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Role of Hypothalamic Transforming Growth Factor-β (TGF-β)/ Smad Signaling in Feeding Regulation in Chickens

Takaoki Saneyasu, Miku Ueda, Kanami Nagata, Jiawei Chai, Kazuhisa Honda and Hiroshi Kamisoyama

Graduate School of Agricultural Science, Kobe University, Kobe 657−8501, Japan

Previous studies in mammalian obesity models have suggested that central transforming growth factor-β (TGF-β) controls the gene expression of appetite-regulating neuropeptides and peripheral energy metabolism. In the present study, we investigated the possible involvement of central TGF-β/Smad signaling in feeding regulation in chickens. Central administration of TGF-β1 resulted in phosphorylation of Smad2 in the hypothalamus of chicks and suppressed feed intake without changing the gene expression of hypothalamic appetite-regulating neuropeptides (neuropeptide Y, agouti-related protein, proopiomelanocortin, and corticotropin-releasing factor). However, neither fasting nor refeeding induced the phosphorylation of hypothalamic Smad2. These findings suggest that the activation of hypothalamic TGF-β/Smad signaling suppresses feed intake in chicks but it might not occur in response to feeding status.

Key words: chickens, food intake, hypothalamus, Smad, transforming growth factor-β

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Introduction

Feed intake is one of the key factors influencing the productivity of poultry industry. Hence, realizing the regulatory mechanisms underlying feed intake in commercial chicken is essential. Hypothalamic cellular signaling pathways, such as insulin, Akt, mTOR, ERK, JAK/STAT, and AMPK signaling, are involved in feeding regulation in mammals (Ono, 2019; Minokoshi *et al*., 2004; Cota *et al*., 2006; Morton *et al*., 2006; Belgardt *et al*., 2009). These pathways control the transcription of appetite-regulating neuropeptides, such as neuropeptide Y (NPY), an orexigenic peptide; agouti-related peptides (AgRP), an orexigenic peptide; and proopiomelanocortin (POMC), the precursor of α-melanocyte-stimulating hormone (α-MSH), an anorexigenic peptide (Morton *et al*., 2006; Belardt *et al*., 2009). Recent studies in chickens have suggested the involvement of central cellular signaling pathways in feeding regulation (Saneyasu *et al*., 2018, 2019a; Song *et al*., 2012; Adeli *et al*., 2020).

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Correspondence: Takaoki Saneyasu, PhD., Department of Bioresource Science, Graduate School of Agricultural Science, Kobe University 1−1, Rokkodai-cho, Nada-ku, Kobe 657−8501, Japan.
(E-mail: saneyasu@phoenix.kobe-u.ac.jp)

Smads are intracellular proteins that act as transcription factors to regulate gene expression (Kamato *et al*., 2013) and are known to mediate the actions of members of the transforming growth factor-β (TGF-β) superfamily, such as TGF-βs, activin, myostatin, and bone morphogenetic proteins (BMP) (Shi and Massagué, 2003; Kamato *et al*., 2013). Smads are divided into three groups: receptor-regulated Smads (R-Smads), common mediator Smads (Co-Smads), and inhibitory Smads (Shi and Massagué, 2003; Kamato *et al*., 2013). R-Smads, Smad2/3 are phosphorylated in response to TGF-β, whereas Smad1/5/8 is phosphorylated in response to BMP (Shi and Massagué, 2003; Kamato *et al*., 2013). Phosphorylated Smad2/3 binds to the co-Smad, Smad4, to form a heteromeric Smad complex (Shi and Massagué, 2003; Kamato *et al*., 2013). The Smad complex enters and accumulates in the nucleus to initiate transcription of target genes (Shi and Massagué, 2003; Kamato *et al*., 2014). A previous study in mice showed that a high-fat diet significantly increased the levels of TGF-β, along with phosphorylation of Smad 2/3 proteins and POMC mRNA in the hypothalamus. Further, the downregulation of TGF-β expression in the arcuate nucleus by injection of lentiviral shRNA particles significantly increased hypothalamic POMC expression (Mendes *et al*., 2018). Another study in mice showed that central TGF-β1 injection significantly decreased POMC mRNA levels in the hypothalamus (Yan *et al*., 2014), and in chickens, central α-MSH suppresses feed intake (Kawakami *et al*., 2000; Saneyasu *et al*., 2011). These findings indicate the possibility of involvement of the TGF-β/Smad signaling pathway in the regulation of feed intake via the transcrip-

tional control of POMC in chickens.

In the present study, we investigated the role of hypothalamic TGF-β/Smad signaling in feeding regulation in chicks.

Materials and Methods

Identification of Smad2 and Smad3 mRNA in Chicken Hypothalamus

To confirm the expression of *Smad2* and *Smad3* in chicken hypothalamus, reverse transcription-PCR was performed. First-strand cDNA synthesized in previous studies (Saneyasu *et al*., 2019a, b) was used as the template. The genes encoding Smad2 and Smad3 were amplified using PrimeSTAR® HS DNA Polymerase (Takara Bio Inc., Otsu, Japan) and primers (Table 1) in a thermal cycler, as per the manufacturer's instructions. Primers were designed in the N-terminal domain (Mad-homology 1 domain) as the length of the domain differs between Smad2, Smad2 variant, and Smad3 (Fig. 1A). The PCR products were separated on a 1.2% agarose gel containing GelRed® nucleic acid gel stain (Biotium, Inc., Fremont, CA, USA) and visualized in a transilluminator. The bands were excised, and PCR products were extracted from the gel using the FastGene Gel/PCR Extraction Kit (Nippon Genetics Co., Ltd., Tokyo, Japan). The extracted PCR products were cloned using the Mighty TA-cloning Reagent Set for PrimeSTAR (Takara Bio Inc., Otsu, Japan) and Competent high DH5α (Toyobo Co. Ltd., Osaka, Japan). The plasmid was purified using the FastGene Plasmid Mini Kit (Nippon Genetics Co., Ltd., Tokyo, Japan), and the insert sequences were analyzed using Fasmac Co., Ltd. (Kanagawa, Japan).

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee, and conducted according to the Kobe University Animal Experimental Regulation (permission number: 2019-07-03).

Newly hatched male layers (White Leghorn, Julia) and broiler chicks (Ross 308) were purchased from a local hatchery (layer, Japan Layer K. K., Gifu, Japan; broiler, Yamamoto Co. Ltd, Kameoka, Japan) and maintained in a room with an automatically controlled 23 h light/1 h dark cycle (23:00−24:00 dark). They were provided free access to water and a commercial chicken starter diet (Nichiwa Sangyo Co. Ltd., Kobe, Japan).

Experimental Design

Effects of Central Administration of TGF-β on the Phosphorylation of Smad in Chicken Hypothalamus

A previous study in rats showed that intracerebroventricular (ICV) injection of activin-A, but not TGF-β, induced nuclear accumulation of Smad2 and Smad3 immunoreactivity in the hypothalamus (Nakajima *et al*., 2018). However, in the preliminary study, bands of phosphorylated Smad2 and 3 were not detected in Western blot analysis using protein extracts from the hypothalamus of 9-day-old chicks that were administered activin-A (1 µg/chick) intracerebroventricularly. Therefore, we administered TGF-β in the present study.

Eight-day-old layer chicks were weighed and allocated to two groups based on their body weights (eight birds per group). The chicks were provided with free access to water and feed. Recombinant human TGF-β1 (cyt-716) was purchased from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel). The amino acid sequence had 82.5% homology with that of chickens. TGF-β1 was dissolved in a saline solution containing 0.1% Evans blue and 10 mM hydrochloric acid. TGF-β1 (1 µg/chick) or vehicle (control) was administered intracerebroventricularly at a volume of 10 µL, according to the method described by Davis *et al*. (1979). The chicks were euthanized by decapitation 30 min post-administration. The hypothalamus was excised based on a reference to the stereotaxic atlas drawn by Kuenzel and Masson (1988), immediately frozen in liquid nitrogen, and stored at −80℃ until use for Western blot analysis. Injections were verified by observing the presence of Evans blue dye in the lateral ventricle. Four successfully injected samples were randomly selected for Western blotting analysis.

Effects of Central Administration of TGF-β on the Feed Intake of Chicks

Eight-day-old layer chicks were allocated to two groups based on their body weights (eight birds per group). After 3 h of fasting, TGF-β1 (1 µg/chick) or vehicle (as a control) was administered intracerebroventricularly, as described above. Feed intake was measured 30, 60, and 120 min after administration. At the end of the experiment, chicks were euthanized to verify the success of injection. Data from chicks without Evans blue dye in the lateral ventricle have been omitted.

Effects of Central Administration of TGF-β on the Gene Expressions of Appetite-regulating Neuropeptides in Chicks

TGF-β1 (1 µg/chick) or vehicle (control) was administered intracerebroventricularly to layer chicks after 3 h of fasting, as described above (eight birds per group). The chicks were kept under continuous fasting condition. Although no significant difference was observed (P=0.13), food intake in the TGF-β1 group was suppressed to be about 60% of that in the control group at 60 min after administration (Fig. 2). Therefore, the chicks were euthanized by decapitation 60 min after administration. The hypothalamus was excised, immediately frozen in liquid nitrogen, and stored at −80℃ until use for real-time PCR analysis. Seven successfully injected samples from each group were used for the real-time PCR analysis.

Table 1. **Primer sequences used for PCR**

Gene name	Forward primer	Reverse primer	Accession number
Smad2	5′-atg tca tcc att ctg cca ttc act-3′	5′-tag tgg tat gga ttc aca cac act tca-3′	NM_001396710.1
Smad3	5′-atg tcc tcc atc ctgccg ttc acc-3′	5′-taa tga tag gga ttc aca cag act tcg-3′	NM_ 204475.2

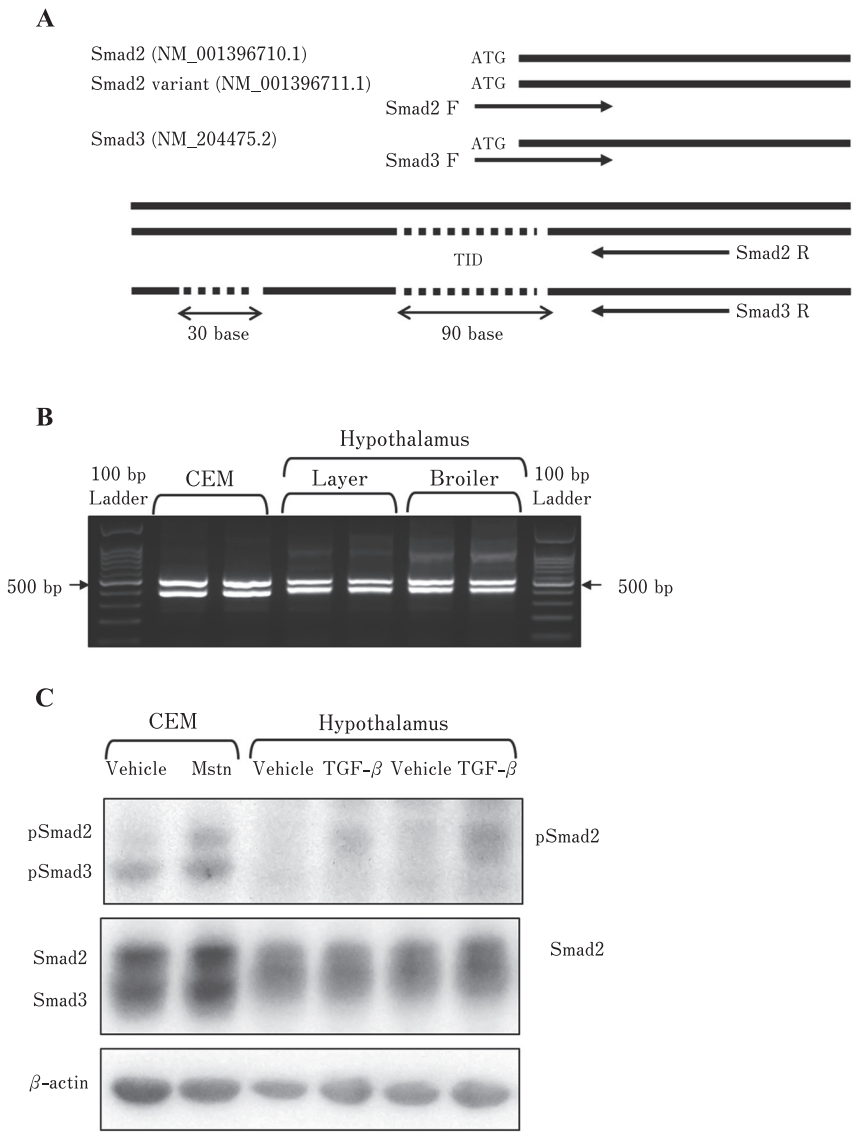

Fig. 1. **Expression of the gene and protein of Smad2/3 in the hypothalamus of chicks.** A: Schematic diagram of position of Smad2- and Smad3-specific primers. B: PCR products of Smad2/3 mRNA. C: Western blot analysis of Smad2/3. The lysates of chicken embryonic myotubes were prepared in a previous study (Saneyasu *et al*., 2019b) and used as a positive control in the present study. CEM, chicken embryonic myotubes; Mstn, myostatin.

Effects of Central Administration of TGF-β on Plasma Concentrations of Glucose and Non-esterified Fatty Acids in Chicks

Eight-day-old layer chicks were allocated to two groups (eight birds per group) and given free access to water and feed until administration. Either TGF-β1 (1 µg/chick) or vehicle (control) was administered intracerebroventricularly, and the chicks were reared under fasting conditions. We confirmed the phosphorylation of Smad2 in the hypothalamus of chicks that were administered TGF-β1 after 30 min (Fig. 1C). Thereafter, the chicks were euthanized by decapitation 30 min after administration, and their blood was collected. The plasma was separated immediately by centrifugation at 1,900×*g* for 10 min at 4℃. Plasma concentrations of glucose and non-esterified fatty acids (NEFA) were measured using commercial kits (LabAssay^{TM} Glucose and NEFA; FUJIFILM Wako Pure Chemical Corp., Osaka, Japan). Data from chicks without Evans blue dye in the lateral ventricle have been omitted.

Effects of Feeding Conditions on Smad Phosphorylation in Chicken Hypothalamus

Seven-day-old chicks (broilers and layers) were weighed and allocated to three groups based on their body weights (six birds per group). One group was administered a diet *ad libitum*, and the other groups were deprived of their diet for

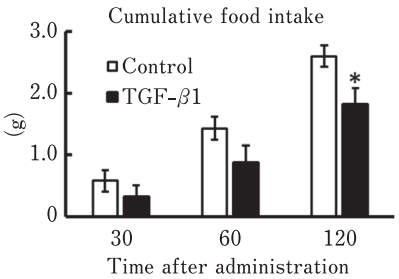

Fig. 2. **Effects of central injection of TGF-β1 on feed intake in chicks.** Data are expressed as mean±SEM of seven birds in each group. The Student's *t*-test was used to analyze the differences between groups. * Significance with respect to the vehicle group (*, $P<0.05$).

24 h. The *ad libitum* group and one fasting group (8-day old) were euthanized, and the other fasting group was refed for 1 h prior to euthanasia. The hypothalamus was excised, immediately frozen in liquid nitrogen, and stored at −80℃ until use for Western blot analysis.

Western Blot Analysis

Western blotting was performed as previously described (Saneyasu *et al*., 2018, 2019a). Frozen tissue samples were ultrasonicated in a lysis buffer containing 150 mM sodium chloride, 10 mM tris(hydroxymethyl)aminomethane, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid, 1% Triton X-100, 0.5% NP-40, 100 mM sodium fluoride, 23 mM sodium phosphate, 2 mM sodium orthovanadate, and protease inhibitor cocktail (Nacalai Tesque, Inc., Kyoto, Japan). Homogenates were centrifuged at 17,900×*g* for 15 min at 4℃, and the supernatants were stored at −80℃. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). The lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting using HorizeBlot (ATTO Co., Tokyo, Japan), according to the manufacturer's instructions. Lysates of chicken embryonic myotubes prepared in a previous study (Saneyasu *et al*., 2019b) were used as the positive control. Bands were detected using Chemi-Lumi One Super (Nacalai Tesque, Inc., Kyoto, Japan), visualized with LumiCube (Liponics Inc., Tokyo, Japan), and quantified using the CS Analyzer software (ATTO Co., Tokyo, Japan). Anti-pSmad2 (Ser465/467)/ Smad3 (ser423/425) (#8828), anti-Smad2/3 (#8685), anti-β-actin (#8457), and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (#7074) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-β-actin was used as the loading control. When detecting pSmad2/3, the antibodies were diluted with Can Get Signal (Toyobo Co. Ltd., Osaka, Japan).

Real-time PCR Analysis

Real-time PCR was performed as previously described (Honda *et al*., 2007; Fujita *et al*., 2019; Saneyasu *et al*., 2019a, b). Briefly, total RNA was extracted from the hypothalamus using Sepazol-RNA I Super G (Nacalai Tesque, Inc., Kyoto, Japan), according to the manufacturer's instructions. First-strand cDNA was synthesized from total RNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo Co. Ltd., Osaka, Japan), as per the manufacturer's instructions. The mRNA levels of target genes were analyzed by the relative standard curve method. The Thermo Scientific PikoReal Real-Time PCR System (Thermo Fisher Scientific Oy, Vantaa, Finland) was set up for each primer (Fujita *et al*., 2019) with SYBR Premix Ex Taq II (Tli RNaseH Plus; Takara Bio Inc., Otsu, Japan), according to the manufacturer's recommendations: 95℃ for 30 s, 40 cycles of 95℃ for 5 s, and 60℃ for 31 s. The expression levels of the target genes were normalized to those of ribosomal protein S17. The results are shown as relative to those in the saline group.

Statistical Analysis

Data were subjected to *t*-test using Excel 2016 (Microsoft, USA). A *P* value less than 0.05 was considered statistically significant.

Results and Discussion

We first identified the types of Smad molecules expressed in the chicken hypothalamus. As shown in Fig. 1B, two bands were observed in all lanes of the gel. The sizes of the upper bands were the same, indicating the presence of Smad2, whereas those of the lower bands were slightly different between chicken embryonic myotubes (CEM) and the hypothalamus. Sequence analysis revealed that the sequences of the upper band were identical to Smad2 (NM_001396710.1) in both the CEM and hypothalamus, whereas those of the lower bands were identical to Smad3 (NM_ 204475.2) and Smad2 variants (NM_001396711.1) in the CEM and hypothalamus, respectively. Furthermore, we examined the effects of central injection of TGF-β1 on Smad proteins in chicken hypothalamus. Total Smad2 was detected in the hypothalamus of the chicks. Phosphorylated Smad2 was detected in the hypothalamus of the TGF-β1 administered chicks, but not in the vehicle. (Fig. 1C). In contrast, total and phosphorylated Smad3 were not detected in the hypothalamus (Fig. 1C). These results suggest that Smad2 phosphorylation may play a role in TGF-β signaling in chicken hypothalamus.

In the N-terminal domain of Smad3, two lost sequences in Smad2 are present (Fig. 1A, Kamato *et al*., 2013). The larger sequence, called TID, prevents Smad2 from directly binding to DNA and activating transcription (Dennier *et al*., 1999; Kamato *et al*., 2013). The chicken Smad2 variant lacks a TID sequence, similar to Smad3 (Fig. 1A). Therefore, the transcriptional properties of the Smad2 variant appear to be similar to those of Smad3, suggesting that the Smad2 variant may play an alternative role to Smad3 in the chicken hypothalamus.

Central administration of TGF-β1 significantly decreased feed intake at 120 min in chickens (Fig. 2), although no significant changes were observed in the mRNA levels of NPY, POMC, AgRP, or corticotropin-releasing factor in the hypothalamus (Fig. 3). Therefore, it is likely that upregulation of the central TGF-β/Smad2 signaling pathway suppresses feed intake, independent of the gene expression of hypothalamic

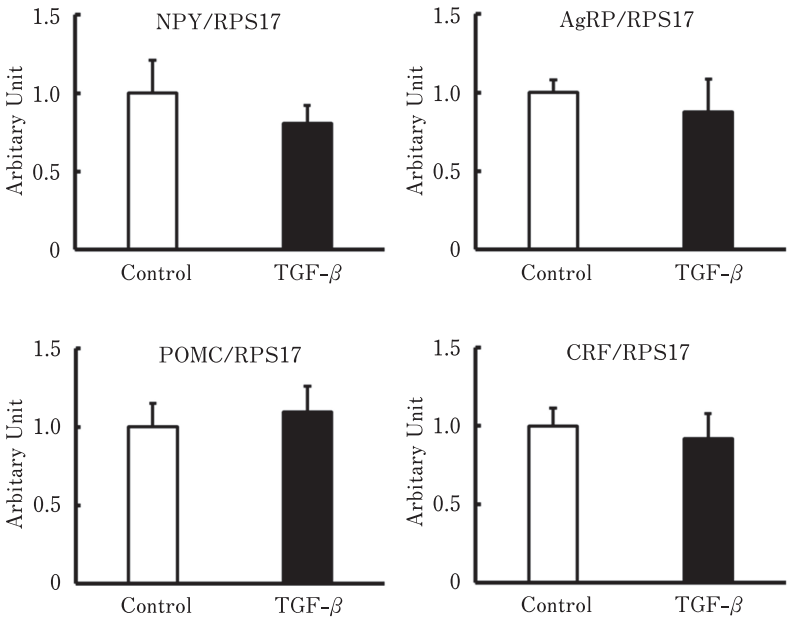

Fig. 3. **Effects of central injection of TGF-β1 on the mRNA levels of neuropeptide Y, agouti-related protein, proopiomelanocortin, and corticotropin-releasing factor in chicken hypothalamus.** Data are expressed as mean±SEM of seven in each group. The Student's *t*-test was used to analyze the differences between groups.

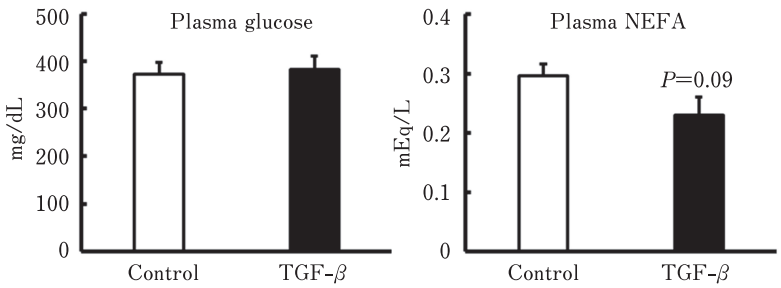

Fig. 4. **Effects of central injection of TGF-β1 on plasma concentrations of glucose and non-esterified fatty acids in chicks.** Data are expressed as mean±SEM of seven and six birds in the vehicle and TGF-β1 group, respectively. The Student's *t*-test was used to analyze the differences between groups.

appetite-regulating neuropeptides in chickens.

A previous study in mice showed that although no significant change was observed in food intake for 24 h after an injection of TGF-β1 (4 ng/mouse) into the third ventricle, hypothalamic POMC expression was significantly decreased by the injection of TGF-β1 (Yan *et al*., 2014). In another study, hypothalamic POMC expression was significantly increased by the downregulation of TGF-β expression in the arcuate nucleus using shRNA (Mendes *et al*., 2018). Therefore, these findings in mice and our results suggest that the roles of TGF-β1 in the central nervous system may differ between mammals and chickens.

A previous study in mice showed that the injection of TGF-β1 into the third ventricle leads to hyperglycemia and insulin resistance (Yan *et al*., 2014). However, no significant change was observed in the plasma glucose concentration in chicks after central administration of TGF-β. In addition, the plasma NEFA concentration tended to decrease (Fig. 4). Insulin is an antilipolytic hormone in mammals. In chickens, insulin immune neutralization elevates plasma NEFA concentrations (Dupont *et al*., 2008). Therefore, it is possible that the activation of the central TGF-β/Smad signaling pathway does not induce insulin resistance in chickens, and the reason for the low plasma NEFA concentration in the TGF-β group is unclear. Additionally, TGF-β1 was injected *ad libitum*, and blood was obtained 30 min after injection in our experiment. Although the chicks were fasted for 30 min, the plasma NEFA concentration is likely to reflect the balance

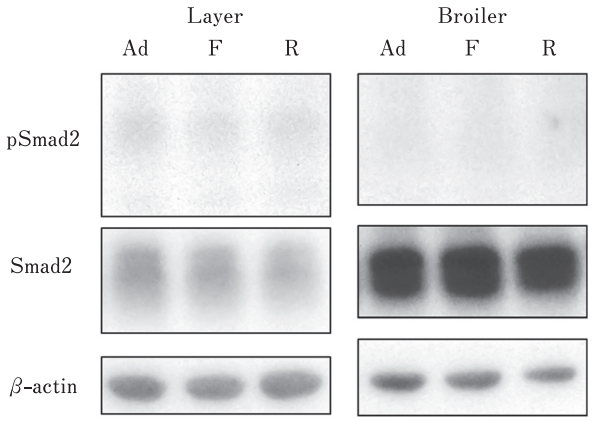

Fig. 5. **Effects of the feeding conditions on phosphorylation of Smad2/3 in chicken hypothalamus.** Representative images of Western blot analysis are shown. Ad, ad libitum feeding conditions; F, fasting conditions; R, refeeding conditions.

between the release of NEFA into the blood stream by basal lipolysis in the adipose tissue and the uptake of plasma NEFA into tissues such as the liver and skeletal muscles. Tachibana *et al*. reported that the central injection of neuropeptides modifies lipid metabolism in chicks (Tachibana *et al*., 2006, 2007). Further studies are warranted to clarify whether the central TGF-β/Smad signaling pathway regulates peripheral lipid metabolism.

Finally, we examined whether feeding conditions affected Smad2/3 phosphorylation in the hypothalamus of chicks. Surprisingly, phosphorylated Smad2/3 was not detected under any conditions in the hypothalamus of broiler and layer chicks (Fig. 5). In mice, a high-fat diet increased the weight of white adipose tissue and phosphorylated Smad2/3 protein in the hypothalamus (Mendes *et al*., 2018). Therefore, we further analyzed the phosphorylated Smad2/3 in the hypothalamus of 4- and 7-week-old broiler chickens, which store more abdominal fat compared to 1-week old chicken, but no signal was observed (unpublished data). These findings suggest that the hypothalamic Smad signaling pathway is not involved in the regulation of appetite, in response to feeding status in chickens.

In the present study, we only administered 1 µg/chick of TGF-β, and collected tissue samples 30 or 60 min after administration. Therefore in further studies, administering several doses and collection at several points in time would provide a better understanding of the roles of the hypothalamic TGF-β/Smad signaling pathway in feeding regulation in chickens.

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Author Contributions

Takaoki Saneyasu designed and conducted the experiments, analyzed the data, and wrote the paper. Miku Ueda and Kanami Nagata conducted the experiments and analyzed the data. Jiawei Chai analyzed the data. Kazuhisa Honda and Hiroshi Kamisoyama designed the experiments, analyzed the data, and edited the paper.

Conflict of Interest

The authors declare no conflicts of interest that could inappropriately influence the work reported in this study.

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