Correlation of ghrelin concentration and ghrelin, ghrelin-*O*-acetyltransferase (GOAT) and growth hormone secretagogue receptor 1a mRNAs expression in the proventriculus and brain of the growing chicken

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Abstract

To determine mechanisms for age-related decrease of *GHS-R1a* expression in the chicken proventriculus, changes in mRNA expression of ghrelin and ghrelin-*O*-acetyltransferase (GOAT) as well as ghrelin concentrations in the proventriculus and plasma were examined in growing chickens. Changes in expression levels of *ghrelin*, *GOAT* and *GHS-R1a* mRNAs were also examined in different brain regions (pituitary, hypothalamus, thalamus, cerebellum, cerebral cortex, olfactory bulb, midbrain and medulla oblongata). Ghrelin concentrations in the proventriculus and plasma increased with aging and reached plateaus at 30 to 50 days after hatching. High level of *ghrelin* mRNA decreased at 3 days after hatching, and it became stable at half of the initial level. Expression levels of *GHS-R1a* and *GOAT* decreased 3 or 5 days after hatching and became stable at low levels. Significant negative correlations were found between plasma ghrelin and mRNA levels of *GOAT* and *GHS-R1a*. Expression levels of *ghrelin* mRNA were different in the brain regions, but a significant change was not seen with aging. *GHS-R1a* expression was detected in all brain regions, and age-dependent changes were observed in the pituitary and cerebellum. Different from the proventriculus, the expression of *GOAT* in the brain increased or did not change with aging. These results suggest that decreased *GHS-R1a* and *GOAT* mRNA expression in the proventriculus is due to endogenous ghrelin-induced down-regulation. Expression levels of *ghrelin*, *GOAT* and *GHS-R1a* in the brain were independently regulated from that in the proventriculus, and age-related and region-dependent regulation pattern suggests a local effect of ghrelin system in chicken brain.

Key words: ghrelin, ghrelin-*O*-acetyltransferase, growth hormone secretagogue receptor, chicken, growth, mRNA expression

1. Introduction

Ghrelin, a natural ligand for growth hormone secretagogue-receptor 1a (GHS-R1a), has been identified in many species of mammalian and non-mammalian vertebrates. GHS-R1a is a G-protein-coupled receptor linked to intracellular Ca^{2+} mobilization. It has been reported that ghrelin exists in the stomach and hypothalamus and that it regulates growth hormone (GH) release from the pituitary **[10, 11, 12, 24, 25].** In addition to its effect on GH secretion, ghrelin is now recognized to be an important regulator of glucose homeostasis, food intake and fat utilization during periods of growth and negative energy balance. Moreover, ghrelin may be involved in the regulation of endocrine and exocrine pancreatic function, cardiac function, anxiety, and gastrointestinal (GI) functions. The possible multiple functional roles of ghrelin are supported by evidence that ligand binding sites and *GHS-R1a* mRNA are ubiquitously distributed in the brain and in several peripheral tissues **[10, 25]**.

In the chicken, ghrelin is composed of 26 amino acids and shares about 50% total sequence identity to human ghrelin and 100% identity to the N-terminal region (Gly^1-Pro^7) of human ghrelin **[13].** Chicken ghrelin mRNA and ghrelin immunoreactivity are mainly distributed in the proventriculus **[13, 15, 39, 41]**. Two types of chicken GHS-R have been characterized: GHS-R1a is a functional receptor, and GHS-R1aV (GHS-R1c) is a splice variant lacking the transmembrane domain-6 **[7, 38]**. In functional studies focusing on GI motility, it was shown that chicken ghrelin caused a region-dependent contraction of the non-stimulated GI tract and that expression of *GHS-R1a* mRNA is heterogeneous, being consistent with the amplitude of ghrelin-**induced** contraction **[19, 20]**. Because increased tonus of non-stimulated GI muscle by ghrelin itself has rarely been observed in mammalian species (rat, mouse and guinea-pig) **[3, 4, 18, 28]**, and *GHS-R1a* mRNA is expressed heterogeneously depending on the region of GI tract in the chicken unlike that in the rat, human and guinea-pig **[20, 21, 37]**, the chicken is a unique animal species for investigating the physiological roles of ghrelin in GI motility **[19, 20].**

Ghrelin is thought to participate in body growth of animals through regulation of GH release, food intake, glucose and fat metabolism, body weight gain and digestive function **[10, 11, 12, 15, 25].** Using chicken of different ages (from 1 to 100 days after hatching), we have demonstrated age-dependent reduction of ghrelin-induced contraction and expression level of *GHS-R1a* mRNA in the proventriculus but not in the crop, ileum or colon **[22]**. Since ghrelin or GHS-R1a agonists have been reported to influence *GHS-R1a* mRNA expression in mammals **[7, 23],** endogenous ghrelin and a synthetic enzyme (ghrelin-*O*-acetyltransferase, GOAT) are possible **candidate** molecules to regulate *GHS-R1a* mRNA expression in the growing chicken proventriculus. However, there have been few studies in which changes in ghrelin concentration and expression of *GHS-R1a, GOAT* and *ghrelin* mRNAs in growing chickens were compared.

In the present study, to examine the underlying mechanisms of age-dependent changes in the expression of *GHS-R1a*, ghrelin contents in the proventriculus and plasma ghrelin concentrations were measured in chickens of different ages (1, 3, 5, 10, 20, 30, 50 and 100 days after hatching). In addition to *GHS-R1a* expression, *ghrelin* and *GOAT* mRNAs in the proventriculus were also investigated in growing chickens to examine their relationships. We also examined age-related changes in *ghrelin*, *GOAT* and *GHS-R1a* mRNA expression levels in eight parts of the brain (pituitary, hypothalamus, thalamus, cerebellum, cerebral cortex, olfactory bulb, midbrain and medulla oblongata) to compare difference in changes of these ghrelin factors in the brain with those in proventriculus. Although the expression of *ghrelin* and *GHS-R1a* mRNAs has already been investigated in several brain regions **[7, 30, 38]**, chickens at different ages were **used** in each experiment. It was possible to clarify age-related changes in the present study.

2. Materials and methods

All experiments were performed in accordance with Institutional Guidelines for Animal

Care at Rakuno Gakuen University.

2.1. Chickens

Male white Leghorn chickens (1–100 days after hatching, Hokuren, Yuni, Japan) were used. Blood was collected by heart puncture under anesthesia in order to measure the plasma levels of ghrelin. Each blood sample was immediately centrifuged at 9000×*g* for 5 min at 4 ◦C, and plasma was collected. For the measurement of acylated ghrelin, plasma was acidified with a one-tenth volume of 1N HCl. After drawing blood, the chickens were stunned and bled to death. The proventriculus was removed after a midline incision, and luminal contents were flushed out using an ice-cold nutrient solution (NaCl, 118 mM; KCl, 4.75 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; $CaCl_2$, 2.5 mM; NaH_2CO_3, 25 mM; glucose, 11.5 mM). One half of each proventriculus was immediately soaked in liquid nitrogen for measurement of ghrelin contents, and the other half was stored in a RNA stabilizing solution, RNAlater (Ambion Inc., Austin, TX) for analysis of *ghrelin*, *GOAT* and *GHS-R1a* mRNAs expression.

Age-related changes in *ghrelin, GOAT* and *GHS-R1a* mRNAs were also examined in eight regions of brain, including the pituitary, hypothalamus, thalamus, cerebellum, cerebral cortex, olfactory bulb, midbrain and medulla oblongata, to compare with changes in ghrelin-related factors in the proventriculus. Each brain region was manually dissected out under a microscope. Each tissue was soaked in RNAlater, frozen, and stored in a deep freezer until use.

2.2.Measurement of chicken ghrelin in the plasma and proventriculus

The proventriculus was boiled with 5 volumes of Milli-Q-grade water relative to tissue weight for 10 min. After cooling on ice, acetic acid was added to adjust to 1 M. The boiled tissue was homogenized and centrifuged at 13,600 ×*g* for 10 min, and the supernatant was collected. Chicken plasma was acidified as described previously **[14]** or supernatant prepared from the proventriculus was purified using a Sep-Pak C18 cartridge (Waters). The cartridge was washed with 3 ml chloroform, 5 ml methanol, and 3 ml 60% acetonitrile containing 0.1%

trifluoroacetic acid (TFA). After being equilibrated with 3 ml 0.1% TFA, a plasma sample or supernatant was loaded onto the conditioned cartridge. The cartridge was washed with 3 ml 10% acetonitrile containing 0.1% TFA, and then the absorbed substances were eluted with 60% acetonitrile containing 0.1% TFA into a tube containing 50 μl 0.1% Triton X-100. The percent recovery of synthetic ghrelin during this procedure was 67% (n = 5).

Ghrelin concentration was measured using the specific radioimmunoassay (RIA) for acylated ghrelin (N-RIA) that has been validated for chicken ghrelin **[14]**. Lyophilized plasma samples were reconstituted with 250 μl of RIA buffer, and tissue extracts were diluted appropriately. Ghrelin concentration in100 μl of the sample was measured in duplicate. A primary antibody that recognizes the N-terminal portion of octanoylated rat ghrelin (Gly^{1}-Arg^{11}) was used at a final dilution of 1/5,000,000. Octanoylated chicken ghrelin (chicken ghrelin-26-C8), synthesized at Daiichi Suntory Pharma Co. Ltd., Institute for Medicinal Research and Development (Gunma, Japan), was used to create a standard curve for estimation of ghrelin concentration instead of rat ghrelin. ^{125}I-(Tyr^{29})-rat ghrelin was used as a tracer. The intra-assay coefficient of variation was 4.9%.

2.3.Quantitative real-time PCR (qPCR) for chicken *ghrelin, GHS-R1a* and *GOAT* mRNAs

Total RNA was extracted from tissues using Trizol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was transcribed from 1 μg total RNA with a QuantiTect RT Kit (QIAGEN GmbH, Hilden, Germany) using Oligo-dT primers. One tenth of the cDNA solution was used as a template. Quantitative real-time PCR analysis was performed using the LightCycler 480 system (Roche Applied Science, Mannheim, Germany). A QuantiFast SYBR Green PCR Kit (QIAGEN GmbH) was used. The reaction mixture consisted of 250 nM each of the primer and template (100 ng total RNA equivalent) in 1× master mix. Amplification conditions were an initial incubation at 95°C for 5 min followed by 35 cycles of 94°C for 10 sec and 60°C for 30 sec. To estimate mRNA copy numbers, qPCR samples were run with a serially diluted

pCRII plasmid vector that contained an *Xba-I* linearized full-length target cDNA from 10^3 to 10^6 copies or from 10^4 to 10^7 copies. After the amplification reaction, the samples were electrophoresed on 1.5% agarose gels containing ethidium bromide to confirm the amplicon size. Primer sets used in this experiment are listed in Table 1.

2.4. Statistical analysis

The results are expressed as means ± S.E.M. To examine changes in ghrelin parameters (ghrelin contents, *ghrelin*, *GOAT* and *GHS-R1a* mRNAs) with aging, one-way analysis of variance (ANOVA) followed by Dunnett's test was used for comparison in chickens at different post-hatching days with that at the first day. Comparison of region-dependent expression of *ghrelin*, *GOAT* and *GHS-R1a* mRNAs in the chicken brain was performed using one-way ANOVA followed by Bonferroni's test. Correlation between ghrelin contents and ghrelin-related mRNAs expression was determined by simple regression analysis. Significance was accepted at the 5% level.

3. Results

3.1. Changes in endogenous ghrelin concentrations with aging

As shown in Fig. 1A, ghrelin contents in the proventriculus were negligible immediately after hatching (0.3 ± 0.2 fmol/mg tissue, n=5). However, the contents increased gradually with growth. The contents reached a plateau at 30 days (73 ± 9.7 fmol/mg tissue, n=5) and **stayed constant levels until** 100 days after hatching (90 ± 10 fmol/mg tissue, n=5).

Plasma ghrelin concentrations increased with increase in the number of post-hatching days and reached a plateau at 50 days (92.4 ± 17.7 fmol/ml, n=5) (Fig. 1B). The correlation coefficient between plasma ghrelin concentrations and ghrelin contents in the proventriculus was 0.85, and it was significant (p=0.0075) (Fig. 1C).

3.2. Changes in ghrelin-related genes in the proventriculus

Expression level of *ghrelin* mRNA in the proventriculus was examined from 1 to 100 days

after hatching (Fig. 2A). *Ghrelin* mRNA expression (1 day: 87,000 ± 4,250 copies/100 ng total RNA, n=5) was decreased significantly at 3 and 5 days (about 60% of that of the first day) and became constant from 10 to 100 days after hatching. At 100 days after hatching, *ghrelin* mRNA expression was 44,300 ± 2,780 copies/100 ng total RNA (n=5, 53% of that of the first day, p=0.0015).

As previously reported **[22]**, *GHS-R1a* mRNA in the proventriculus decreased suddenly at 3 days after hatching. The level remained constant until 10 days after hatching and then decreased gradually (Relative expression levels were 41.8% at 3 days, 26.3% at 5 days, 32.1% at 10 days, 23.4% at 20 days, 16% at 30 days, 8.2% at 50 days and 5.8% at 100 days) (Fig. 2B). In our previous study, we **saw** that ghrelin-induced **proventriculus** contraction decreased depending on the age **[22]. A** significant correlation between ghrelin-induced contraction and expression level of *GHS-R1a* mRNA **was detected** in the proventriculus (R=0.88 and p=0.002) (Fig. 3).

GOAT is an enzyme for producing acylated ghrelin (biologically active form), which catalyzes **addition of** fatty acid on Ser^3 ghrelin **[40]**. *GOAT* mRNA had been already expressed in the proventriculus at the first day after hatching (440 ± 77 copies/100 ng total mRNA, n=9). The expression level decreased with increase in the number of post hatching days (3 days: 431 ± 1 95 copies/100 ng total RNA, n=9; 5 days: 157 ± 12 copies/100 ng total RNA, n=10; 10 days: 148 ± 19 copies/100 ng total RNA, n=9; 20 days: 163 ± 17 copies/100 ng total RNA, n=9; 30 days: 167 ± 26 copies/100 ng total RNA, n=8; 50 days: 106 ± 16 copies/100 ng total RNA, n=5; 100 days: 76 ± 5 copies/100 ng total RNA, n=4). However, this reduction was not statistically significant (p=0.07 to 0.13, Fig. 2C).

Correlation among endogenous ghrelin concentrations and each mRNA expression was analyzed. A significant correlation was not seen between plasma ghrelin and *ghrelin* mRNA (p=0.15) or between ghrelin contents in the proventriculus and *ghrelin* mRNA levels (p=0.55) (Fig. 4A). On the other hand, significant negative correlations were seen between plasma

ghrelin concentrations and *GHS-R1a* mRNA expression (p=0.0047) and between tissue ghrelin contents and *GHS-R1a* mRNA expression (p=0.037) (Fig. 4B). **Negative** correlations were also observed between plasma ghrelin concentration and *GOAT* mRNA expression (p=0.0043) and between tissue ghrelin contents and *GOAT* mRNA expression (p=0.025) (Fig. 4C). There was no significant correlation between *ghrelin* mRNA and *GOAT* mRNA expression in the proventriculus (p=0.24).

3.3. Changes in ghrelin-related genes in the brain regions.

Ghrelin mRNA expression in the brain was heterogeneous at the first day after hatching and **the expression level** was markedly lower than that in the proventriculus. Expression levels in the cerebral cortex and olfactory bulb were high compared with those in other brain regions (Fig. 5). During growth, the expression level in most regions did not change significantly from 1 to 100 days after hatching. However, expression levels in the midbrain and medulla oblongata were decreased at 3 days after hatching and then remained constant until 100 days. Therefore, the region-related heterogeneous expression of *ghrelin* mRNA was almost the same as that at 100 days after hatching, i.e., the expression levels in the cerebral cortex and olfactory bulb were markedly higher than those in the other six regions of the brain (Fig. 5).

Region-specific expression of *GHS-R1a* mRNA was observed at the first day after hatching (Fig. 6). The expression level in the cerebellum was higher than the levels in the other regions of the brain. Although the expression levels in the olfactory bulb and cerebral cortex were slightly lower, there was no significant difference compared with the levels in the hypothalamus, thalamus, pituitary gland, midbrain and medulla oblongata. Age-dependent change in *GHS-R1a* mRNA expression was observed only in the cerebellum; the expression level of *GHS-R1a* in the cerebellum was decreased significantly at 3 days and then remained constant until 100 days after hatching. The time course of change in *GHS-R1a* expression in the cerebellum was comparable to that in the proventriculus (R=0.95). A negative correlation

was also found between *GHS-R1a* mRNA expression in the cerebellum and plasma ghrelin contents (p=0.024, R=-0.77). On the other hand, *GHS-R1a* expression in the other regions of the brain did not change markedly during growth from 1 to 100 days after hatching despite an unexpected transient decrease in *GHS-R1a* expression in the medulla oblongata at 10 days, and unexpected transient increases in *GHS-R1a* expression in the hypothalamus at 50 days and midbrain at 5 days. Due to the region-related changes in expression of *GHS-R1a* mRNA during development, expression level was highest in the hypothalamus and expression levels were almost the same in other brain regions at 100 days after hatching (Fig. 6).

As shown in Fig. 7, expression levels of *GOAT* mRNA varied from region to region of the brain at the first day after hatching. However, no significant difference was seen in the expression levels of *GOAT* among the eight regions examined. During growth, expression levels of *GOAT* in brain regions including the olfactory bulb, thalamus, cerebellum, midbrain and medulla oblongata were increased significantly at 20 days or 30 days after hatching, and the expression levels except for the thalamus reached plateaus. On the other hand, expression levels in the cerebral cortex, pituitary gland and hypothalamus did not change during growth. At 100 days after hatching, *GOAT* mRNA was expressed heterogeneously in the brain: the expression levels were high in the midbrain, moderate in the medulla oblongata, thalamus, cerebellum and olfactory bulb, and low in the hypothalamus, cerebral cortex and pituitary gland (Fig.7).

4. Discussion

We have reported an age-dependent reduction of ghrelin-induced contraction as well as *GHS-R1a* mRNA expression in the proventriculus **[22]**. At first, we focused on ghrelin concentration in the proventriculus and plasma, in addition to the expression levels of *ghrelin* and *GOAT* mRNAs in different-aged chickens, to investigate the underlying mechanisms of reduction of *GHS-R1a* mRNA expression. Ghrelin concentrations in the proventriculus and

plasma increased with aging. Negative correlations between plasma ghrelin concentration and *GOAT* or *GHS-R1a* mRNA expression levels **would be** indicative of a feedback mechanism by endogenous ghrelin. Expression of *ghrelin* did not correlate with either tissue contents or plasma ghrelin concentrations, suggesting discrepancy between mRNA expression and ghrelin concentration. The discrepancy may be related to an intermediation of GOAT between them. Furthermore, we examined expression levels of *ghrelin*, *GHS-R1a* and *GOAT* mRNAs in various brain regions. Age-related changes in expression of these ghrelin-related factors were observed, indicating that physiological roles for the ghrelin system would locally change during growth in the chicken brain.

4.1. Proventriculus

4.1.1. *Ghrelin*

Ghrelin-immunopositive cells are detected in the proventriculus of hatching chickens, and the number increases gradually toward the adult stage **[39]**. In the present study, ghrelin levels in the proventriculus and plasma increased with aging and reached plateaus from 30 to 50 days after hatching. **Kaiya et al. [14] have already measured proventriculus and plasma ghrelin concentrations in 6 days young chickens and both plasma ghrelin concentration (20-30 fmol/ml) and tissue ghrelin concentration (20-40 fmol/mg tissue) were comparable with the present results. However, Date et al. [2] measured both stomach and plasma ghrelin concentrations in rats and indicated the tissue content of ghrelin was about 20 times higher than that in the plasma, and this is different from the case in the chicken. Although underlying mechanisms of the difference between rat and chicken in ghrelin concentrations are not clear at present, species difference in synthetic pathway, storage including the number of producing cells and releasing mechanisms of ghrelin might explain the difference.**

Plasma ghrelin concentration showed a positive correlation with ghrelin content in the proventriculus, suggesting that ghrelin synthesized in the proventriculus is the main source of

circulating plasma ghrelin. However, *ghrelin* mRNA expression in the proventriculus of growing chickens was decreased 5 days after hatching, and the level became almost constant until 100 days after hatching. Chen et al. also reported constant expression of *ghrelin* in the chicken proventriculus with the exception of a transient decrease at 44 days after hatching [1]. In the Peking duck, *ghrelin* expression increases during embryonic stages, but the expression level remains unchanged after hatching **[33]**. These results indicate that *ghrelin* mRNA expression in the proventriculus of birds does not greatly change with aging, although it is partly regulated.

In the present study, ghrelin level in the proventriculus or plasma did not show a significant correlation with expression of *ghrelin* mRNA in the proventriculus. This means that ghrelin contents and the plasma concentration increased, though expression of *ghrelin* mRNA was almost constant, indicating a discrepancy between *ghrelin* mRNA expression and ghrelin peptide level in the growing chicken. Wada et al. **[39]** and Yamato et al. **[41]** reported that the number of ghrelin-immunopositive cells was similar to that of *ghrelin* mRNA-expressing cells in adult chickens, whereas the number of *ghrelin* mRNA-expressing cells was greater than that of ghrelin-immunopositive cells just after hatching. In the proventriculus of the Peking duck, *ghrelin* mRNA expression was detected at 14 embryonic days, whereas ghrelin immnoreactivity was not observed at that time but was detected at 21 embryonic days **[33].** These histological results also suggested a dissociation of ghrelin gene expression and protein expression as seen in the present study. A similar discrepancy between ghrelin gene expression and protein level has been reported in the rat heart **[8]**. There are three possiblities for this discrepancy: (1) *ghrelin* mRNA synthesizes unacylated ghrelin first and then GOAT attaches the medium-chain fatty acids (mainly octanoic acid) at Ser^3 of ghrelin, resulting in production of acylated ghrelin. Therefore, *ghrelin* mRNA expression may correlate more with the amount of unacylated ghrelin than with that of acylated ghrelin. To clarify this possibility, it is necessary to measure unacylated ghrelin concentrations in the

proventriculus and plasma in developing chickens; (2) there is a possibility of lack of substrates for acyl modification of ghrelin. Yamato et al. **[41]** reported a marked increase of ghrelin-immunoreactive cells by application of octanoic acid in the early hatching chicken without affecting *ghrelin* mRNA-expression cells**.** This result suggests an insufficient level of substrate for GOAT to synthesize acylated ghrelin in hatching chickens; and (3) the sensitivity of methods used for detection of *ghrelin* mRNA and ghrelin protein are different. The difference in detection sensitivity might have contributed to the discrepancy between time courses of *ghrelin* mRNA expression and plasma ghrelin concentrations.

4.1.2. *GHS-R1a*

We previously reported that decreased contractile activity of ghrelin in the proventriculus is consistent with reduction of *GHS-R1a* mRNA expression **[22]**. Although it has been reported that *GHS-R1a* mRNA expression in the chicken proventriculus was decreased 16 days after hatching, was transiently increased at 30 days, and then decreased again at 44 days [1]. We confirmed again age-dependent monophasic reduction of *GHS-R1a* mRNA expression in the present study. Similar age-dependent reduction of *GHS-R1a* mRNA expression has been reported in the mouse pituitary from 1 to 30 months of age **[34]**. What are changes in the receptor mRNA expression like these caused by? In rats, infusion of L-692,585 (non-peptidyl GHS-R1a ligand) induced 50% reduction of *GHS-R1a* mRNA level in the pituitary without affecting *GH-releasing hormone receptor* mRNA *in vivo* **[23]**. Down-regulation of *GHS-R1a* mRNA expression by ghrelin has also been reported in cultured chicken pituitary cells **[7]** and porcine pituitary cells *in vitro* **[27].** These facts suggest that the increased endogenous ghrelin might reduce *GHS-R1a* mRNA expression in the proventriculus as a feedback response. **To establish the definite evidences for ghrelin-induced down regulation of *GHS-R1a* mRNA expression in the developing chicken, it is necessary to inject or infuse exogenous ghrelin to the chicken and to determine changes in the *GHS-R1a* mRNA expression level of the proventriculus. On**

the other hand, expressions levels of *GHS-R1a* mRNA in the crop, ileum and colon did not decrease with aging **[22]**, suggesting that down-regulation of *GHS-R1a* mRNA expression occurred only in the proventriculus. The underlying mechanism is unclear, but possible explanations are: (i) since the proventriculus is the main organ to synthesize and release ghrelin in the chicken **[11, 13, 39]**, a local high concentration of ghrelin might affect the expression of *GHS-R1a* mRNA in the proventriculus in an autocrine/paracrine manner and (ii) GHS-R1a is distributed on smooth muscle cells in the crop, while it is expressed on both enteric neurons and smooth muscle cells in the proventriculus **[19].** Neural *GHS-R1a* genes might be more sensitive to affect down-regulation by endogenous ghrelin than myogenic *GHS-R1a* genes.

4.1.3. *GOAT*

GOAT post-translationally modifies Ser^3 of unacylated ghrelin with octanoic acid **[40].** *GOAT* mRNA transcripts and GOAT protein exist in the same mucosal oxyntic cells containing ghrelin in mammals **[31].** In this study, *GOAT* mRNA was identified and found to be expressed abundantly in the chicken proventriculus as in the case of *ghrelin* mRNA, suggesting that GOAT also plays a role in ghrelin acylation in the chicken proventriculus. In the present study, *GOAT* mRNA expression tended to decrease with growth and did not correlate with *ghrelin* mRNA expression (P=0.24) as previously reported **[6]**. Interestingly, *GOAT* mRNA expression in the proventriculus showed a negative correlation with increased ghrelin concentration (tissue ghrelin, P=0.025, plasma ghrelin, P=0.002). These results suggested negative feedback of *GOAT* mRNA expression by endogenous ghrelin, and ghrelin itself regulates its own production through affecting the expression of synthetic enzyme *GOAT* mRNA. Actually, in normal and cancer prostate cell lines, ghrelin decreases *GOAT* mRNA expression **[32].** In contrast, however, there are reports that ghrelin increased *GOAT* mRNA expression in cultured mouse pituitary cells **[5]** and that ghrelin treatment did not affect *GOAT* mRNA expression in cultured human and mouse chondrocytes **[9]**. These results

suggested that the expression of *GOAT* mRNA is regulated by ghrelin, in a different manner in each tissue. Consistent with this, we observed that *GOAT* mRNA expression increased depending on the age with concomitant increase in endogenous ghrelin in most regions of the chicken brain. Therefore, the relation between ghrelin concentration and *GOAT* expression should be treated carefully depending on cell types and tissue types examined.

4.2. Brain

4.2.1. Brain *Ghrelin*

Heterogeneous expression of *ghrelin* mRNA has been reported in the chicken brain **[29, 30].** Strong expression of the *ghrelin* gene was found in the corpus striatum, followed by the cerebellum, optic lobes and brainstem **[30]**. However, these results were obtained from only non-quantitative **RT**-PCR. In the present study, we quantified *ghrelin* mRNA expression in various brain regions and found that the cerebral cortex and olfactory bulb highly express *ghrelin* mRNA, followed by the cerebellum. Unexpectedly, there was also slight expression in the hypothalamus, the feeding center. It is noteworthy that change in *ghrelin* mRNA expression was not variable throughout development in almost all regions of the brain. This is different from *ghrelin* mRNA expression change in the proventriculus. In the midbrain, medulla oblongata and pituitary, a sudden decrease in expression was observed 3-5 days after hatching. We do not have any idea about the physiological relevance of these changes. Although ghrelin regulates appetite, heat production, behavior and energy production in chickens **[11, 12],** relatively high expression of *ghrelin* mRNA in the cerebral cortex and olfactory bulb suggests novel physiological actions of ghrelin in chickens.

4.2.2. Brain *GHS-R1a*

GHS-R1a expression in the chicken brain has been reported in 10-day-old **[7],** 8-week-old **[38],** 4-day-old chickens **[30]** and in growing chickens (from 2 to 58 days after hatching) [1]. These studies showed that *GHS-R1a* expression levels were high in the pituitary, brainstem

and cerebellum. In the present study, for the first time, we quantified changes in *GHS-R1a* expression in various brain regions during growth of chickens. A marked change was not observed in most of the brain regions including the olfactory bulb, thalamus and cerebral cortex throughout the development for 100 days. This is consistent with results reported by Chen et al. [1]. However, a great change was observed in some brain regions. The highest expression level was detected in the cerebellum from 1 to 5 days after hatching, being similar to the observation by Saito et al. **[30]**. The level gradually decreased to 10 days and was stable thereafter. This change is the same as that reported by Geelissen et al. **[7]** and suggests that ghrelin affects functions of the cerebellum such as movement and maintenance of body balance, intimately in the restricted early stage of growth after hatching. In addition, transient but significant changes in *GHS-R1a* expression were detected in the midbrain at 5 days (increase) and in the medulla oblongata at 10 days (decrease). Different regulation of *GHS-R1a* expression in brain regions in aging animals has already been reported in rats: *GHS-R1a* expression is up-regulated in the hypothalamus but down-regulated in the hippocampus by aging **[17]**. Taken together, these results suggest that there is a case that expression of *GHS-R1a* is regulated independently with aging as necessary.

The expression level of *GHS-R1a* in the hypothalamus was higher than the levels in other regions (after 20 days), as described previously **[7].** *Ghrelin* mRNA expression was detected throughout the brain during the development of chickens, as observed in this study. Intracerebroventricular injection of ghrelin affects pituitary hormone release, feeding, drinking and sleeping behavior in chickens **[13, 14, 30, 35, 36]**. The high expression level of *GHS-R1a* in the hypothalamus reflects these actions and suggests that the hypothalamus is one of the most important targets for ghrelin in the chicken.

4.2.3. Brain *GOAT*

This is the first study showing the expression of *GOAT* mRNA in the chicken brain during growth. *GOAT* mRNA was expressed homogenously in almost all brain regions just after

hatching. Since it has been shown that GOAT is a specific enzyme to modify ghrelin **[40],** expression of *ghrelin* and *GOAT* mRNAs in each brain region suggests local and independent regulation of brain function by the ghrelin system.

GOAT mRNA expression did not change in the cerebral cortex, thalamus, hypothalamus or pituitary but increased in the olfactory bulb, medulla oblongata, midbrain and cerebellum with aging in contrast to results in the proventriculus, where *GOAT* mRNA expression decreased. However, the pattern of change in expression is different between *ghrelin* and *GOAT* in the olfactory bulb, cerebral cortex, and cerebellum. This dissociation is similar to that for the expression pattern of *GOAT* and *ghrelin* in the proventriculus as discussed earlier. A possible reason why expression of *ghrelin* does not accord with existence of *GOAT* may be the amount of substrate (fatty acids) for GOAT. Manipulation of fatty acid contents in nutrition (feed) could modify the expression of *ghrelin* and/or *GOAT* mRNAs and their relationships.

Reduction of *GOAT* mRNA expression observed in the proventriculus was not seen in the brain. As described earlier, we hypothesized that gastric reduction of GOAT may be due to a feedback regulation by ghrelin. However, such a feedback regulation may not occur in the brain. As previously discussed, the regulation pattern of *GOAT* mRNA expression by ghrelin was different depending on the cell type expressing *GOAT* **[5, 9, 32].** It is likely that regulation of GOAT mRNA by ghrelin is different in the proventriculus and brain neurons. Another possibility is that the absolute level of ghrelin in the brain is lower than that in the proventriculus because of low production level of brain ghrelin and low permeability of ghrelin through the blood-brain barrier. Low ghrelin concentration might regulate *GOAT* mRNA expression in a different manner from that in the proventriculus.

The expression levels of *GOAT* mRNA in chicken brain were comparable to *ghrelin* mRNA in the brain and were similar with those in the proventriculus, even though *ghrelin* mRNA levels in the proventriculus were 100 times higher than those of brain.

Lin et al. [26] carried out cloning of porcine GOAT and investigated the expression of *ghrelin* and *GOAT* mRNAs in various pig tissues including gastrointestinal tract, brain and liver. The expression levels of *ghrelin* and *GOAT* were correlate in ghrelin producing organs (such as stomach, duodenum and pancreas), but dissociation was seen in the liver, lung, testis and ovary, in which *GOAT* mRNA expression was higher than that of *ghrelin*. Dissociation of the expression level between *ghrelin* and *GOAT* mRNAs was comparable with present results of the growing chicken. Broad expression of *GOAT* throughout the pig organs suggested other functional roles of GOAT in addition to the acylation of ghrelin [26]. The differences in phenotypes between ghrelin knockout mice and GOAT knockout mice also suggested the additional substrates besides ghrelin for GOAT [16]. Therefore, measurement of *GOAT* and *ghrelin* mRNAs in respective organs is necessary to accumulate knowledge about the relationships between both molecules. Discrepant distribution of *ghrelin* and *GOAT* mRNAs might indicate the specific function of GOAT that is independent of ghrelin.

5. Conclusion

In chickens, changes in ghrelin levels in the proventriculus and plasma were parallel with aging. A distinct inverse correlation between ghrelin concentration and *GHS-R1a/GOAT* mRNA expression in the proventriculus indicates the possibility that endogenous ghrelin down-regulates *GHS-R1a* and *GOAT* expression probably due to homeostatic feedback regulation of the ghrelin **system.** Age- and region-specific changes in the expression of *ghrelin*, *GOAT* and *GHS-R1a* were observed in the brain of chickens, suggesting that physiological roles of the ghrelin system in growing chickens might change during growth.

Especially, from 1 to 5 days after hatching, the ghrelin-induced contraction, ghrelin concentration and mRNAs expression of *ghrelin, GOAT* and *GHS-R1a* changed greatly in the present and previous studies [22]. The marked changes suggest that functional

demands of ghrelin might change from a just hatching to adult chicken at respective organs. This period is consistent with switching time for nutritional condition of chicken from internal nutrients to external nutrients. An egg yolk of non-absorption remains after hatching in the abdominal cavity of newborn chicks, but the egg yolk lump is generally reduced within 3 days after hatching and newborn chickens start to eat feeds for growth. Since ghrelin regulates food intake and energy balance of various animals [10, 24], marked changes in ghrelin responses and ghrelin-related molecule expression are suggested to be related to this switching phenomena.

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The authors declare no conflict of interest.

Authors contributions:

T.K. and H.K. designed research; T.K., T.H., N.Y., H.T. and H.K. performed research; T.K., N.Y., H.T. and H.K. analyzed data; and T.K., H.T. and H.K. wrote the paper.

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Figure Legends

Fig. 1. Changes in endogenous ghrelin levels of proventriculus and plasma in the growing chicken. Ghrelin concentrations in the proventriculus (A, fmol/mg tissue) and plasma (B, fmol/ml) were measured using a specific radioimmunoassay in chickens at different ages (1, 3, 5, 10, 20, 30, 50 and 100 days after hatching). Ghrelin in the proventriculus and that in plasma showed a significant correlation (C. R=0.85, P=0.007). *; Significant increase compared with the value at 1 day (A and B). Values are means ±S.E.M. of more than 5 experiments.

Fig. 2. Age-dependent changes in expression levels of *ghrelin, GOAT and GHS-R1a* mRNAs in the proventriculus of growing chickens. mRNA expression levels of *ghrelin* (A), *GHS-R1a* (B) and *GOAT* (C) in the proventriculus obtained from chickens at different ages (1, 3, 5, 10, 20, 30, 50 and 100 days after hatching). Values are means ±S.E.M. of 4 or 5 experiments. * Significantly different from the value at 1 day.

Fig. 3. Correlation of *GHS-R1a* mRNA expression and ghrelin-induced contraction in the proventriculus of chickens at different ages. *GHS-R1a* mRNA expression level showed a significant correlation with amplitude of contraction (R=0.88, P=0.002). The data of ghrelin-induced contraction were obtained from Kitazawa et al. [20]. Values are

means ±S.E.M. of 4 or 5 experiments.

Fig. 4. Correlations of plasma ghrelin concentration and mRNA expression levels of ghrelin-related molecules (*ghrelin, GHS-R1a and GOAT*) in the proventriculus of chickens at different ages. The figures show relationships between plasma ghrelin concentration (X-axis) and *ghrelin* mRNA (A), *GHS-R1a* mRNA (B) and *GOAT* mRNA (C) (Y-axis) in the proventriculus obtained from chickens at different ages (1, 3, 5, 10, 20, 30, 50 and 100 days after hatching). Correlation coefficient (R) and probability (P) are shown in each figure. Values are means ±S.E.M. of more than 4 experiments.

Fig. 5. Age-dependent changes in expression levels of *ghrelin* mRNAs in eight different regions of the growing chicken brain (1, 3, 5, 10, 20, 30, 50 and 100 days after hatching). (A) shows mRNA expression levels of ghrelin in the olfactory bulb, cerebral cortex, thalamus and hypothalamus. (B) shows mRNA expression levels of ghrelin in the pituitary gland, midbrain, cerebellum and medulla oblongata. *Ghrelin* expression level significantly decreased only in the midbrain and medulla oblongata of growing chickens from 3 to 100 days. Values are means ±S.E.M. of 4 or 5 experiments.

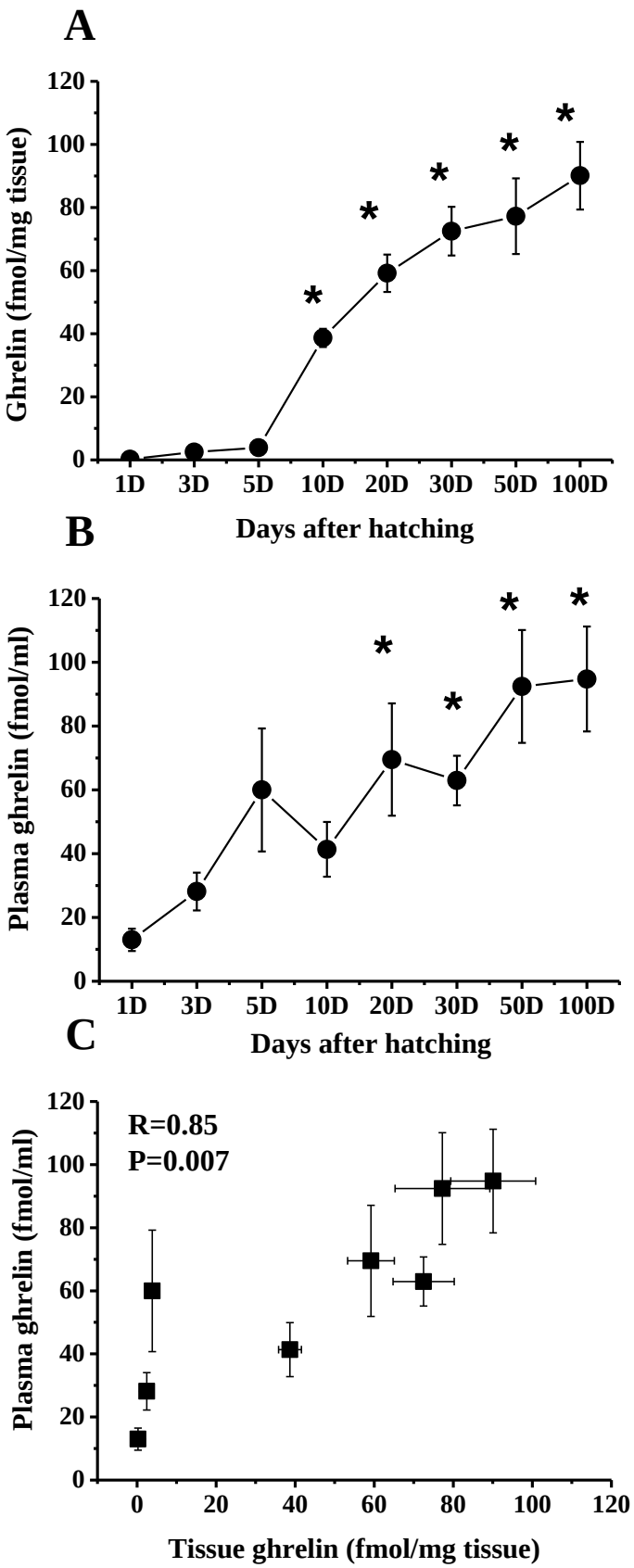
Fig. 6 Age-dependent changes in expression levels of *GHS-R1a* mRNAs in eight

different regions of the growing chicken brain (1, 3, 5, 10, 20, 30, 50 and 100 days after hatching). (A) shows mRNA expression levels of GHS-R1a in the olfactory bulb, cerebral cortex, thalamus and hypothalamus. (B) shows mRNA expression levels of GHS-R1a in the pituitary gland, midbrain, cerebellum and medulla oblongata. *GHS-R1a* expression level was significantly decreased only in the cerebellum from 3 to 100 days. Transient significant increases were observed in the hypothalamus at 50 days and in the midbrain at 5 days, and a transient significant decrease was observed in the medulla oblongata at 10 days. Values are means ±S.E.M. of 4 or 5 experiments.

Fig. 7. Age-dependent changes in expression levels of *GOAT* mRNAs in eight different regions of the growing chicken brain ((1, 3, 5, 10, 20, 30, 50 and 100 days after hatching). (A) shows mRNA expression levels of GOAT in the olfactory bulb, cerebral cortex, thalamus and hypothalamus. (B) shows mRNA expression levels of GOAT in the pituitary gland, midbrain, cerebellum and medulla oblongata. *GOAT* mRNA expression was increased significantly in the olfactory bulb at 20, 30, 50 and 100 days, in the midbrain at 20, 30, 50 and 100 days, in the cerebellum at 30 days and in the medulla oblongata at 30, 50 and 100 days compared with the expression level at 1 day. Values are means ±S.E.M. of 4 or 5 experiments.

Figure

Fig. 1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Ghrelin (fmol/mg tissue)
120
100
80
60
40
20
0
1D
3D
5D
10D
20D
30D
50D
100D
Days after hatching
B
Plasma ghrelin (fmol/ml)
120
100
80
60
40
20
0
1D
3D
5D
10D
20D
30D
50D
100D
Days after hatching
C
Plasma ghrelin (fmol/ml)
R=0.85
P=0.007
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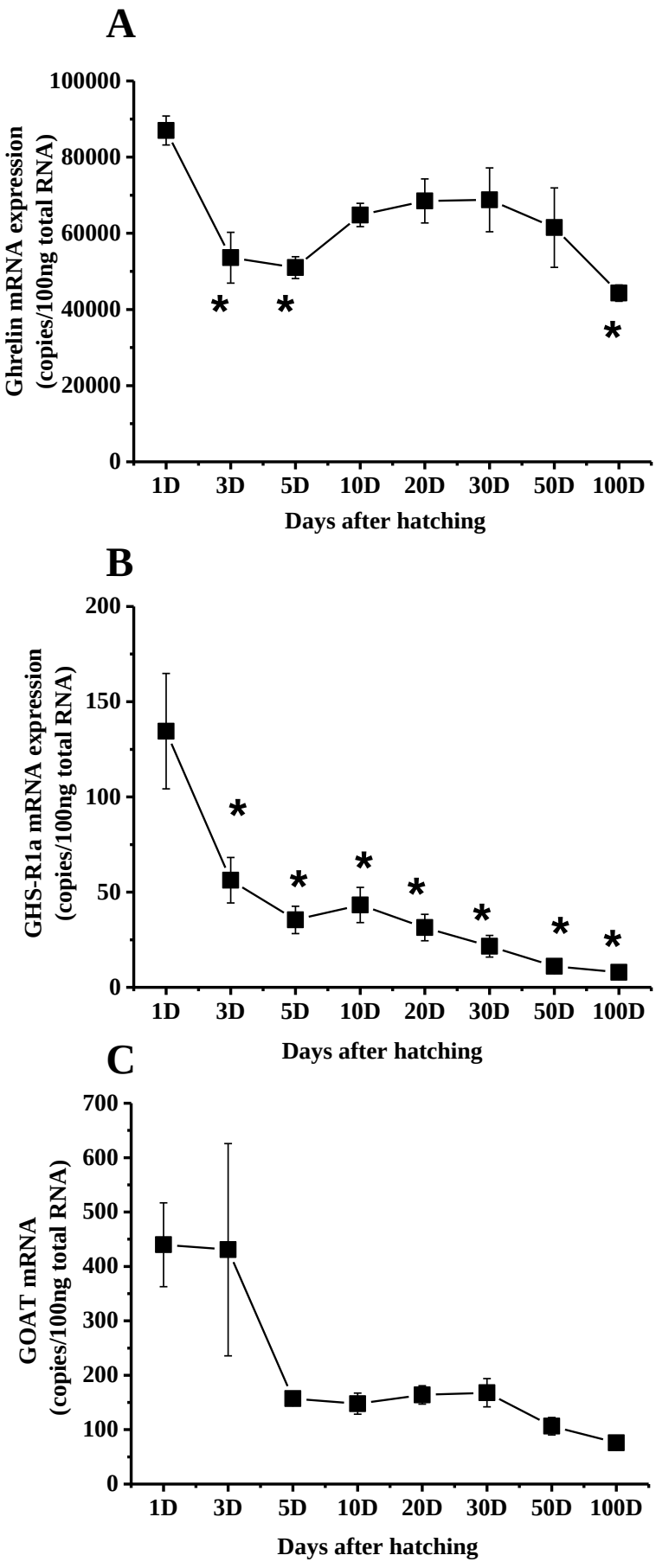
100
80
60
40
20
0
0
20
40
60
80
100
120
Tissue ghrelin (fmol/mg tissue)

Fig. 2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(copies/100ng total RNA)
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80000
60000
40000
20000
0
1D
3D
5D
10D
20D
30D
50D
100D
Days after hatching
B
GHS-R1a mRNA expression
(copies/100ng total RNA)
200
150
100
50
0
Days after hatching
C
GOAT mRNA
(copies/100ng total RNA)
700
600
500
400
300
200
100
0
Days after hatching

Figure 3

Fig. 3

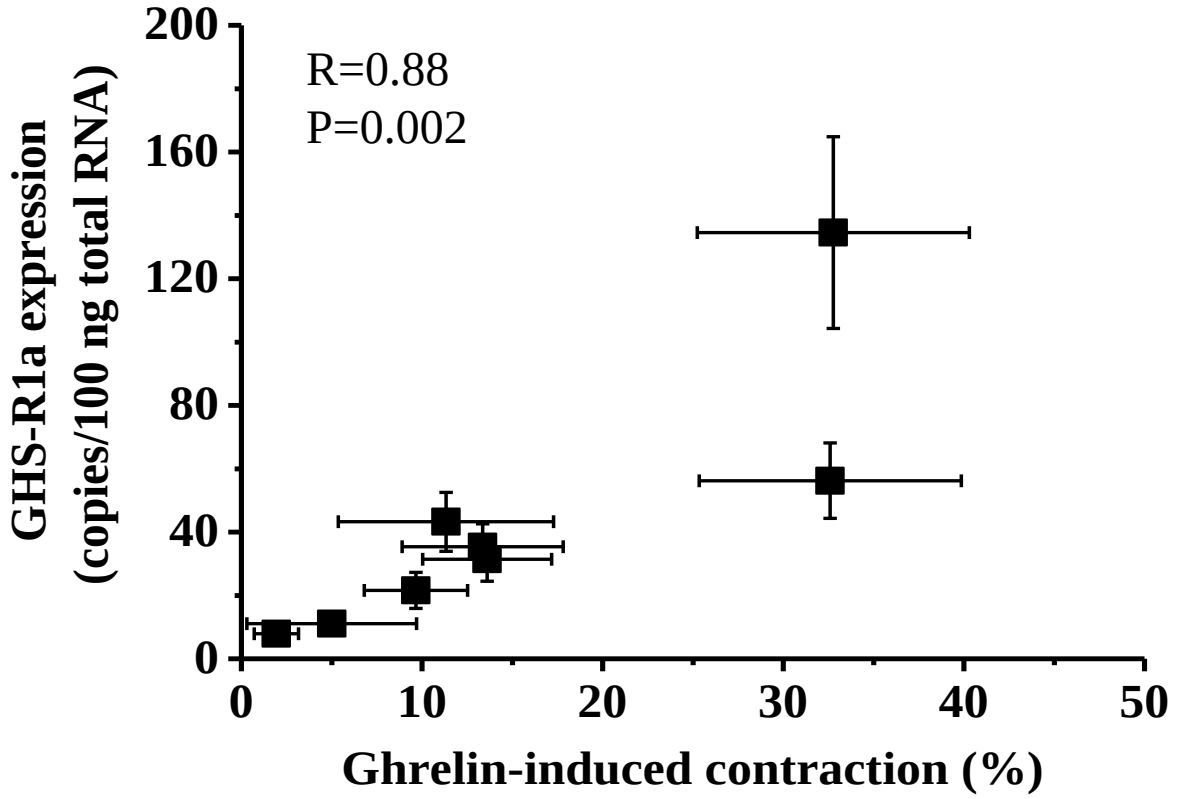


Figure 4

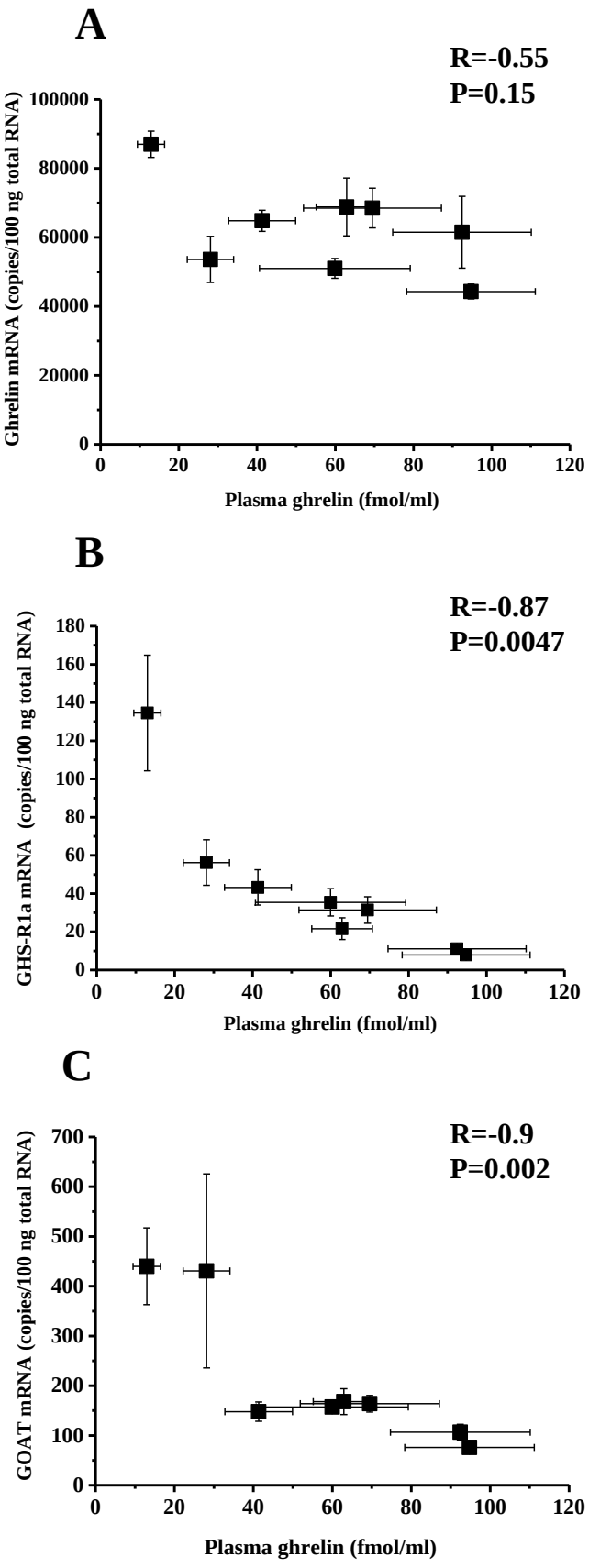


Fig. 4

Figure 5

Fig. 5

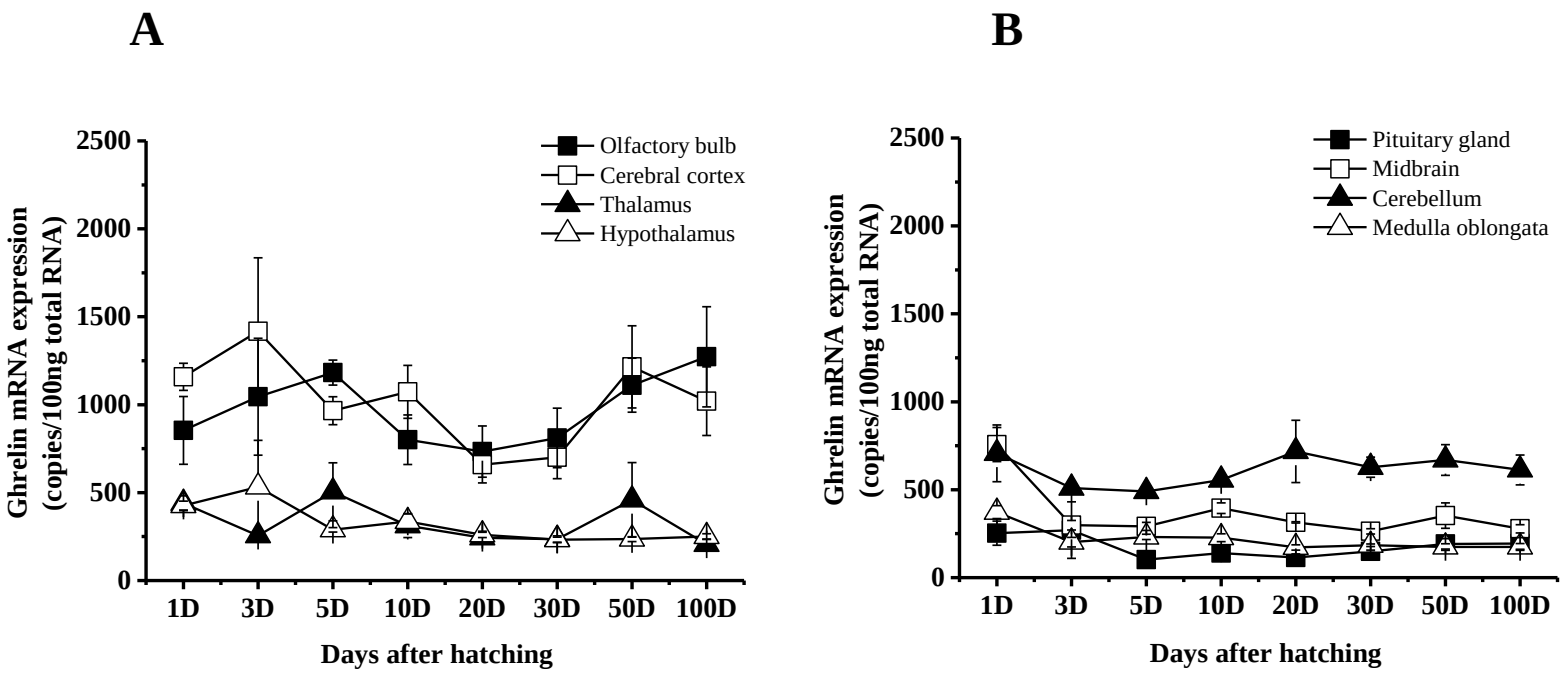


Figure 6

Fig. 6

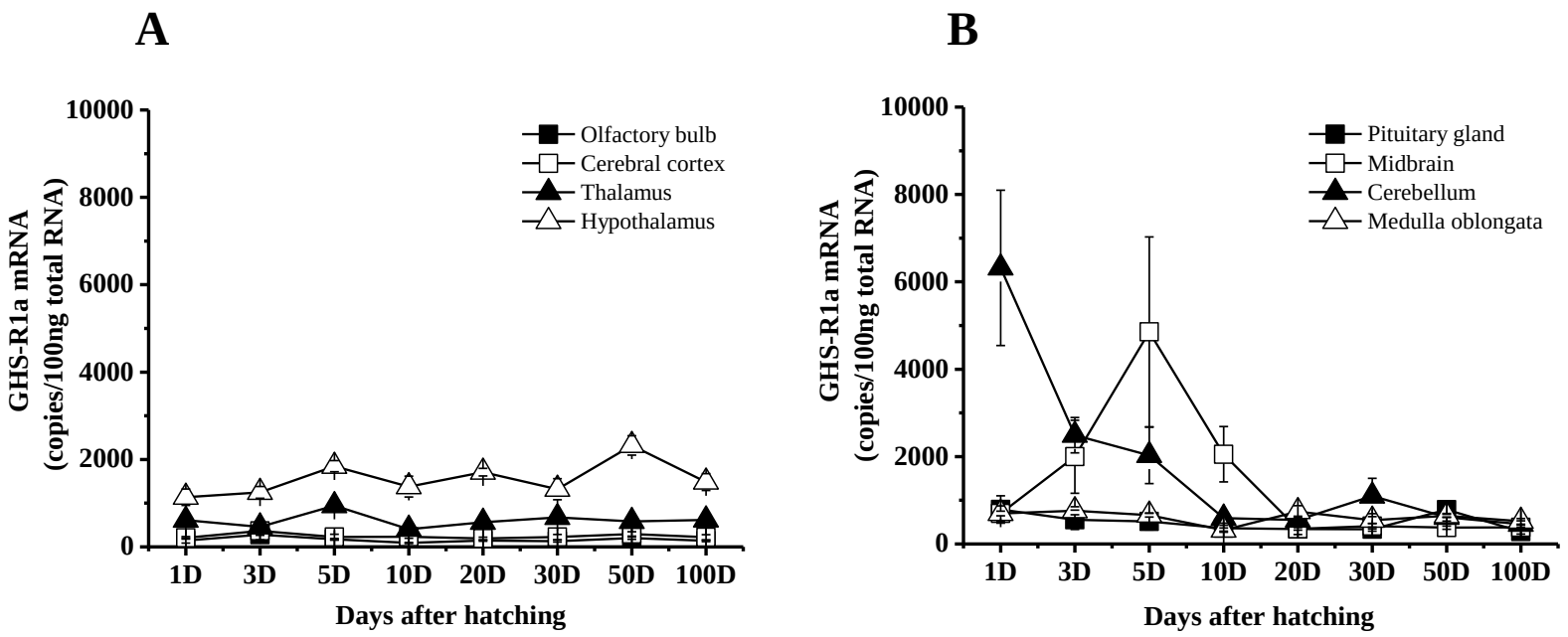
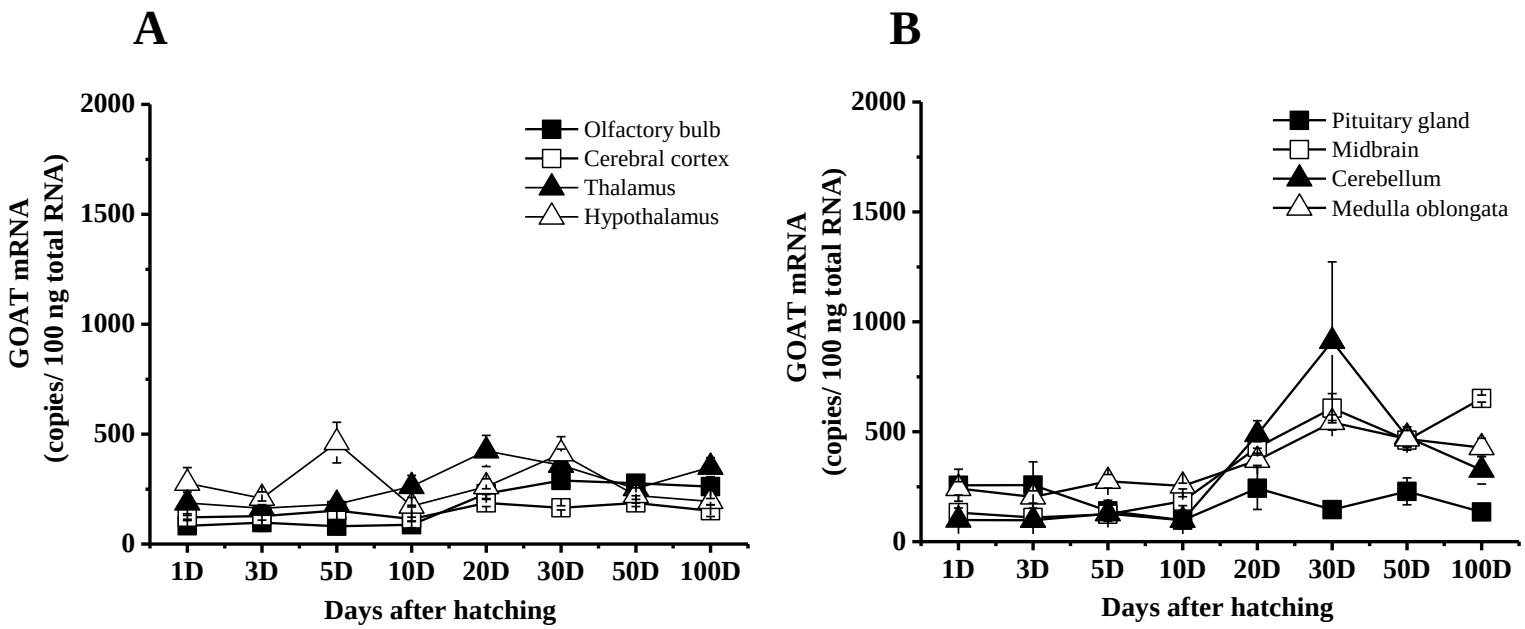


Figure 7

Fig. 7



Table

Table 1. Primers used in this study

Name	Sequence (5' to 3')	Amplicon size (bp)
GHRL-s2	GAA ACA GAG GGA GAA GAT GAC AAT	
GHRL-AS2	TTT GTC TGA GTT TCT TCA GCA TTC	146
GHSR-Q-s	GGG CCG TCT CCT TCA TTA GTG CTG	
GHSR-Q-AS	TTC CTC TTC CTC CTC CAC AGC TTC	232
GOAT-Q-s	GGT ACC TCG GGG TGC TGG TGC TG	
GOAT-Q-AS	AAA GTG GCA AGG AGT GGC TGA GC	337
B-actin-Q-s	CCC TGA ACC CCA AAG CCA ACA	
B-actin-Q-AS	GGA CTC CAT ACC CAA GAA AGA	488