (w/v) Evans Blue. The peptides were ICV-administered according to the method of Davis et al. (1979) at a volume of 10 μl after three hours of fasting. Chicks were administered with either a vehicle (control), 30 pmol GLP-1, or 30 pmol GLP-2 at 13:00. At 60 minutes after the ICV injection, the chicks were euthanized by decapitation by a skilled person. Verification of injection was made by observation of the presence of Evans Blue dye in the lateral ventricle. Samples from chicks without Evans Blue dye in

the lateral ventricle were excluded. Diencephalons were collected and preserved in RNAlater tissue storage reagent (Sigma-Aldrich, St. Louis, Mo, USA) for 2 days. The hypothalamus was excised as described previously (Honda et al., 2015a).

Real-time PCR analysis was performed as described in Experiment 1. Complementary DNA of *CRF* (GenBank accession no. NM_001123031.1), *PDK4* (GenBank accession no. NM_001199909.1), *AMPK α1* (GenBank accession no. DQ302133.1) and *AMPK α2* (GenBank accession no. DQ340396.1) were amplified using the following primers: *CRF* sense, 5'-CAT CTC CCT GGA CCT GAC TTT C-3'; *CRF* antisense, 5'-CCG ATG ATT TCC ATC AGT TTC C-3'; *PDK4* sense, 5'-AGTCTG CTT CCA AAC ATT ACC AAA C-3'; *PDK4* antisense, 5'-CAG TCT GCT TTG GAC CTT TAC TTG-3'; *AMPK α1* sense, 5'-CGG CGG CAG ATA AAC AGA A-3'; *AMPK α1* antisense, 5'-CAG AAT GTA ATG CCC AAT CTT CAC-3'; *AMPK α2* sense, 5'-CGC CTT TTC CAG CAG ATT CT-3'; *AMPK α2* antisense, 5'-GAC AAC CAT GTG T CG GTG ACA-3'. Complementary DNA of *NPY*, *AgRP*, and *POMC* were amplified using primers as described previously (Fujita et al., 2019). Complementary DNA of *GLP1R* and *GLP2R* was amplified using primers as described in Experiment 1.

2.4 Experiment 3: Effects of central administration of GLP-1 and GLP-2 on AMPK protein levels in the chick hypothalamus

Either a vehicle, 30 pmol GLP-1, or 30 pmol GLP-2 was administered as described in Experiment 2. At 60 minutes after administration, the chicks were euthanized by decapitation by a skilled person, and the hypothalamus was excised, immediately frozen in liquid nitrogen, and stored at −80 °C.

Western blot analysis was performed as described previously (Fujita et al., 2019). Anti-AMPK (#2532), anti-phospho-AMPK (pAMPK) (Thr172) (#2531), anti-β-actin (#8457),

and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (#7074) were purchased from Cell Signaling Technology (Danvers, MA, USA). An anti-β-actin antibody was used to detect a loading control.

2.5 *Experiment 4: Effects of central administration of GLP-1 and GLP-2 on sleep-like behavior in chicks.*

Forty-two 4-day-old chicks were weighed and allocated to three groups based on body weight (14 birds in each group). Either a vehicle, 30 pmol GLP-1, or 30 pmol GLP-2 was administered at 13:00 as described in Experiment 2. The postures were observed at 30 minutes after the ICV injection. As described in the previous study (Bungo et al., 1999), four postures were categorized: (1) active wakefulness; (2) standing/sitting with eyes open; (3) standing motionless with eyes closed; (4) sitting motionless with head drooped (sleeping posture). After observation, chicks were euthanized by decapitation. Verification of injection was made by observation of the presence of Evans Blue dye in the lateral ventricle. Data from chicks without Evans Blue dye in the lateral ventricle were excluded.

2.6 Data analysis

Data from Experiment 1, 2, and 3 were analyzed by one-way analysis of variance, after a significant difference ($P < 0.05$) was detected, data from Experiments 1 were analyzed by the Tukey-Kramer test, whereas data from Experiments 2 and 3 were analyzed by Fisher's protected least significant difference test. Data from Experiment 4 were analyzed by the Games-Howell test. All statistical analyses were performed using a commercial software package (StatView version 5, SAS Institute, Cary, NC, USA, 1998).

3. Results

GLP1R mRNA levels in the brain stem and optic lobes were significantly higher than in other parts of the brain (Fig. 7a), whereas *GLP2R* mRNA was densely expressed in the telencephalon (Fig. 7b). Proglucagon mRNA levels in the caudal part of the brain stem were significantly higher than in other parts of the brain (Fig. 7c).

As shown in Fig. 8, ICV administration of 30 pmol of either GLP-1 or 2 significantly reduced the mRNA levels of *CRF* and *AMPKα1*, whereas that of *POMC* was significantly increased, and those of *AMPKα2* and *GLP2R* were significantly decreased by ICV injection of GLP-2, but not GLP-1. On the other hand, only GLP-1 significantly increased the mRNA level of *PDK4* and decreased that of *GLP1R*. However, neither GLP-1 nor GLP-2 affected the protein levels or phosphorylation of AMPK (Supplementary file).

ICV administration of 30 pmol of either GLP-1 or 2 significantly induced sleep-like behavior in chicks, and there was no significant difference between the GLP-1 and GLP-2 groups (Fig. 9).

4. Discussion

In the present study, we showed different distributions of proglucagon, *GLP1R*, and *GLP2R* mRNAs in the chick brain. This is the first evidence showing statistical differences in the mRNA levels of these genes between brain parts. Kuenzel (1989) reported that three neural pathways, including the trigeminal sensorimotor system, visual system/basal ganglia pathway, and olfactory pathway, play different roles in controlling food intake in birds. The telencephalon is involved in these pathways and optic lobes are involved in the visual system/basal ganglia pathway. In mammals, the NTS receives peripheral signals, such as vagal afferent activation, and GLPs producing neurons in the

NTS convey these signals to several areas, including the hypothalamus, in mammals (van Bloemendaal et al., 2014). We demonstrated that the caudal part of the brain stem, which includes NTS, may be the major production area of GLP-1 and GLP-2 in chicken brains and various expression sites of *GLP1R* and *GLP2R* mRNAs throughout the brain. These findings raise the hypothesis that GLPs in NTS may convey peripheral signals to several areas in the brain, which in turn results in anorexia in chickens as well as in mammals. Our findings suggest the importance of determining the distribution of GLP-producing neurons throughout the brain.

Guan (2014) reported that ICV administration of GLP-2 in mice significantly suppressed food intake and significantly increased *POMC* mRNA levels in the ARC in the hypothalamus. In addition, the anorexigenic effects of GLP-2 were abolished in melanocortin receptor-4 knockout mice. They also found that POMC neuron-specific *GLP2R* knockout mice exhibited hyperphagia (Guan, 2014). In the present study, we showed that ICV administration of GLP-2 significantly increased *POMC* mRNA levels in the hypothalamus, suggesting that POMC neurons are involved in the anorexigenic pathway of GLP-2 in chicks. We also showed that proglucagon mRNA levels are densely expressed in the caudal part of the brain stem. GLP-2 producing neurons in the NTS project to the hypothalamus and directly influence POMC neurons in mice (Guan, 2014). Therefore, further study is needed to clarify whether GLP-2-producing neurons in the NTS directly project to the POMC neurons in the infundibular nucleus in the chicken hypothalamus.

ICV administration of GLP-1 and GLP-2 significantly reduced the mRNA levels of *GLP1R* and *GLP2R*, respectively, suggesting the desensitization of these receptors. It is well known that agonist exposure downregulates G protein coupled receptor activity via several mechanisms, including transcriptional regulation (Pitcher et al., 1998). GLP-1 significantly suppresses *GLP1R* expression in rat insulinoma cells (Fehmann et al.,

1996). In the present study, ICV administration of GLP-1 and GLP-2 differently affected the mRNA levels of *POMC* and *PDK4* in the hypothalamus. These findings suggest that both GLP-1 and GLP-2 regulate the transcription of different genes via their receptor.

Fang et al. (2014) found that fasting increased the mRNA levels of *PDK4* in the chicken hypothalamus. They also showed that ICV administration of alpha-lipoic acid, a PDK4 inhibitor, suppressed food intake in chicks. These findings suggest that PDK4 functions as an orexigenic factor in chicks. In the present study, GLP-1 upregulated the gene expression of *PDK4*. It is therefore likely that GLP-1-increased *PDK4* mRNA is not the cause of the anorexigenic action of GLP-1. The mechanism underlying the upregulation of *PDK4* expression in the hypothalamus in GLP-1 group is not clear. However, in the mammalian brain, glucocorticoids upregulate *PDK4* transcription in astrocytes but not in neurons (Juszczak and Stankiewicz, 2018). ICV administration of GLP-1 in chicks elevates plasma corticosterone at 30 min after administration (Tachibana et al., 2006), but not at 60 min (Honda et al., 2014). Therefore, the elevation of the mRNA levels of *PDK4* in the GLP-1 group may be due to the acute and temporal elevation of plasma corticosterone in chicks. It is not clear whether the appetite-regulating role of PDK4 is different between astrocytes and neurons. Further studies are required to clarify the relationships among astrocytes, PDK4, and GLP-1-induced anorexia in chicks.

Previous studies suggest that AMPK is involved in the appetite regulating pathway in chicks. For example, central administration of ghrelin, an anorexigenic peptide in chickens, suppressed the phosphorylation of AMPK in both high and low weight strains of chicks (Xu et al., 2011). Glucocorticoids cause hyperphagia via the AMPK-NPY signaling pathway (Liu et al., 2014). However, in the present study, neither total AMPK nor phosphorylated AMPK were affected by GLPs, although hypothalamic *AMPKα* mRNA levels were reduced by them. It is therefore likely that AMPK may not play an important role in the anorexigenic action of GLPs in chicks, at least in this

experimental condition. Further study is needed to clarify whether GLP-1 and GLP-2 regulate AMPK activity in each hypothalamic nucleus, which in turn regulates food intake in chickens.

In the present study, both 30 pmol of GLP-1 and GLP-2 induced sleep-like behavior in chicks. We previously showed that both 30 pmol of GLP-1 and GLP-2 potently suppressed food intake in chicks (Honda et al., 2015c). Sleep induction is one of the causes of food intake suppression of food intake in chicks (Tran et al., 2019). These findings suggest that GLPs-induced sleep-like behavior is one of the causes of reduced food intake in chicks. GLP-1 induced sleep-like behavior may be induced by the noradrenergic system in chicks (Steinman et al., 1987; Bungo et al., 1999). Interestingly, central administration of noradrenalin in chicks suppressed CRF-induced vocalization and locomotion and induced sleep-like behavior (Zhang et al., 2003). Tachibana et al. (2006) reported that central administration of a CRF receptor antagonist in chicks attenuated the anorexigenic effects of GLP-1 at 30 min after injection. However, in the present study, ICV administration of GLP-1 in chicks reduced the mRNA levels of *CRF* at 60 min after injection and induced sleep-like behavior. Thus, the acute anorexigenic effects of GLP-1 may be partly mediated by CRF, but noradrenergic system-induced sleep-like behavior plays a more important role in decreased food intake in chicks. Further studies are required to clarify whether noradrenalin suppresses hypothalamic *CRF* expression in GLPs-injected chicks.

5. Conclusion

In the present study, we compared the effects of central administration of GLP-1 and GLP-2 on sleep-like behavior and appetite-regulating factors in the chick hypothalamus. Our findings suggest that both GLP-1 and GLP-2-induced behavioral

changes in chicks may be expressed partly through different pathways.

Figure captions

Fig. 7. Distribution of *GLP-1* receptor, *GLP-2* receptor, and proglucagon mRNAs in the chick brain. Data represent means ± SEM of four birds. Groups with different letters are significantly different ($P < 0.05$).

Fig. 8. Effects of central administration of GLP-1 and GLP-2 on the mRNA levels of hypothalamic appetite-regulating factors in chicks. *NPY*, neuropeptide Y; *POMC*, proopiomelanocortin; *AgRP*, agouti-related protein; *CRF*, corticotrophin releasing factor; *AMPKα1*, AMP-activated protein kinase alpha 1; *AMPKα2*, AMP-activated protein kinase alpha 2; *PDK4*, pyruvate dehydrogenase kinase; *GLP1R*, glucagon-like peptide-1 receptor; *GLP2R*, glucagon-like peptide-2 receptor. Data are the means ± S.E.M. of eight birds in each group and are expressed as a percentage of the mean in the control group. * Significant with respect to the control group ($P < 0.05$).

Fig. 9. Central administration of GLP-1 and GLP-2 on chick posture. Numbers of chicks were as follows: saline, 12; GLP-1, 11; GLP-2, 12. *Significance with respect to the control group ($P < 0.05$). (1) active wakefulness, (2) standing/sitting with eyes open, (3) standing motionless with eyes closed, (4) sitting motionless with head drooped.

Fig 7

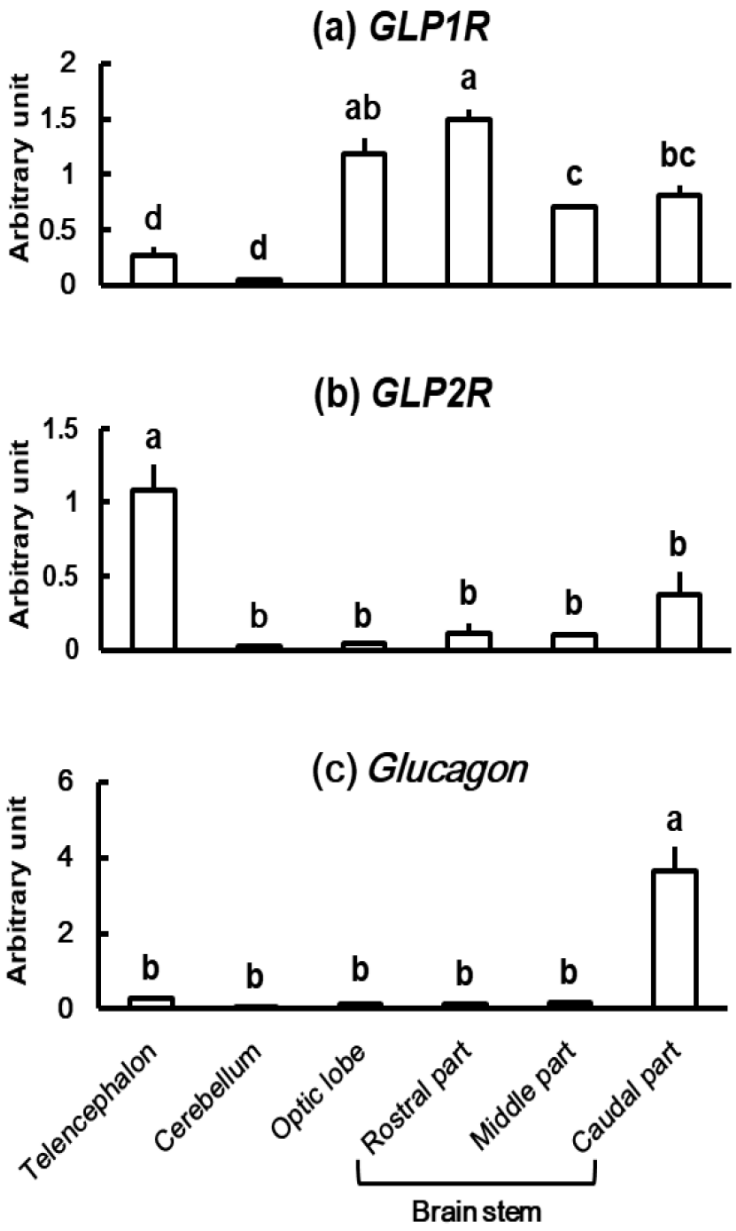

(a) GLP1R
Arbitrary unit
2
1.5
1
0.5
0
d
d
ab
a
c
bc
(b) GLP2R
Arbitrary unit
1.5
1
0.5
0
a
b
b
b
b
b
(c) Glucagon
Arbitrary unit
6
4
2
0
b
b
b
b
b
a
Telencephalon
Cerebellum
Optic lobe
Rostral part
Middle part
Caudal part
Brain stem

Fig. 8

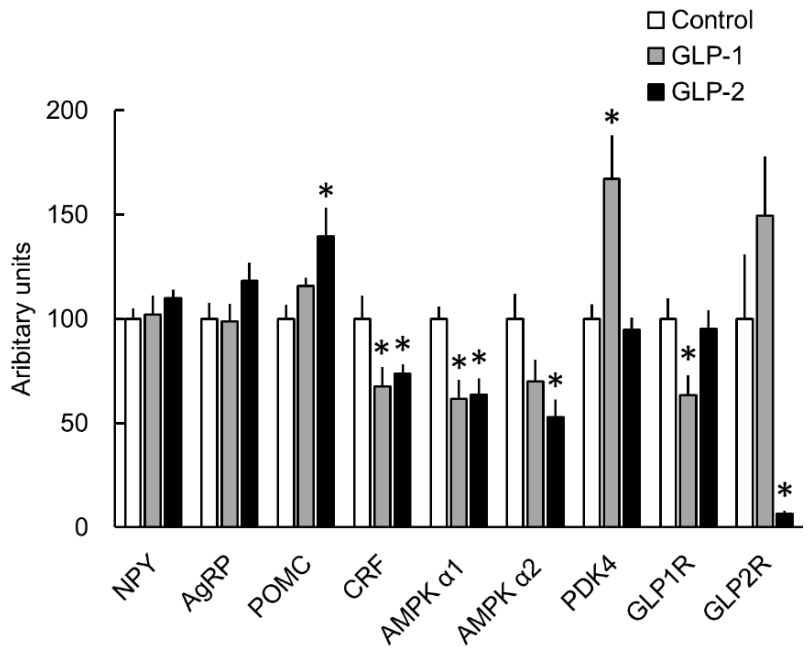
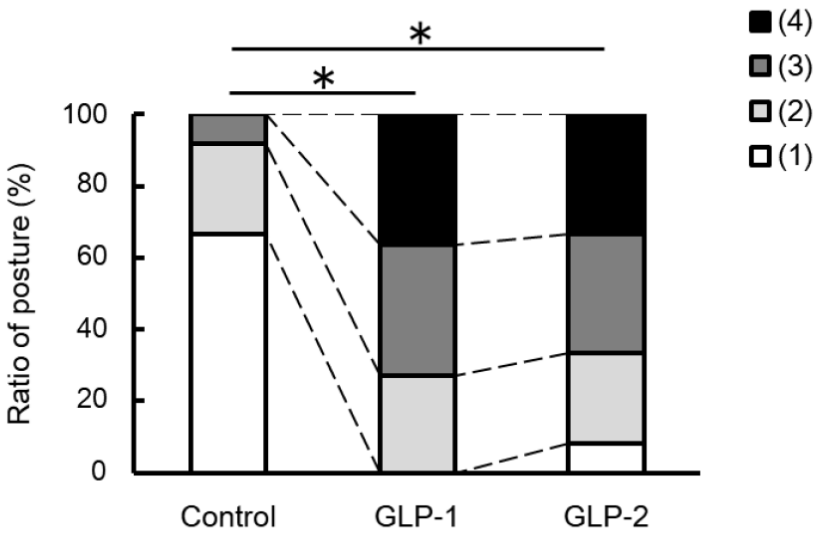
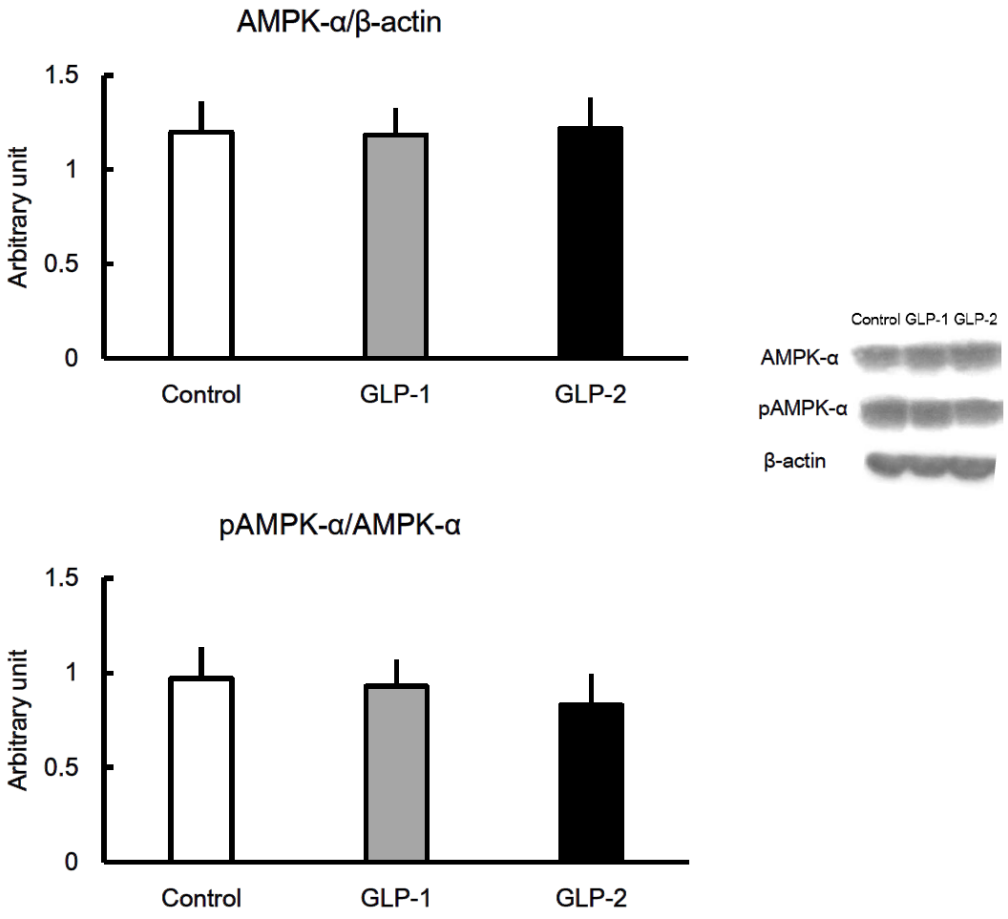

Control
GLP-1
GLP-2
Aribitary units
200
150
100
50
0
NPY
AgRP
POMC
CRF
AMPK α1
AMPK α2
PDK4
GLP1R
GLP2R

Fig. 9



Supplementary file



Summary

In the present study, I investigated the possible roles of satiety signals, such as pancreatic PYY, intestinal CCK, PYY, and their receptors, and hepatic IGF-related proteins. I also examined whether IGF-2 functions as a satiety signal in chicks. Finally, I investigated the mechanism of GLPs anorexigenic action in the hypothalamus in chicks.

In Chapter 1, I introduced the hyperphagia phenomena in modern meat-type chicken and how over-feed consumption contributed to health and metabolic problems in the poultry industry. I also reviewed how some appetite regulation aspects in birds are different than in mammals. I demonstrated the importance of satiety signals such as CCK, PYY, and IGF-1, the possibility that GLPs could deliver satiety signals to the hypothalamus in chickens. Finally, I explained the aim of this study.

In Chapter 2, I showed the effects of fasting and re-feeding on the expression of CCK, PYY, hypothalamic neuropeptides NPY and POMC, and hepatic IGF-related genes in layer and broiler chicks. In layer chicks, 12 h of fasting reduced the mRNA levels of intestinal CCK, PYY, Y2 receptor, and pancreatic PYY, and these changes were reversed by 12 h of re-feeding. On the other hand, in broiler chicks 12 h of fasting reduced the mRNA levels of intestinal PYY and Y2 receptor, but not intestinal CCK and pancreatic PYY, and these changes were reversed by 12 h of re-feeding. Hypothalamic NPY mRNA significantly increased by 12 h of fasting in both chicks, and these changes were reversed by re-feeding. Also, 12 h of fasting significantly increased the mRNA levels of hypothalamic agouti-related protein and reduced the mRNA levels of hepatic IGF-1 only in broiler chicks, and 12 h of re-feeding did not change these. IGFBP-1 and -2 mRNA levels were markedly increased by 12 h of fasting in both chicks, and these changes were reversed by re-feeding. IGFBP-3 mRNA levels were increased by 12 h of fasting only in layer chicks, while re-feeding reduced the mRNA levels of IGFBP-3 in both types of

chicks. These results suggest that several peripheral hormones, such as pancreatic PYY and intestinal CCK, may not play important roles in the regulation of food intake in broiler chicks.

In Chapter 3, I evaluated whether IGF-2 is involved in the regulation of food intake in chicks. I also examined the effects of fasting on the mRNA levels of IGF binding proteins (IGFBPs) in the liver and hypothalamus. Intracerebroventricular administration of IGF-2 significantly suppressed food intake in chicks. The mRNA levels of IGFBPs in the hypothalamus were not affected by six hours of fasting. On the other hand, six hours of fasting markedly increased the mRNA levels of hepatic IGFBP-1 and -2. The mRNA levels of IGFBP-3 were also significantly increased by six hours of fasting, whereas the mRNA levels of IGF-2, IGFBP-4, and -5 were unchanged. These findings suggest that circulating IGF-2 may be involved in satiety signals, but its physiological role may be regulated by IGFBPs production in the liver in chicks.

In Chapter 4, I showed effects of intracerebroventricular administration of glucagon-like peptides 1 and 2 on hypothalamic appetite regulating factors and sleep-like behavior in chicks. GLP1R mRNA levels in the brain stem and optic lobes were significantly higher than in other parts of the brain, whereas GLP2R mRNA was densely expressed in the telencephalon. Intracerebroventricular administration of either GLP-1 or GLP-2 significantly reduced the mRNA levels of corticotrophin releasing factor and AMP-kinase (AMPK) α1. The mRNA level of proopiomelanocortin was significantly increased, and those of AMPKα2 and GLP2R were significantly decreased by GLP-2, whereas the mRNA level of pyruvate dehydrogenase kinase 4 was significantly increased, and that of GLP1R was significantly decreased by GLP-1. Intracerebroventricular administration of either GLP-1 or GLP-2 induced sleep-like behavior in chicks. Our findings suggest that the anorexigenic peptides GLP-1 and GLP-2 induce similar behavioral changes in chicks, but the mechanism may differ between them.

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