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Study on the central and peripheral regulation of food intake in Japanese quail using some novel peptides

(新規ペプチドによるウズラの中枢性および末梢性摂食制御機構の解析)

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GENERAL INTRODUCTION

Food intake regulation is a fundamental concern of poultry producers as the growth rate of broilers or breeders is very important. Feeding is a basic behavior that is necessary for life. Long-term lack of food results in death. It is well accepted that appetite is controlled by the brain and that feeding behavior is regulated by complex mechanisms in the central nervous system, in particular the hypothalamus (Druce & Bloom, 2003; Schwartz *et al.*, 2000; Vettor *et al.*, 2002). There is not a great deal known about the basic physiological mechanisms regulating food intake. Although physical means of regulating intake, i.e., restricting or force-feeding, have been successful, a better understanding of the mechanisms controlling food intake could lead to the development of other methods that are either less labor-intensive or less stressful to the bird.

The performance of domestic avian species has significantly improved as a result of genetic selection. For instance, the growth rate of broilers has increased in recent decades; however, this improvement in growth rate is hardly explained by nutritional modification and/or enhancement of food efficiency. Higher growth rate is always associated with higher food intake. Successful poultry production depends mainly on fast growth rates and efficient feed conversion ratio. Normally the cost of feed represents about one half of the total cost of raising birds.

To either increase or decrease body weight of poultry, an understanding of food intake regulation is essential. Although it is advantageous to increase food intake in market birds, it is desirable to decrease food intake in breeders. Over the last 20 years, due largely to genetic selection but partially to improvements in nutrition, there has been a substantial decrease in the time it takes to grow a broiler to market weight. Much of this improvement is attributed to increased food intake (McCarthy & Siegel, 1983).

An understanding of food intake regulation is important both in attempting to develop methods to increase consumption in market broilers, but also in developing practical methods of restricting intake to control obesity in breeders. Food intake regulation is a complex, multifaceted system involving sites both inside and outside the central nervous system (CNS).

Recent studies have shown that the digestive tract, liver, and brain are all involved in food intake regulation. The role of various neurotransmitters and metabolic substances in food intake regulation, both within the central nervous system as well as in the periphery over the last 2 decades is studied.

It has been well established that the center regulating feeding behavior is the hypothalamus. Removal of the lateral hypothalamus causes hypophagia (decreased feeding), leading to death due to severe weight loss. On the other hand, removal of the ventromedial hypothalamus causes hyperphagia (increased feeding); treated animals

increase both feeding amount and frequency, leading to weight gain and severe obesity. In addition to the VMH, the dorsomedial nucleus (DVN) and the paraventricular nucleus (PVN) are involved in the satiety center (Luiten *et al.*, 1987; Sclafani & Kirchgessner, 1986; Bray, 1984). Thus feeding is regulated by a balance of stimulating and inhibiting forces in the hypothalamus. Recent identification of appetite-regulating humoral factors reveals regulatory mechanisms not only in the central nervous system, but also mediated by factors secreted from peripheral tissues (Neary *et al.*, 2004; Small & Bloom , 2004; Ukkola., 2004;.Wynne *et al.*, 2004.). Leptin, produced in adipose tissues, is an appetite-suppressing factor that transmits satiety signals to the brain (Friedman, 2002.). Hunger signals from peripheral tissues, however, had remained unidentified until the recent discovery of ghrelin. Recent molecular biological approaches have advanced our knowledge of the mechanisms involved in the regulation of feeding in mammals but in case of avian species, more investigations are needed to clear the mechanism of the food regulation.

It has been reported that there are many discrepancies in feeding regulation between mammals and avian species. For examples; orexin-A, orexin-B (Szekely *et al.*, 2002), motilin (Garthwaite, 1985; Rosenfeld & Garthwaite, 1987), melanin-concentrating hormone (Qu *et al.*, 1996; Rossi *et al.*, 1997), ghrelin (Nakazato *et al.*, 2001), and galanin (Akabayashi *et al.*, 1994) have been reported to stimulate food intake in rats, but these peptides failed to either stimulate or inhibit

feeding in the neonatal chick (Ando *et al.*, 2000; Furuse *et al.*, 1999a, 2001). On the other hand, it has been reported that cholecystokinin (Gibbs *et al.*, 1973), glucagon-like peptide-1 (Tang-Christensen *et al.*, 1996; Turton *et al.*, 1996), CRH (Benoit *et al.*, 2000), histamine (Lecklin *et al.*, 1998), and α-melanocyte stimulating hormone (Rossi *et al.*, 1998; Wirth *et al.*, 2001) suppress feeding in rats. The suppression of food intake by these peptides has also been observed in the neonatal chick (Furuse *et al.*, 1997a; 1997b; 1999b; 2000; Kawakami *et al.*, 2000a; 2000b). Therefore, it may be that the mechanisms underlying the inhibition of feeding are well conserved in chicken, but those underlying the stimulatory effects are not.

There are not yet published data regarding the peripheral or central effects of such peptides in adult avian species. Although to date orexigenic and anorexic regulation in bird species has been examined in 1-day- old chicks (Ando *et al.*, 2000; Furuse *et al.*, 1997a, 1997b, 1997c, 2000, 2001; Kawakami *et al.*, 2000a, 2000b), in the study presented here I used adult Japanese quail (*Coturnix coturnix Japonica*). The reasons behind this choice are: (1) the growth curve of chicken is steep and so body weight gain and food intake vary widely every day, whereas in the adult Japanese quail, the growth curve remains consistent, and therefore, body weight gain and food intake are not subject to such wide variations; (2) it is possible to chronically implant an icv cannula into the adult Japanese quail brain. Moreover, raising Japanese quail in approximate comparisons with poultry has

many advantages, that they occupied less space area per bird (200 cm^2 as compared with 1000 cm^2 in chicken), consume less feed with a higher metabolic rate (14 gm/day as compared with 110 gm/day in chicken) , reaching maturity earlier (42 days as compared with 150 days in chicken), produce greater volume of eggs per unit of body weight, apparently more resistant to diseases and develop 3.5 times as fast as domestic fowl (Marsh, 1976). Quails as chickens are used as a suitable source of animal proteins due to their rapid growth rate and its efficiency to convert vegetable proteins and carbohydrates to animal proteins and fats (Ensminger, 1992). The raising of Japanese quail (*Coturnix coturnix Japonica*) is very important to help in solving the problem of a deficiency in animal proteins; this may be due to the rapid life cycle and low productive costs of quail as compared with chicken. They kept mainly for meat production since their meat is palatable and popular (Kovach, 1975).

Because we are studying physiological parameters such as food intake and body temperature, so we used adult male quails to avoid the effect of female reproductive hormones on food intake and the interference of reproductive hormones with the injected peptides on food intake or other parameters. Therefore the purpose of this study is to investigate the central and peripheral regulation of food intake in adult Japanese quail using some novel peptides such as neuromedin (U and S), ghrelin and glucagon-like peptide-1 and -2.

CHAPTER 1

Effect of neuromedin U on feeding regulation in the Japanese quail

Abstract

Nuromedin U (NMU), which is a brain–gut peptide, was first isolated as a smooth – muscle-contracting peptide from the porcine spinal cord in 1985. Intracerebroventricular (icv) injection of NMU into rats significantly reduced food intake during dark period, and increased oxygen consumption, locomotor activity and body temperature, suggested that NMU is an anorectic and catabolic signaling molecule in mammals. In this study, we elucidated the central role of NMU in avian species using Japanese quail. Gene cloning analysis revealed that the amino acid sequence of Japanese quail NMU has high homology with that of chick, and low homology with that of rat except the C-terminal biologically active region. RT-PCR analysis revealed that NMU mRNA was expressed in various central and peripheral tissues. Both intraperitoneal (ip) and icv administration of synthetic Japanese quail NMU resulted in a significant reduction in food intake and increase in both body temperature and gross locomotor activity in Japanese quails. Conversely, the administration of rat NMU into Japanese quail resulted in the opposite effects on food intake, body temperature and locomotor activity. These opposing results suggest that rat NMU might act as an antagonist toward the Japanese quail NMU receptor. If so, this study is the first to show that endogenous NMU plays an important role in the regulation of food intake and body temperature.

1.1. Introduction

Neuromedin U (NMU) is a highly conserved brain-gut peptide that was first isolated from the porcine spinal cord (Minamino *et al.*, 1985), and later from the brain, spinal cord and intestine of other species (Austin *et al.*, 1995; Domin *et al.*, 1989; Domin *et al.*, 1992; Kage *et al.*, 1991; O'Harte *et al.*, 1991). Two molecular forms have been purified: an octapeptide (NMU-8), which constitutes the core active C- terminus of NMU, and the N-terminally extended 25- or 23-amino-acids form in human and rats, respectively (Austin *et al.*, 1995; Conlon *et al.*, 1988; Minamino *et al.*, 1985; 1988). Both forms are biologically active, stimulating contraction of rat uterus in vitro and causing potent vasoconstriction in rats and dogs (Gardiner *et al.*, 1990; Minamino *et al.*, 1985; Sumi *et al.*, 1987). It is thought that the C-terminal region of NMU is the biologically active region of the peptide, and it is widely conserved among various species, such as dog (O'Harte *et al.*, 1991), rabbit (Kage *et al.*, 1991), frog (Domin *et al.*, 1989), human (Austin *et al.*, 1995), and chicken (Domin *et al.*, 1992).

Although the only known physiological role of NMU is smooth muscle contraction of blood vessel, the uterus, and the gastrointestinal tract (Gardiner *et al.*, 1990; Minamino *et al.*, 1985; Sumi *et al.*, 1987), the recent discovery of NMU receptors has prompted a search for new physiological roles for this peptide (Howard *et al.*, 2000; Fujii *et al.*, 2000; Szekeres *et al.*, 2000; Kojima *et al.*, 2000). FM-3 (also called

GPR66) and FM-4 (TGR-1), which are orphan G-protein-coupled receptors that have been identified as cognate receptors of NMU, have been designated as NMU1R and NMU2R, respectively (Howard *et al.*, 2000). NMU1R has a wide distribution in peripheral tissues, including the gastrointestinal tract, lung and adrenal cortex, but little or no expression is seen in the rat or human central nervous system (Howard *et al.*, 2000; Raddatz *et al.*, 2000; Szekeres *et al.*, 2000). On the other hand, NMU2R is mostly restricted to specific regions of the brain, such as the hypothalamic paraventricular nucleus, along the wall of the third ventricle in the hypothalamus, and the CA1 region of the hippocampus in rats and humans (Austin *et al.*, 1995; Howard *et al.*, 2000).

NMU is involved in the central control of feeding (Howard *et al.*, 2000; Nakazato *et al.*, 2000). Intracerebroventricular (icv) administration of NMU to free-feeding rats decreases food intake, body weight and time spent feeding (Howard *et al.*, 2000; Nakazato *et al.*, 2000, Kojima *et al.*, 2000). Also it is reported that icv administration of NMU to 24-h fasted rats resulted in a decrease in subsequent food intake and body weight gain and also was accompanied by an increase in core body temperature (Ivanov *et al.*, 2002; Wren *et al.*, 2002). Moreover, it has been shown that anti-NMU IgG increases dark-phase feeding compared with preimmune serum IgG (Kojima *et al.*, 2000), and NMU knockout mice became obese (Hanada *et al.*, 2004). In addition, fasting induces the downregulation

of NMU mRNA levels (Howard *et al.*, 2000). These results indicate that NMU is a potent endogenous anorexic peptide in rats. In contrast, NMU increases gross locomotor activity, body temperature, and heat production in rats, suggested that NMU is also a catabolic signaling molecule (Nakazato *et al.*, 2000).

Thus far there are no published data regarding the peripheral or central effects of NMU in birds. Although to date orexigenic and anorexic regulation in bird species has been examined in 1-day- old chicks (Ando *et al.*, 2000; Furuse *et al.*, 1997a, 2000, 2001; Kawakami *et al.*, 2000a, 2000b), in the study presented here I used adult Japanese quail.

The purpose of this study was to elucidate the peripheral and central effects of NMU on food intake and body temperature in the Japanese quail. To do this, firstly the DNA sequence of Japanese quail NMU was determined, and the peptide was synthesized. Thereafter, I compared the effects of icv and intraperitoneal (ip) injections of rat and Japanese quail NMU on food intake and body temperature in these birds.

1.2 Materials and methods

1.2.1. Animals

Adult male Japanese Quail (*Coturnix coturnix Japonica*), weighing 110-120 g, were reared in a room with light a 12-h light/12-h dark cycle (lights on at 0700 hours) at a temperature of 28 ± 1 °C , and were given free access to food and water. Rat or Japanese quail NMU was dissolved in 0.9% saline, and either 200 μl of this solution was administered ip, or 10 μl was administered icv to each 6 free-feeding male birds in each experimental group. These experiments were performed in duplicate. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

1.2.2 Surgical procedures

For icv administration, Japanese quails were anesthetized with 5% sodium pentobarbital (1.4 μl / g body weight) and then placed in a stereotaxic frame. A stainless steel guide cannula (outer diameter, OD: 550 μm; length: 14-mm) was implanted into the 3rd cerebral ventricle stereotaxically by the method described previously with minor modification (Bayle *et al.*, 1974). The coordinates were 5 mm anterior to the interaural axis and 6.5 mm below to dura and the midline. One stainless steel anchoring screw was fixed to the skull, and the guide cannula was secured in place by acrylic dental cement. The birds were then returned to their individual cages and allowed to recover for at least 4 days. The birds were then handled every day and housed in individual cages before the start of the experiments. Icv injections

were carried out in conscious animals. At the end of the experiments, proper placement of the cannulae was verified by dye administration. Data for birds lacking Evan's blue dye in the 3rd ventricle were excluded from the analysis.

1.2.3. Measurement of food intake

Before the feeding experiment, birds were weighed and assigned to experimental groups according to body weight, so that the average weight between groups was as uniform as possible. Rat or Japanese quail NMU, or saline (as a control) was administered ip (2.0 or 10.0 nmol/200 μl saline) or icv (0.5 or 1.0 nmol/10 μl saline) at 0700 hours. I preliminary used 0.1 nmol and 0.5 nmol of NMU in icv and ip injections, respectively, but I did not observe any significant effect on food intake.

Food consumption and body weight changes in free-fed birds were measured at 2, 4, and 12 h after administration. In another experiment, which was designed to examine whether pretreatment with rat or quail NMU blocks the effect of subsequent treatment with quail or rat NMU on food intake in Japanese quail, respectively, the birds were pretreated with either saline, or rat or quail NMU (ip; 10.0 nmol NMU/200 μl saline) at 0700 hours. One hour later, the birds were given a further injection of either saline, or quail or rat NMU (ip; 10.0 nmol NMU/200 μl saline), depending on the experimental group to which they had been assigned. Food consumption was measured at 12 h after the first injection.

1.2.4. Measurement of body temperature

Body temperature was measured 0 min (before injection), 5, 10, 20, 40, 60, and 120 min after ip (2.0 or 10.0 nmol NMU/200 μl saline, or saline) or icv (0.5 or 1.0 nmol NMU/10 μl saline, or saline) injection of rat or Japanese quail NMU (n = 6 in each group) by the method described previously (Bayle *et al.*, 1974). Body temperature was measured with a small sensor (measurable range: 25–50 °C; measurement error: 0.05 °C) connected to a line (OD: 0.7 mm; length: 45 cm) and monitor body. The sensor tip was inserted into the cloaca, and part of the line was fixed to the body of the bird.

1.2.5. Measurement of gross locomotor activity

Locomotor activity was measured in each bird under LD condition for 1 week, and thereafter under constant dim light at an intensity of about 30 lux, with a rat locomotor activity recording system (Muromachi Co. Ltd., Tokyo, Japan) comprising infrared sensors, an interface, and a computer (Marumato *et al.*, 1996). The infrared sensors were placed above the cages and measured all locomotion such as eating, and perch hopping. Each cage with its infrared sensor was placed in an isolated chamber box with a controlled light/dark cycle. The data were collected at 15-min intervals and analyzed by CompactACT AMS software (Muromachi Co. Ltd.). Rat NMU (2.0 or 10.0 nmol / 200 μL saline) or vehicle was administered ip at 0730 to birds (n = 8 per group). Rat NMU (0.5 or 1.0 nmol / 10 μL saline) or vehicle was administered icv at 0730 hours to birds. After injection,

the birds were immediately returned to the individual cages. Locomotor activity counts were made every 15 min and summed for 2 h after administration.

1.2.6. Rat experiments

Adult male Wistar rats, weighing 250-300 g, were maintained under 12-h light/12-h dark condition (lights on at 0700 hours). To confirm the previous results that icv injection of NMU reduced food intake during nighttime (Kojima *et al.*, 2000), rats are injected icv with 0.5 and 1.0 nmol rat NMU at 1830 hours. All procedures were conducted using the same methods described earlier. As there is as yet no published report concerning the effects of peripheral injection of NMU, I injected another group of rats with either 5.0 or 10.0 nmol NMU ip at 1830 hours and examined food intake during the dark period.

1.2.7. cDNA cloning of Japanese quail NMU

Total RNA was extracted from the Japanese quail spinal cord with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Ten μg of total RNA was treated with DNase to remove any contaminating genomic DNA, and poly $(A)^+$ RNA was isolated by using an Oligotex-dT30 super mRNA purification kit (Takara Shuzo, Kyoto, Japan). Double-stranded cDNA was synthesized from 2 μg of poly $(A)^+$ RNA with oligo (DT) primers and superscript III RNase H $^-$ reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Two degenerate primers (sense:

5′TTYTTATTYCAYTAYTCNAAGAC-3′; antisense: 5′-CGTGGCCTRAARAAAAARTA-3′) were used for amplification. Polymerase chain reaction (PCR) products were gel-purified (Qiagen gel extraction kit; Qiagen, Tokyo, Japan) and cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA). Plasmid DNA was prepared from bacterial culture with Qiagen Plasmid Mini Kits. The correct identity of the cloned fragments was confirmed by sequencing. DNA sequencing reactions were performed in a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA) and electrophoresed with an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). According to the DNA sequence, Japanese quail NMU consisting of 25 amino acids was synthesized (Shimadzu Biotech, Kyoto, Japan).

1.2.8. Reverse transcriptase (RT)-PCR for NMU mRNA

Total RNA was extracted from various tissues of Japanese quail with TRIzol. First- strand cDNA was synthesized from 2 μg of total RNA with oligo (DT) by reverse transcription. The resulting cDNA was PCR-amplified with the sense and antisense primers each at 2 μM, and 2.5 U *Taq* polymerase (Promega). The PCR primers specific for NMU of Japanese quail (determined by cDNA cloning) were 5′-CTCAGCCTCAGGTGCCCAGTACAC-3′ (sense) and 5′-AAATCCACTGATCTCTTCCCGTTACGTG-3′ (antisense). β-Actin was used as a housekeeping gene. Hot-start PCR was performed on 25 μl in an iCycler (Bio-Rad Laboratories, Richmond, CA, USA). The

program was 2 min at 94 °C followed by 30 cycles of 15 s at 94 °C, and 30 s at 68 °C, and a final extension at 68 °C for 5 min. The PCR products were electrophoresed on 2 % agarose gel.

1.2.9. Statistical analysis

Data were analyzed by analysis of variance and *post hoc* Fisher's test, and the results are expressed as mean ± SEM.

1.3. Results

1.3.1. cDNA cloning and RT-PCR analysis

cDNA cloning revealed that the amino acid sequence of Japanese quail NMU had a high homology with chicken NMU – there was only a two-amino-acid difference. On the other hand, although five amino acids at the C-terminal region were identical between Japanese quail and rat NMU, the rest of the protein shared a low homology (Fig. 1A). RT-PCR analysis revealed that NMU mRNA is expressed in a wide range of tissues. Abundance was relatively high in the interstitial tract and spinal cord (Fig. 1B).

1.3.2. Food intake and body temperature

The ip and icv administration of synthetic Japanese quail NMU to quails resulted in a significant ($P < 0.05$) decrease in food intake in comparison with saline injection (Fig. 2). This reduction in food intake was apparent at 4 h after injection and continued for 12 h after either ip injection of 10.0 nmol (saline, 20.3 ± 2.4; NMU, 14.78 ± 2.99 g) or icv injection of 1.0 nmol (saline, 20.3 ± 2.4; NMU, 11.1 ± 1.76 g) ($P < 0.05$ vs saline). Also, body weight gain decreased significantly ($P < 0.05$ vs saline) after both ip and icv of quail NMU (Fig. 3).

In contrast, both icv and ip injections of rat NMU into Japanese quail increased food intake over the subsequent 12-h period (Fig. 4). The administration of rat NMU to quails, either 1.0 nmol icv or 10.0

nmol ip, resulted in a significant ($P < 0.05$) increase in food intake within 4 h of the injection and continued for 12 h. The increase of food intake was apparent during the first 2 h (saline, 4.4 ± 0.4; NMU, 7.1 ± 0.9 g) after icv injection, and continued for 12 h (saline, 20.3 ± 2.4; NMU, 31.2 ± 3.6 g) (Fig. 4). This effect was not observed the following day (Fig. 5). Also a significant increase in body weight gain was measured at 4 and 12 h after ip and icv injection of 10.0 and 1.0 nmol, respectively. Body weight gain increased more than food intake did. By 12 h, a large increase (saline, 3.6 ± 1.3; NMU, 13.3 ± 1.7 g) of body weight gain was observed in the rat NMU-treated group relative to the saline-injected group (Fig. 6).

I examined the effect of Japanese quail and rat NMU on the body temperature of Japanese quail. Body temperature was increased by both ip and icv injection of Japanese quail NMU (Fig. 7). This effect was observed sooner following the icv injection than following the ip injection. On the other hand, both the icv and ip administration of rat NMU decreased body temperature in Japanese quail (Fig. 8). Body temperature decreased by 1.8 °C 1 h after icv injection of 1.0 nmol NMU and 1.9 °C after ip injection of 10.0 nmol NMU.

As it is reported previously that icv injection of rat NMU into rats causes a rapid increase of locomotor activity and body temperature for 1 to 2 h, therefore I tried to examine the effect of rat and Japanese quail NMU on the locomotor activity and body temperature of Japanese quail. In the quail, both ip and icv administration of Japanese

quail NMU resulted in a significant ($P < 0.05$) increase in gross locomotor activity. Increase of locomotor activity measured 132.4% during 2 h after icv injection of 1.0 nmol and 144.6 % after ip injection of 10.0 nmol of Japanese quail NMU (Fig. 9). On the other hand, rat NMU resulted in a significant ($P < 0.05$) decrease in gross locomotor activity. Decreases of locomotor activity measured 34.1 % during 2 h after icv injection of 1.0 nmol and 41.8 % after ip injection of 10.0 nmol rat NMU (Fig. 10).

As the effects of Japanese quail and rat NMU on food intake in Japanese quail were opposing, I examined the possibility that the rat peptide (rat NMU) that was used here had degenerated prior to its administration. When administered to rats, however, icv, but not ip, injections of rat NMU inhibited food intake, as demonstrated previously (Fig. 11 A, B). Pretreatment with rat NMU completely blocked the quail NMU-induced inhibition of food intake in Japanese quail (Fig. 12). No significant difference was observed between the groups treated with saline followed by rat NMU (pre-post treatments) and rat NMU followed by quail NMU. On the other hand, pretreatment with quail NMU completely blocked the rat NMU-induced stimulation of food intake in Japanese quail. No significant difference was observed between the groups treated with saline followed by quail NMU and quail NMU followed by rat NMU (Fig. 12).

A

Rat	**YKV-** NEY**QG**P- VAPSGGF**FLFRPRN-**
NH2	
Chicken	**YKVDEDLQGAGGIQSRGY<u>FFFRPRN</u>-**
NH2	
Japanese quail	**FKVDEDLQGTGGIQSRGY<u>FFFRPRN</u>-**
NH2	

B

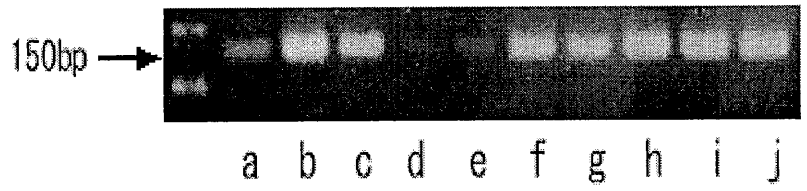

Figure 1. (A) Comparison of the amino acid sequences of neuromedin U (NMU) in rat, chicken, and Japanese quail. The dark bold letters show the homology of amino acid sequences of NMU between these species. Solid underline letters are conserved C-terminal biologically active regions. (B) NMU mRNA expression in various tissues from the Japanese quail, as demonstrated by RT-PCR analysis. a, lung; b, kidney; c, jejunum; d, heart; e, liver; f, duodenum; g, proventriculus; h, gizzard; i, spinal cord; j, brain.

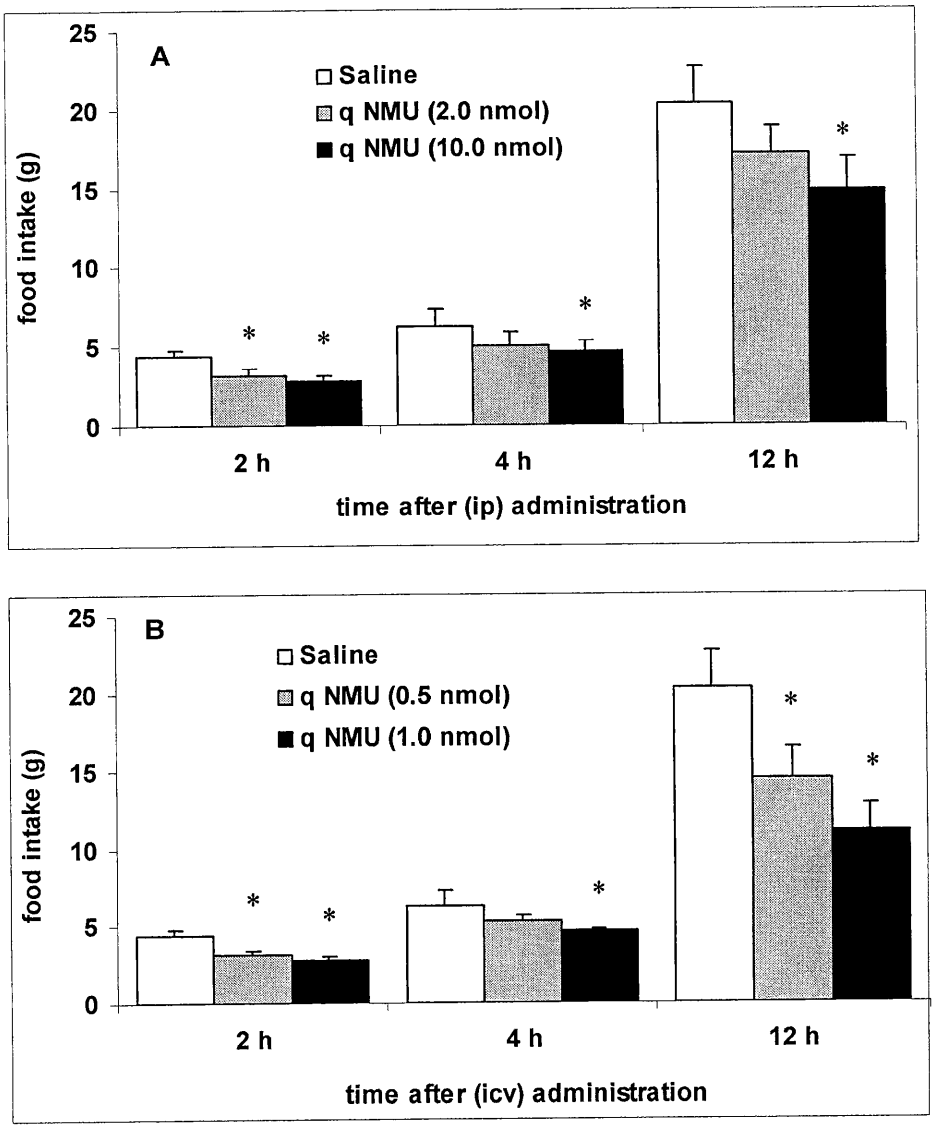

Figure 2. The effect of (A) ip administration of saline, 2.0 or 10.0 nmol, or (B) icv administration of saline, 0.5 or 1.0 nmol of Japanese quail NMU on food intake in Japanese quail. Each bar and vertical line represents the mean ± SEM (n = 12). *P < 0.05 vs the saline-treated group.

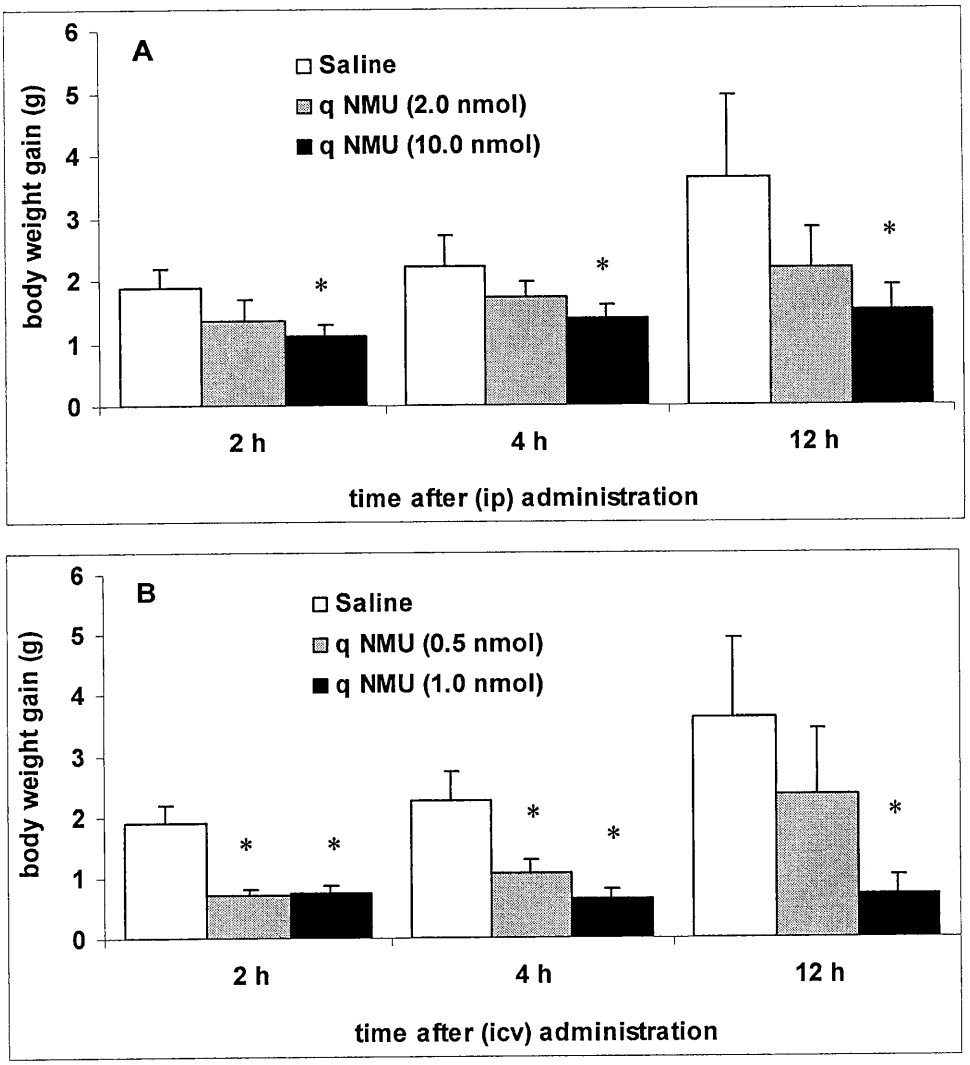

Figure 3. The effect of (A) ip administration of saline, 2.0 or 10.0 nmol, or (B) icv administration of saline, 0.5 or 1.0 nmol of Japanese quail NMU on body weight change in Japanese quail. Each bar and vertical line represents the mean ± SEM (n = 12). *P < 0.05 vs the saline-treated group.

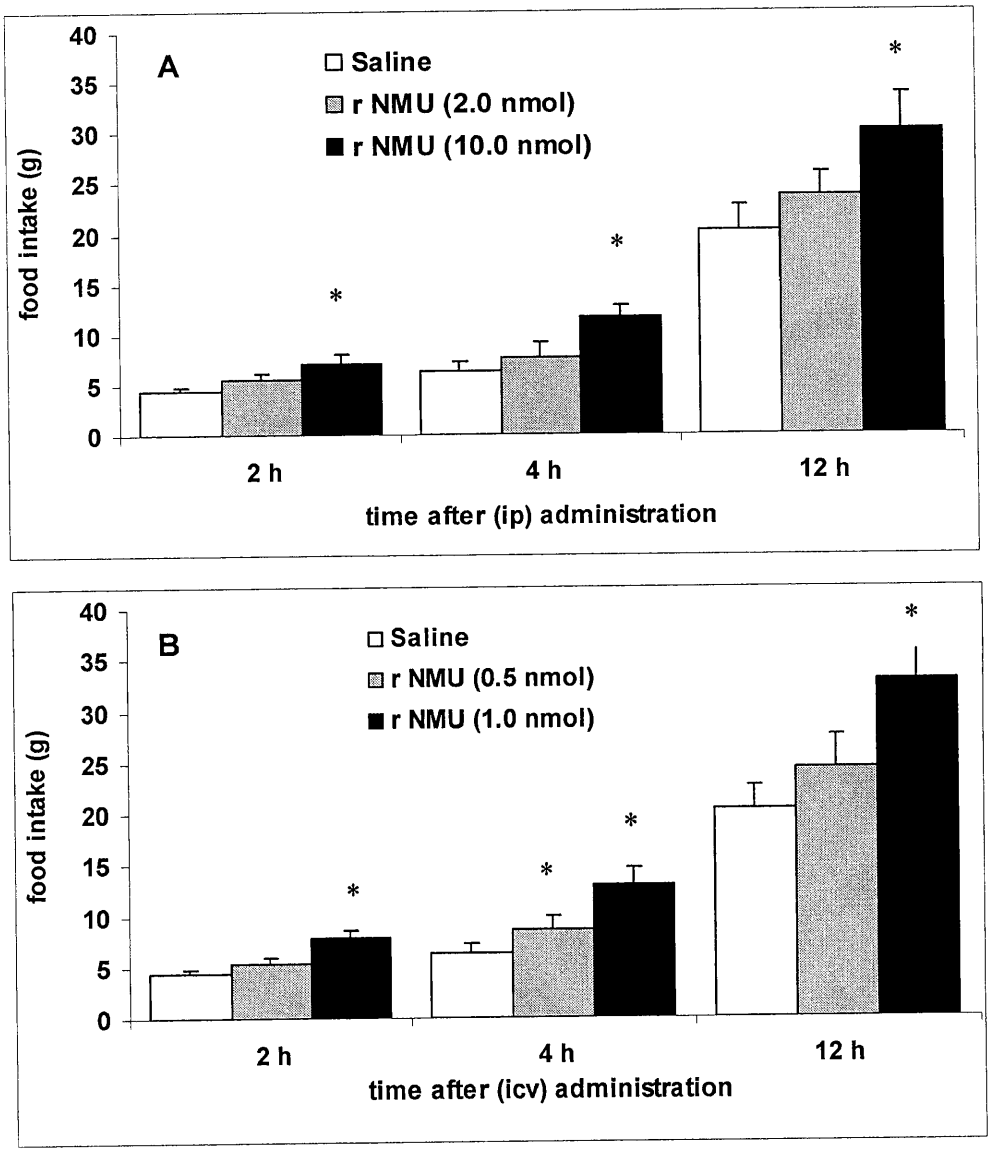

Figure 4. The effect of (A) ip administration of saline, 2.0 or 10.0 nmol, or (B) icv administration of saline, 0.5 or 1.0 nmol of rat NMU on food intake in Japanese quail. Each bar and vertical line represents the mean ± SEM ($n = 12$). *$P < 0.05$ vs the saline-treated group.

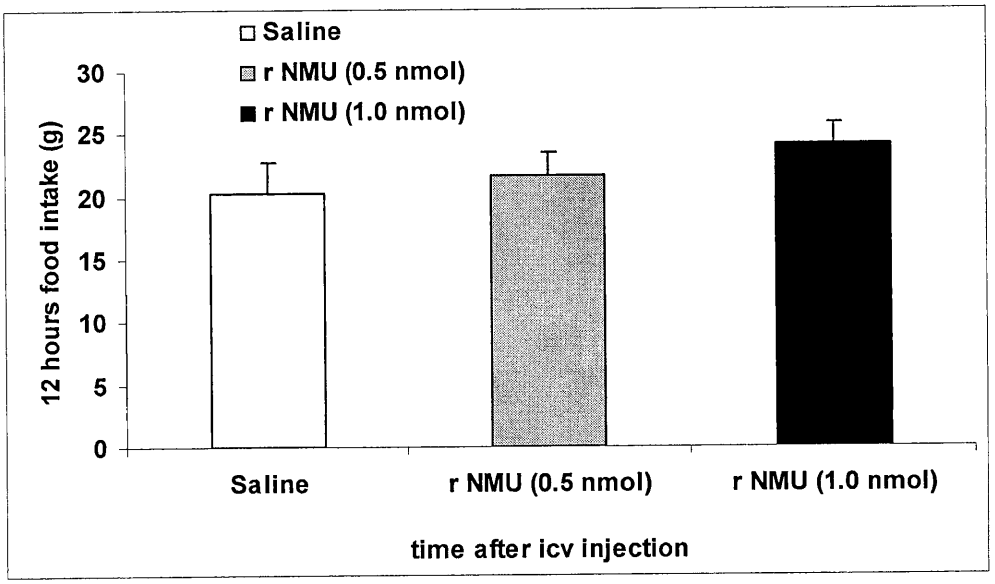

Figure 5 . Food intake in Japanese quail in the following day after icv administration of saline, 0.5 or 1.0 nmol of rat NMU. Each bar and vertical line represents the mean ± SEM (n = 12). *P < 0.05 vs the saline-treated group.

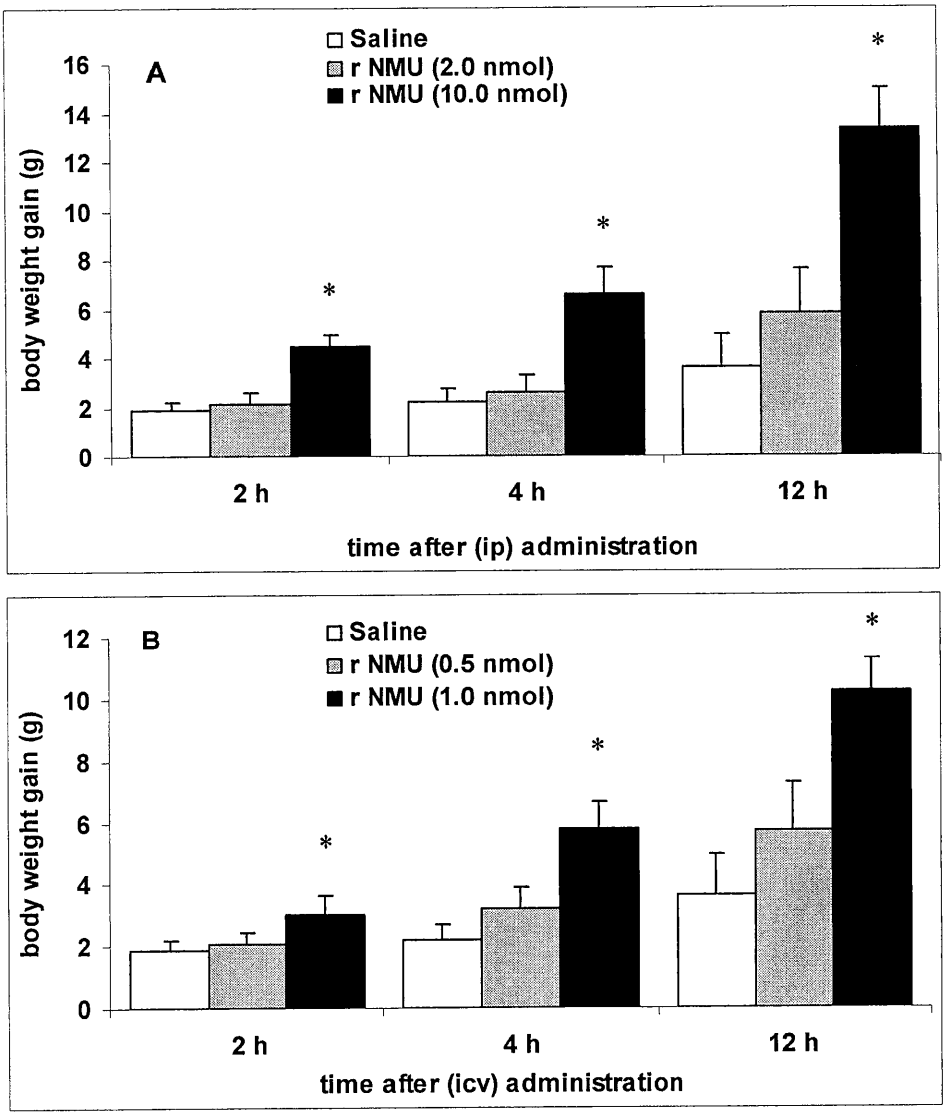

Figure 6. The effect of (A) ip administration of saline, 2.0 or 10.0 nmol, or (B) icv administration of saline, 0.5 or 1.0 nmol of rat NMU on body weight change in Japanese quail. Each bar and vertical line represents the mean ± SEM (n = 12). *P < 0.05 vs the saline-treated group.

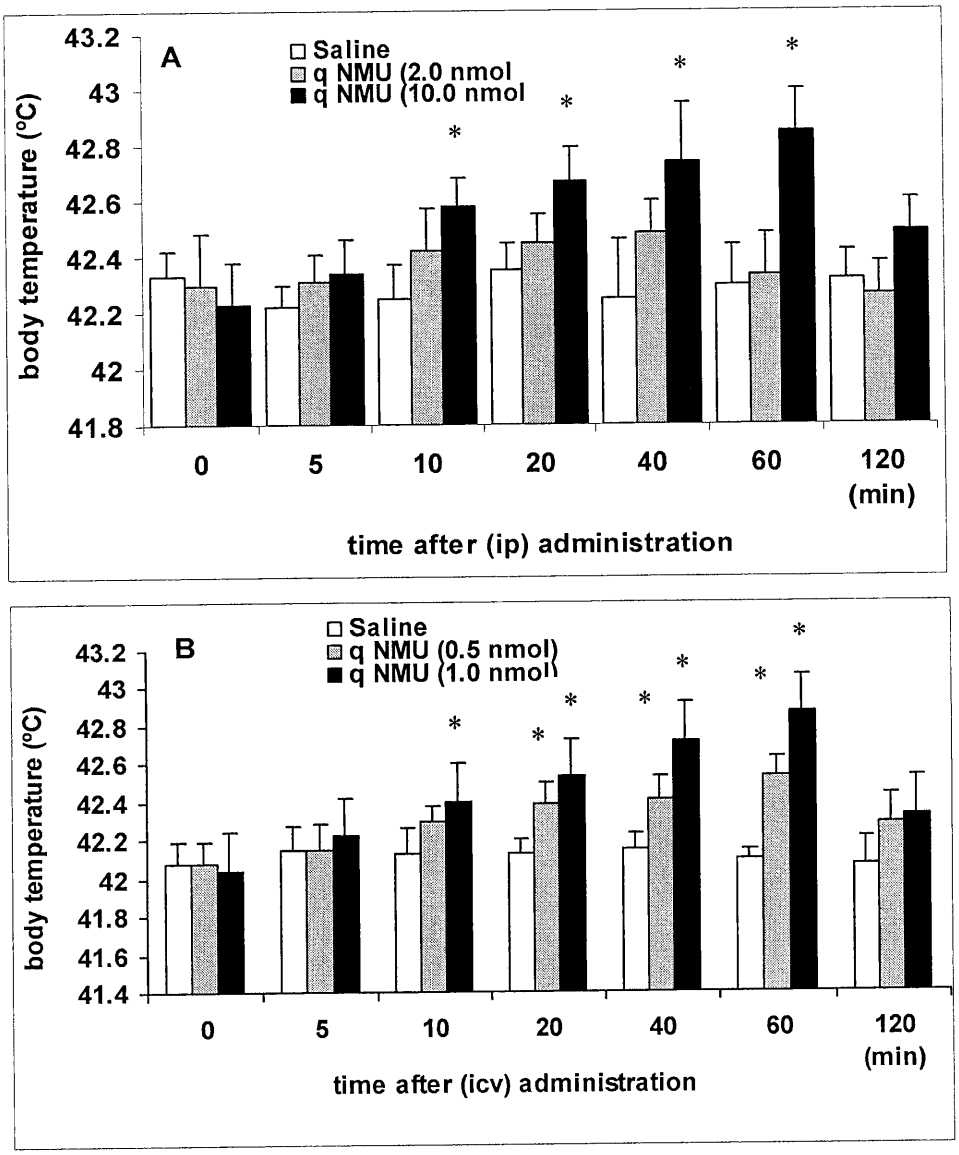

Figure 7. The effect of (A) ip administration of saline, 2.0 or 10.0 nmol, or (B) icv administration of saline, 0.5 or 1.0 nmol of Japanese quail NMU on body temperature in Japanese quail. Each bar and vertical line represents the mean ± SEM (n = 12). *P < 0.05 vs the saline-treated group.

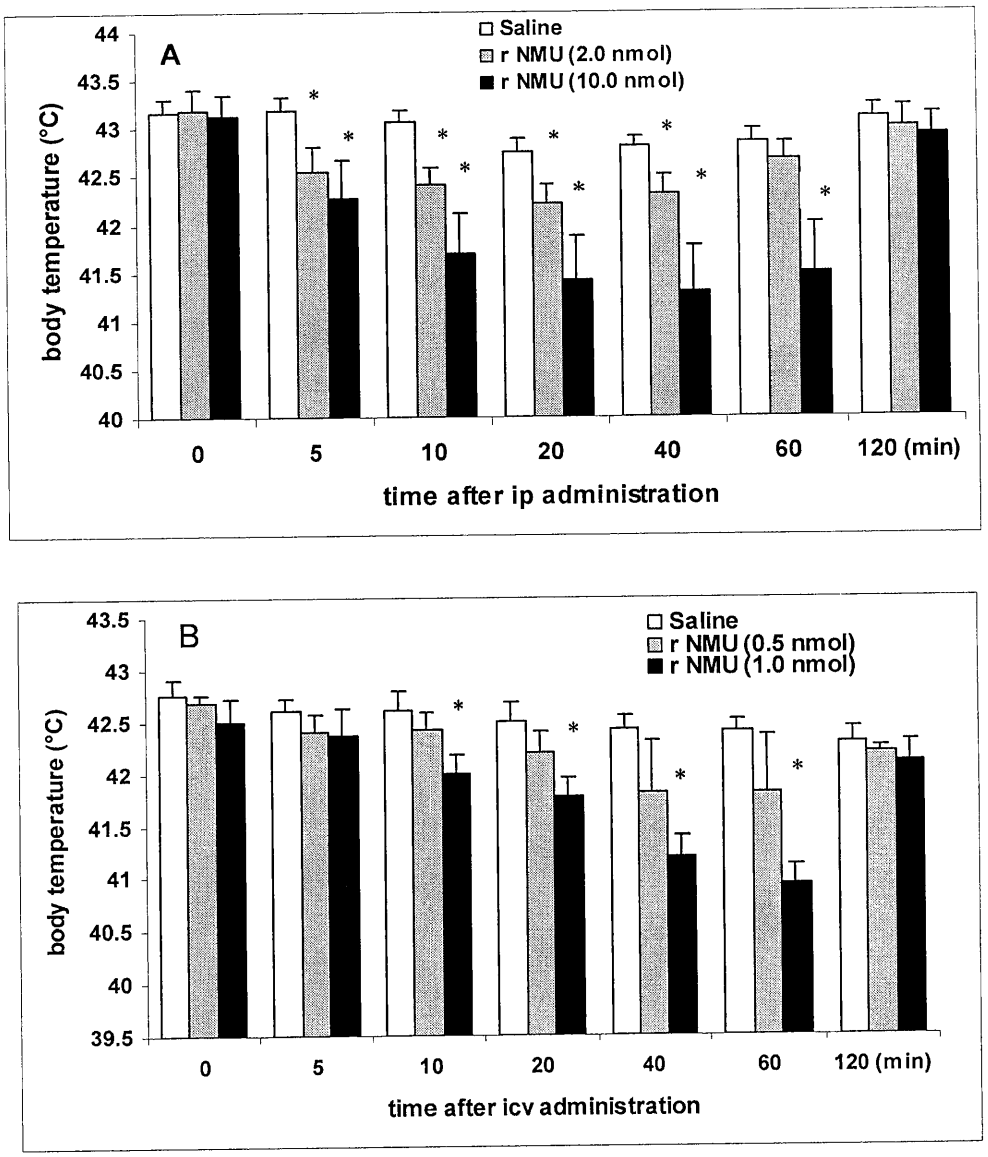

Figure 8. The effect of (A) ip administration of saline, 2.0 or 10.0 nmol, or (B) icv administration of saline, 0.5 or 1.0 nmol of rat NMU on body temperature in Japanese quail. Each bar and vertical line represents the mean ± SEM (n = 12). *$P < 0.05$ vs the saline-treated group.

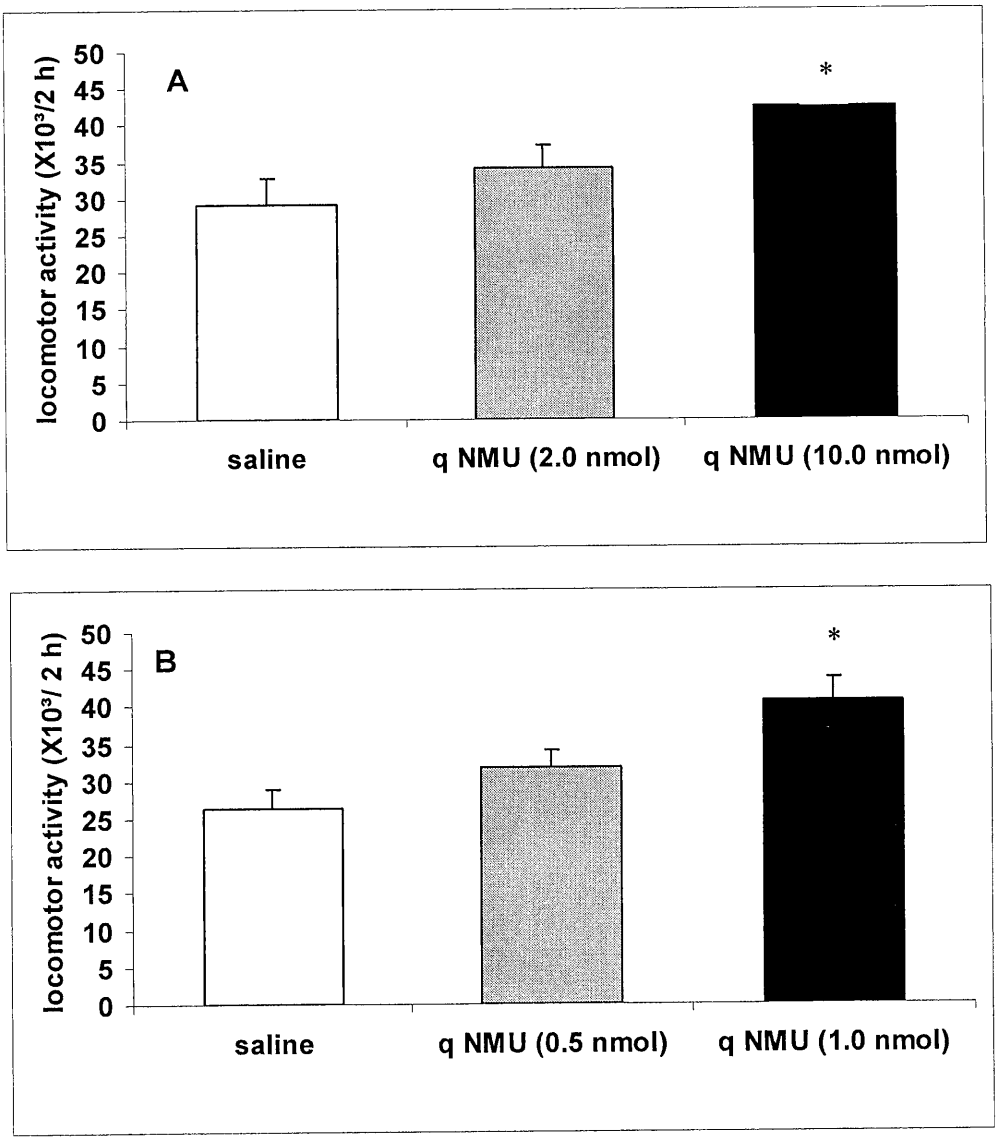

Figure 9. The effect of (A) ip administration of saline, 2.0 or 10.0 nmol, or (B) icv administration of saline, 0.5 or 1.0 nmol of Japanese quail NMU on gross locomotor activity in Japanese quail. Each bar and vertical line represents the mean ± SEM ($n = 8$). *$P < 0.05$ vs the saline-treated group.

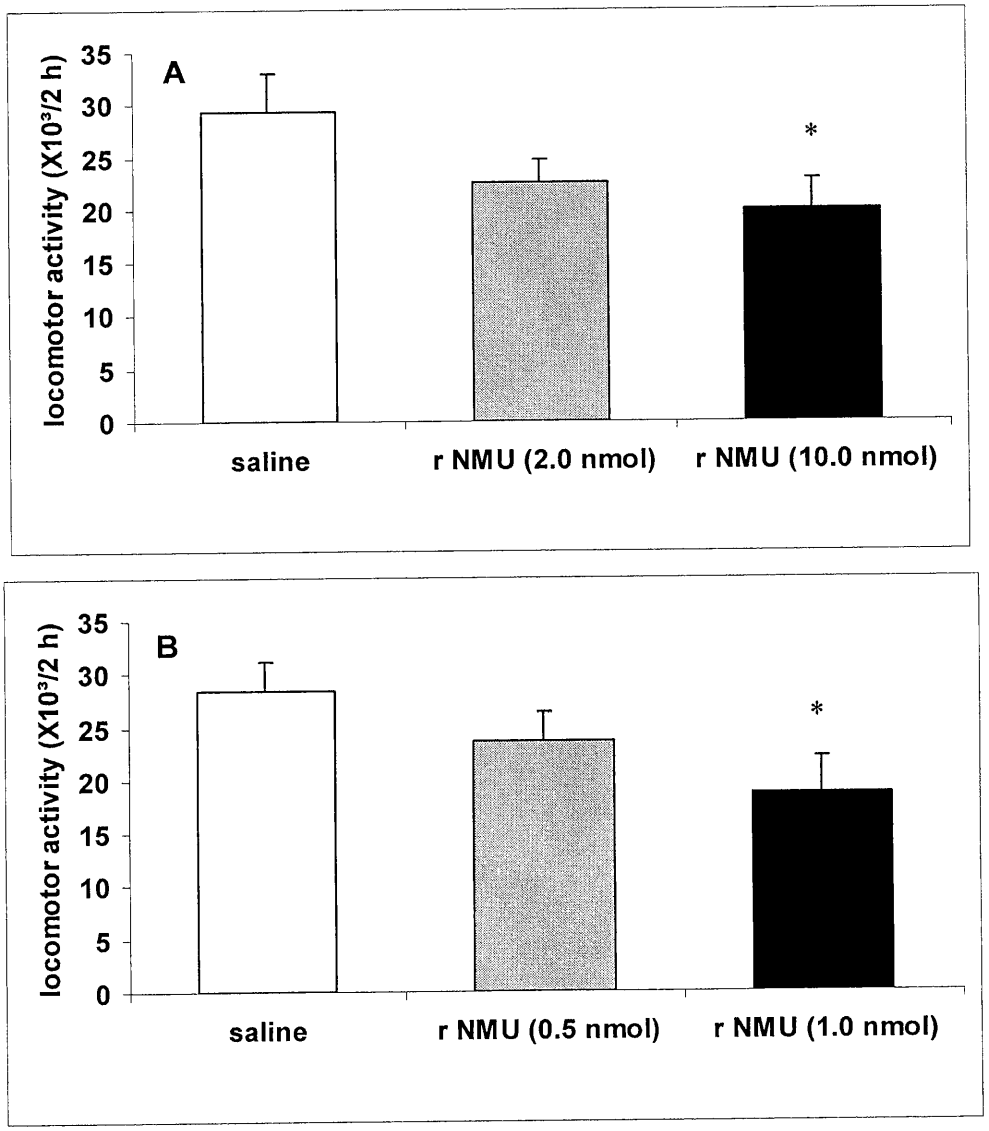

Figure 10. The effect of (A) ip administration of saline, 2.0 or 10.0 nmol, or (B) icv administration of saline, 0.5 or 1.0 nmol of rat NMU on gross locomotor activity in Japanese quail. Each bar and vertical line represents the mean ± SEM (n = 8). *P < 0.05 vs the saline-treated group.

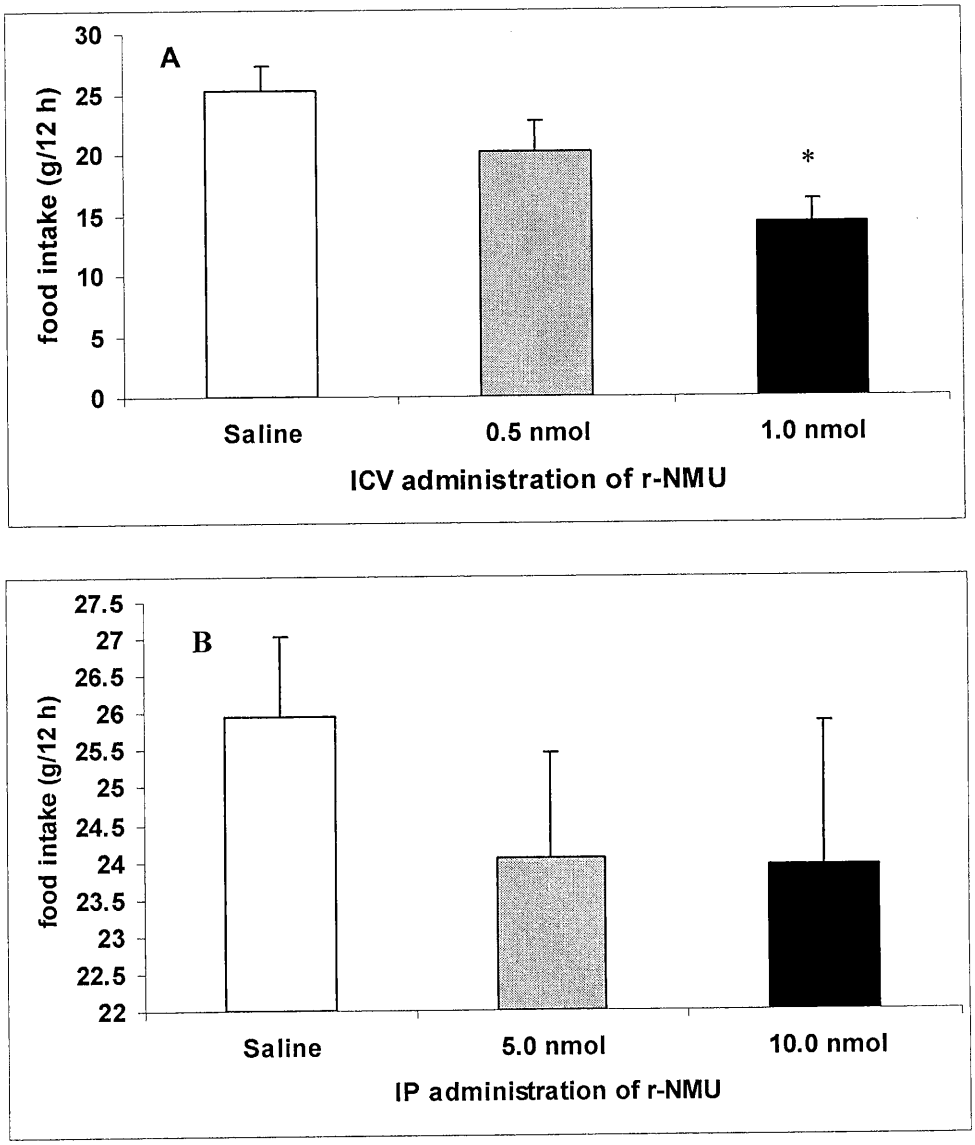

Figure 11. The effect of (A) icv administration of saline, 0.5 or 1.0 nmol of rat NMU, and (B) ip injection of saline, 5.0 or 10.0 nmol of rat NMU on food intake during the dark period in rats. Each bar and vertical line represents the mean ± SEM (n = 6). *P < 0.05 vs the saline.

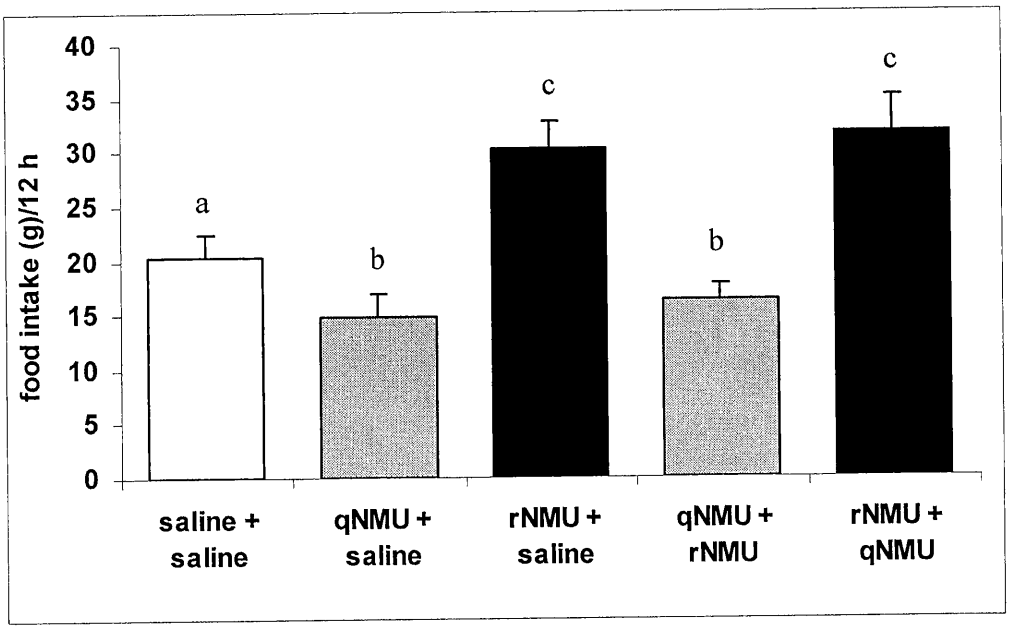

Figure 12. The effect of pretreatment with rat or quail NMU on the quail and rat NMU-induced suppression and stimulation of food intake in Japanese quail. Each bar and vertical line represents the mean ± SEM (n = 6). Columns of different letters are significantly different. $P < 0.05$ vs the saline-saline treated group.

1.4. Discussion

The effects of feeding-related peptides on food intake in birds have thus far been examined by icv injections into 1-day-old chicks (Ando *et al.*, 2000; Furuse *et al.*, 1997a, 2000; Kawakami *et al.*, 2000a, 2000b). It has yet to be established whether 1-day-old chicks can control the central regulation of various physiological functions, such as feeding, body temperature, and the sleep–wake cycle. In the study presented here, therefore, I used adult Japanese quails, which have an almost constant daily food intake and body temperature.

NMU mRNA was expressed in many central and peripheral tissues in these birds with a special abundance in the interstitial tract and spinal cord. These results are almost identical to those gleaned from rats (Austin *et al.*, 1994; Howard *et al.*, 2000). The high homology between the amino acid sequences of Japanese quail and chicken NMU (Domin *et al.*, 1992) suggests conservation between bird species. On the other hand, with the exception of five amino acid residues at the C-terminal region, there was little homology between the NMU of Japanese quail and rat (Minamino *et al.*, 1988). Although it is thought that the C-terminal region of NMU is the biologically active region of the peptide, and it is certainly widely conserved among species including dog (O'Harte *et al.*, 1991), rabbit (Kage *et al.*, 1991), frog (Domin *et al.*, 1989), human (Austin *et al.*, 1995), and chicken (Domin *et al.*, 1992), the findings of the present study demonstrate that rat and Japanese quail NMU have opposing effects

on feeding and body temperature in the Japanese quail. Therefore, although conserved, the biologically active region of this peptide may not necessarily have the same activity in different species.

Both icv and ip injections of quail NMU decreased food intake and increased both body temperature and gross locomtor activity in Japanese quail. These results are consistent with those obtained in rats following injections of rat NMU. Therefore, these results indicate that NMU acts both anorectically and catabolically in avian species as well as in mammals.

The icv or ip administration of rat NMU resulted in a significant increase in food intake and body weight gain, concomitant with transient decrease in body temperature and decrease in gross locomotor activity (Fig. 4, 5, 8, 10) in Japanese quail, suggesting that rat NMU acts both orexigenically and anabolically in Japanese quail. These reverse effects (compared with Japanese quail NMU) indicate that rat NMU decreases energy expenditure in this bird. The fact that the body weight gain was more prominent than the change in food intake during the 12 h following ip administration of NMU (Fig. 4A, 5A) and the significant decrease in gross locomotor activity may be due to both the orexigenic and anabolic actions of rat NMU. I presume that the rat NMU might act as an antagonist by competing for the endogenous Japanese quail NMU receptors. Pretreatment with either rat or quail NMU blocked the effect of subsequent treatment with either quail or rat NMU, respectively, on food intake. This

observation indicates that the NMU administered first occupied the NMU receptors so that the subsequently administered NMU could not bind to them. If this is the case, then these results suggest strongly that endogenous NMU plays an important role in the regulation of food intake and body temperature under normal conditions. However, binding studies would be required to show that the two peptides are antagonising at the same receptor.

How NMU affects food intake in rats remains to be elucidated. NMUR2 is abundant in the paraventricular nucleus, which is involved in satiety signaling (Hanada *et al.*, 2003). Recently, it is demonstrated that the absence of NMU-induced suppression of food intake in corticotrophin releasing hormone (CRH)-knockout mice (Hanada *et al.*, 2003). NMU-induced increases in oxygen consumption and body temperature were also attenuated in these mice. As CRH is synthesized predominantly in the paraventricular nucleus, these results suggest that quail NMU plays a role in feeding behavior and catabolic functions via CRH. The icv injection of CRH into chicken suppressed food intake, as it does in rats (Furuse *et al.*, 1997b). Further studies are required to elucidate the mechanisms underlying the action of NMU in birds.

It has been reported that there are many discrepancies in feeding regulation between mammals and avian species. Although orexin-A, orexin-B (Szekely *et al.*, 2002), motilin (Garthwaite, 1985; Rosenfeld & Garthwaite, 1987), melanin-concentrating hormone (Qu *et al.*, 1996;

Rossi *et al.*, 1997), ghrelin (Nakazato *et al.*, 2001), and galanin (Akabayashi *et al.*, 1994) have been reported to stimulate food intake in rats, these peptides failed to either stimulate or inhibit feeding in the neonatal chick (Ando *et al.*, 2000; Furuse *et al.*, 1999a, 2001). It is possible that the differences between rats and neonatal chicks may be due to antagonistic actions of the rat peptide in the neonatal chick, since the chick experiments were performed using rat peptides. On the other hand, it has been reported that cholecystokinin (Gibbs *et al.*, 1973), glucagon-like peptide-1 (Tang-Christensen *et al.*, 1996; Turton *et al.*, 1996), CRH (Benoit *et al.*, 2000), histamine (Lecklin *et al.*, 1998), and α-melanocyte stimulating hormone (Rossi *et al.*, 1998; Wirth *et al.*, 2001) suppress feeding in rats. The suppression of food intake by these peptides has also been observed in the chicken (Furuse *et al.*, 1997a, 1999b, 2000; Kawakami *et al.*, 2000a, 2000b). Therefore, it may be that the mechanisms underlying the inhibition of feeding are well conserved in chicken, but those underlying the stimulatory effects are not.

I do not know why the effect of NMU on body temperature is shorter than its effect on food intake. Further studies are required to elucidate the mechanisms underlying the action of NMU in avian species. After either ip or icv administration of NMU, changes in feeding may produce a transient change in body temperature. The mechanism of this transient thermal change is still unclear, but may be related to the changes in metabolism observed after NMU

administration because the regulatory center of body temperature is located close to feeding center in same hypothalamus. In addition, 2-hour food restriction every day in rats induced anticipatory increase of body temperature (Boulos & Terman, 1980), suggesting that both regulatory mechanisms for temperature and feeding may link each other but, I have no data concerning about the shorter effect on body temperature than the effect on food intake in avian species.

Due to the abundant expression of NMU mRNA in the interstitial tract, it might be that NMU acts peripherally through a receptor which may be found in the duodenum or jejunum or stomach either muscular or glandular part. I think the peripheral distribution of NMU mRNA and NMU receptors could explain any differences in the results.

In conclusion, endogenous NMU plays an important role in the regulation of food intake and body temperature in avian species. The Japanese quail is thought to be a good model with which to examine the regulation of feeding by the central or peripheral administration of test substances.

CHAPTER 2

Effect of ghrelin on feeding regulation in the Japanese quail

Abstract

When rat or human ghrelin is administered to chickens or rats it stimulates the release of growth hormone; however, its effects on food intake differ between the two species. To investigate this discrepancy, plasma ghrelin concentrations before and after food intake were measured and the effects of central (icv) and peripheral (ip) injections of various ghrelin doses on food intake and body temperature in the Japanese quail were determined. An immunohistochemical study, performed with an antibody that recognizes the N-terminal region of ghrelin, revealed that immunostained cells were located in the stomach, but they were few in number compared with what is usually observed in rats and other animals. In control quails, plasma ghrelin levels were significantly increased in the fasting state; subsequent feeding produced a reduction, suggesting that ghrelin may act as an orexigenic signal in Japanese quails as well as mammals. Food intake was stimulated by ip, but not icv, injections of small doses of ghrelin, whereas both ip and icv injections of larger doses inhibited feeding. A large dose of ghrelin also increased body temperature. These results suggest that an increase in peripheral ghrelin may act as a hunger signal to induce food intake through stimulation of the afferent vagal nerve in the Japanese quail, as in rats, whereas central ghrelin may inhibit feeding in different ways in the two species.

2.1. Introduction

Ghrelin, a novel 28-amino acid peptide, was originally isolated from the stomachs of rats and humans as an endogenous ligand for the growth hormone (GH) secretagogue receptor (GHS-R) (Kojima *et al.*, 1999). It has a unique structure, containing a Ser^3 residue that is modified by *n*-octanoic acid. This octanoyl modification is essential for receptor binding and the subsequent expression of biological activity (Kojima *et al.*, 1999). Ghrelin has also been isolated from chickens. This form is composed of 26 amino acids, has an octanoylated Ser^3 and shows 54 % total sequence identity and 100 % N-terminal-region identity [Gly1-Pro7] with rat and human ghrelin (Kaiya *et al.*, 2002). In addition, two isoforms of the chicken GHS-R (cGHS-R1a and cGHS-R1aV) have been generated by alternative splicing of a primary transcript. cGHS-R1a shows strong amino acid sequence identity (68 %) with the corresponding parts of the mammalian GHS-R1a cDNA product, while cGHS-R1aV lacks the transmembrane-6 domain due to a 48-bp deletion (Tanaka *et al.*, 2003).

Ghrelin stimulates the release of GH from the pituitary gland both *in vivo* and *in vitro*; the *n*-octanoyl modification is essential for this activity (Kojima *et al.*, 1999). In addition, central and peripheral injection of ghrelin stimulates food intake in rats (Nakazato *et al.*, 2001; Tschop *et al.*, 2000). The central effect is mediated by neuropeptide Y and agouti-related peptide secretion from the arcuate

nucleus in the hypothalamus (Nakazato *et al.*, 2001). On the other hand, the appetite-stimulating activity of peripheral ghrelin is produced via stimulation of the afferent vagal nerve (Date *et al.*, 2002).

Although accumulating evidence suggests that ghrelin plays many important roles in various physiological functions in rats and humans, for example, fetal development (Hayashida *et al.*, 2002), gastric acid secretion (Date *et al.*, 2001) and improvements in cardiovascular function (Nagaya *et al.*, 2001), little is known about ghrelin in non-mammalian vertebrates. In avian species, ghrelin mRNA is expressed predominantly in the stomach, where it is present in the proventriculus but absent in the gizzard (Wada *et al.*, 2003). In the chicken, administration of either human or chicken ghrelin stimulates GH release (Ahmed & Harvey, 2002; Kaiya *et al.*, 2002) but strongly inhibits feeding (Furuse *et al.*, 2001; Saito *et al.*, 2002a). In particular, central (intracerebroventricular, icv) injection of chicken ghrelin suppresses food intake in neonatal chicks (Saito *et al.*, 2002a), the opposite effect to that seen in rats.

To investigate these discrepant effects of ghrelin on feeding, I examined the distribution of ghrelin immunoreactive cells in the stomach, measured plasma ghrelin concentrations before and after food intake, and determined the effects of both icv and peripheral (intraperitoneal, ip) injection of ghrelin on food intake, in the Japanese quail. If ghrelin is indeed orexigenic in this species, it would expect

plasma ghrelin levels to be increased by fasting and decreased by subsequent feeding, as is the case in mammals.

In addition to its effects on feeding, it has been reported that central injection of ghrelin into rats transiently reduced the core body temperature (Lawrence *et al.*, 2002) and the temperature of brown adipose tissue (Yasuda *et al.*, 2003). Therefore, it is highly probable that ghrelin regulates adiposity by influencing both energy intake and energy expenditure. Although neonatal chicks have been reported to become hyperactive within 30 min of an icv injection of ghrelin (Saito *et al.*, 2002b), and to exhibit sleep-like behavior thereafter (Tachibana *et al.*, 2001), to the best of our knowledge there are no published reports concerning thermoregulation by ghrelin in adult avian species. Therefore, I also measured the effects of peripheral and central ghrelin on body temperature in Japanese quails.

2.2. Materials and methods

2.2.1. Animals

Adult male Japanese quail (*Coturnix coturnix japonica*) were housed in individual net cages (W: 14 x L: 26 x H: 17 cm) in a room with a 12-h light (300 Lx) /12-h dark (dim light, 25 Lx) period (lights on at 07:00 hours) and a temperature of 28 ± 1 °C, and were given free access to food and water. Before the feeding experiment, the birds were weighed and assigned to an experimental group (6 birds in each group) based on their body weight. The average body weight (110–120 g) in each group was kept as uniform as possible.

To examine the orexigenic or anorexic effect of ghrelin, rat ghrelin (Peptide Institute, Osaka, Japan) or 0.9 % saline (vehicle control) was administered ip or icv at either 0700 h (i.e., during the birds' active phase) or 19:00 h (i.e., during the birds' resting phase). The doses injected ip were 0.5 (1.66 μg), 1.0 (3.31 μg) and 3.0 (9.94 μg) nmol/200 μl saline, while the doses injected icv were 0.05 (0.16 μg), 0.5 (1.66 μg) and 1.0 (3.31 μg) nmol/10 μl saline.

These experiments were performed in duplicate. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

2.2.2. Surgical procedures

For implantation of the icv cannula, each bird was anesthetized with 5 % sodium pentobarbital (1.4 μl/g body weight) and placed in a stereotaxic frame. The method of cannulation had been reported in

Chapter 1. The birds were returned to their individual cages and allowed to recover for at least 4 days. They were acclimatized to handling every day before the start of the experiments.

The icv injections were administered through the implanted guide cannulae without anesthesia or restraining of the birds.

At the end of the experiments, proper placement of the cannulae was verified by administering Evans Blue dye (10 μl), followed by sacrifice and brain sectioning (20 μm intervals). Data for birds lacking dye in the third ventricle were excluded from the analysis.

2.2.3. Measurement of food intake

Food consumption in free-fed birds was determined 2, 4 and 12 h after administration of ghrelin or saline by measuring the disappearance of food from the pre-weighed feeder. Any spillage was also collected and weighed.

2.2.4. Measurement of body temperature

The quails' body temperature was measured 0 (before injection), 5, 10, 20, 40, 60 and 120 min after administering ghrelin or saline at the doses stated above ($n = 6$ in each group) at 1000 h, using a previously reported method (Bayle *et al.*, 1974). Briefly, the temperature was measured electronically with a small sensor (measurable range: 25–50 °C; measurement error: 0.05 °C) connected to a line (outer diameter 0.7 mm; length 45 cm) which is connected to the monitor body. The sensor tip was inserted into the cloaca, and part of the line was fixed

to the bird's body, with the digital signal transferred to the monitor body.

2.2.5. Measurement of plasma ghrelin

Plasma acyl-ghrelin concentrations were determined by radioimmunoassay using a specific antibody that recognizes the N-terminal region, including the octanoylated Ser^3 residue (Hosoda *et al.*, 2000). Sixteen Japanese quails were fasted for 24 h (from 1000 h): 8 were sacrificed by decapitation after 24 h of fasting; the remaining 8 were supplied with food after the fast and then sacrificed 3 h later. Untreated, free-feeding quails were sacrificed at 1000 h and 1300 h as controls.

Following decapitation, blood from the neck was drawn into chilled polypropylene tubes containing a proteinase inhibitor, aprotinin (500 kIU/ml; Sigma-Aldrich, St. Louis, USA), and 2Na-EDTA (2 mg/ml), then centrifuged immediately before adding 0.1 N HCl (10 % of the plasma volume). The samples were stored at –80 °C until required.

For determination of ghrelin levels, the plasma was diluted in 0.9 % saline and applied to a Sep-Pak C-18 cartridge (Waters, Milford, MA, USA) that had been pre-equilibrated with 0.9 % saline. The cartridge was washed with saline and 10 % CH_3CN solution containing 0.1 % trifluoroacetic acid (TFA). The adsorbed peptides were eluted with 60 % CH_3CN solution containing 0.1 % TFA,

lyophilized and radioimmunoassayed. The intra- and interassay coefficients of variation were 6.7 % and 3.2 %, respectively.

2.2.6. Immunohistochemistry

Adult free-fed male Japanese quails were decapitated and their stomachs removed. The tissues were immediately rinsed with saline, fixed in 4 % paraformaldehyde and 0.2 % picric acid in 0.1 M phosphate buffer for 2 days, and then incubated in 0.1 M phosphate buffer containing 20 % sucrose for 24 h at 4 °C. The glandular stomach was cut into 12-μm-thick sections with a cryostat at –20 °C. The sections were thaw-mounted on gelatin-coated glass slides and air-dried for 10 min. After pretreatment with 0.3 % hydrogen peroxide for 1 h to inactivate endogenous peroxidases, the sections were incubated with normal goat serum for 1 h to block any nonspecific binding. The polyclonal antibody used in this study was raised in rabbits against the N-terminal fragment of rat ghrelin. Details of the preparation and characterization of the antibody have been described previously (Date *et al.*, 2000). The rat anti-ghrelin antibody (antiserum no. G606) specifically recognizes ghrelin with *n*-octanoylated Ser^3, and does not recognize des-acyl ghrelin. The final dilution of anti-ghrelin antiserum used in the immunohistochemistry was 1/10,000. The sections were incubated for 1 day with the anti-ghrelin antiserum at 4 °C. After washing in phosphate-buffered saline, the slides were stained at room temperature by the avidin-biotin-peroxidase complex method (Vectastain Elite ABC Kit; Vector

Laboratories, Burlingame, CA, USA) with a diaminobenzidine substrate kit (Vector Lab). Control studies were carried out with anti-ghrelin antiserum that had been preincubated with 10 μg of synthesized rat ghrelin. For immunofluorescence staining for ghrelin, after incubation with the anti-rat ghrelin antiserum, a part of slides were incubated with Alexa 546-labeled goat anti-rabbit IgG antibody (Molecular Probes, Inc., dilution 1:400). Samples were observed under an Olympus AX-70 fluorescence microscope (Olympus Co. Ltd., Tokyo, Japan).

2.2.7. Statistical analysis

All results are expressed as the mean ± SEM. Data were analyzed by analysis of variance and the *post hoc* Fisher's test.

3. Results

2.3.1. Food intake

In the ip administration group, 3.0 nmol ghrelin significantly ($P<0.05$) decreased food intake during both the light (Fig. 3A) and dark periods (Fig. 3B) compared with the saline-control group. Conversely, 0.5 and 1.0 nmol ghrelin stimulated feeding (Fig. 3A, B). This orexigenic action of small doses of ghrelin was more evident during the dark period (Fig. 3B) than the light period (Fig. 3A). During the first 2 h after ip injection, ghrelin at dose of 1.0 nmol stimulated food intake more potently during the dark period (Fig. 3B) whereas 3.0 nmol inhibited food intake more markedly during the light period (Fig. 3A). Also a significant decrease in body weight gain was measured at 4 and 12 h after ip injection of 3 nmol during both the light and dark period but more markedly during the dark period. Body weight gain decreased more than food intake did. By 12 h, a large decrease of body weight was observed in the 3.0 nmol treated group relative to the saline-injected group during both the light and dark periods (Fig. 4A, B).

No significant increase in food intake was observed after icv injections of ghrelin (Fig. 5A, B). Indeed, 0.5 and 1.0 nmol ghrelin inhibited food intake during both the light and dark periods (Fig. 5A, B). No significant difference was observed between the saline group and the 0.05 nmol ghrelin-treated group (Fig. 5A, B).

The effects of smaller doses of ghrelin (0.005 nmol icv and 0.01 nmol ip) were examined, but these doses effected no significant change in food intake (n = 6; data not shown). Also a significant decrease in body weight gain was measured at 2, 4 and 12 h after icv injection of 0.5 and 1.0 nmol during both the light and dark periods but more markedly during dark period. Body weight gain decreased more than food intake did. By 12 h, a large decrease of body weight was observed in the 1.0 nmol treated group relative to the saline-injected group during both the light and dark periods (Fig. 6A, B).

2.3.2. Body temperature

Both ip and icv injections of ghrelin increased body temperature in a dose-dependent manner (Fig. 7). At 3.0 nmol ip, ghrelin significantly (P<0.05) increased body temperature 10, 20 and 40 min after treatment. However, no such change was observed with 0.5 and 1.0 nmol ip (Fig. 7A). Similarly, 0.5 and 1.0 nmol ghrelin icv produced a significant (P<0.05) increase in body temperature 10–60 min and 40–60 min after injection, respectively (Fig. 7B). Although 0.05 nmol ghrelin also caused an increase in body temperature, the change was not significantly different from that seen with saline alone (i.e., control birds).

2.3.3. Plasma ghrelin

Plasma ghrelin levels increased about 5-fold in quails that had been fasted for 24 h compared with the free-feeding birds. The levels were decreased 3 h after refeeding (Fig. 8). Ghrelin levels differed

significantly between the two groups; levels in both groups were also significantly different from those in the free-feeding quail.

2.3.4. Immunohistochemistry

Anti-rat ghrelin immunolabeling of the quail gastrointestinal tract revealed the presence of immunostained cells in the proventriculus (glandular stomach). Ghrelin-immunoreactive cells were not so abundant in the quail stomach compared with that has been reported in the stomach of rat (Hayashida *et al.*, 2001) or chicken (Wada *et al.*, 2003), but were distributed widely and scattered in the mucosal layer of the proventriculus (Fig. 9A). Although the immuno-stained cells are small and like the erythrocyte, the shapes of erythrocyte in avian species are ellipse, nucleated and erythrocyte is not stained by immunofluorescence. Therefore, small black spot in (Fig. 9A, C) is immunostained cells for ghrelin. No immunostained cells were observed in sections that had been incubated with synthetic-ghrelin-preadsorbed anti-ghrelin antiserum (Fig. 9 B). Also ghrelin immunopositive cells have been shown by immnofluorescence staining (Fig. 9D). These observations are consistent with the distribution of ghrelin-immunoreactive cells that has been described previously for the hatching and adult chicken, that is, in the mucosal layer of the proventriculus but not in the myenteric plexus (Wada *et al.*, 2003). In the present study, I have demonstrated the existence of ghrelin-immunopositive or immunoreactive cells in the proventriculus, a region that is homologous to the stomach fundus in other species,

and in the duodenum of the Japanese quail. However, it is likely that number of immunostained cells in Japanese quail is few in comparison with those in rats (Hayashida *et al.*, 2001) and chicken (Wada *et al.*, 2003).

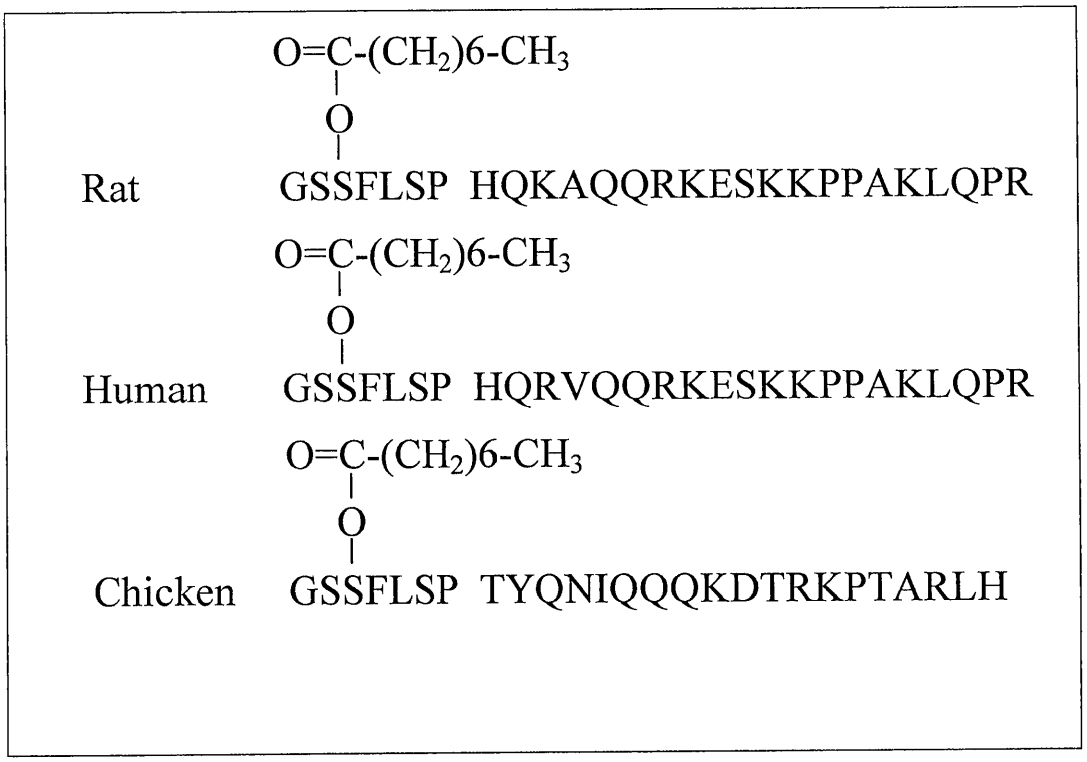

Figure 1. Comparison of amino acid sequence of ghrelin in Rat, Human, and Chicken. (Kaiya et al., Endocrinology, 2002)

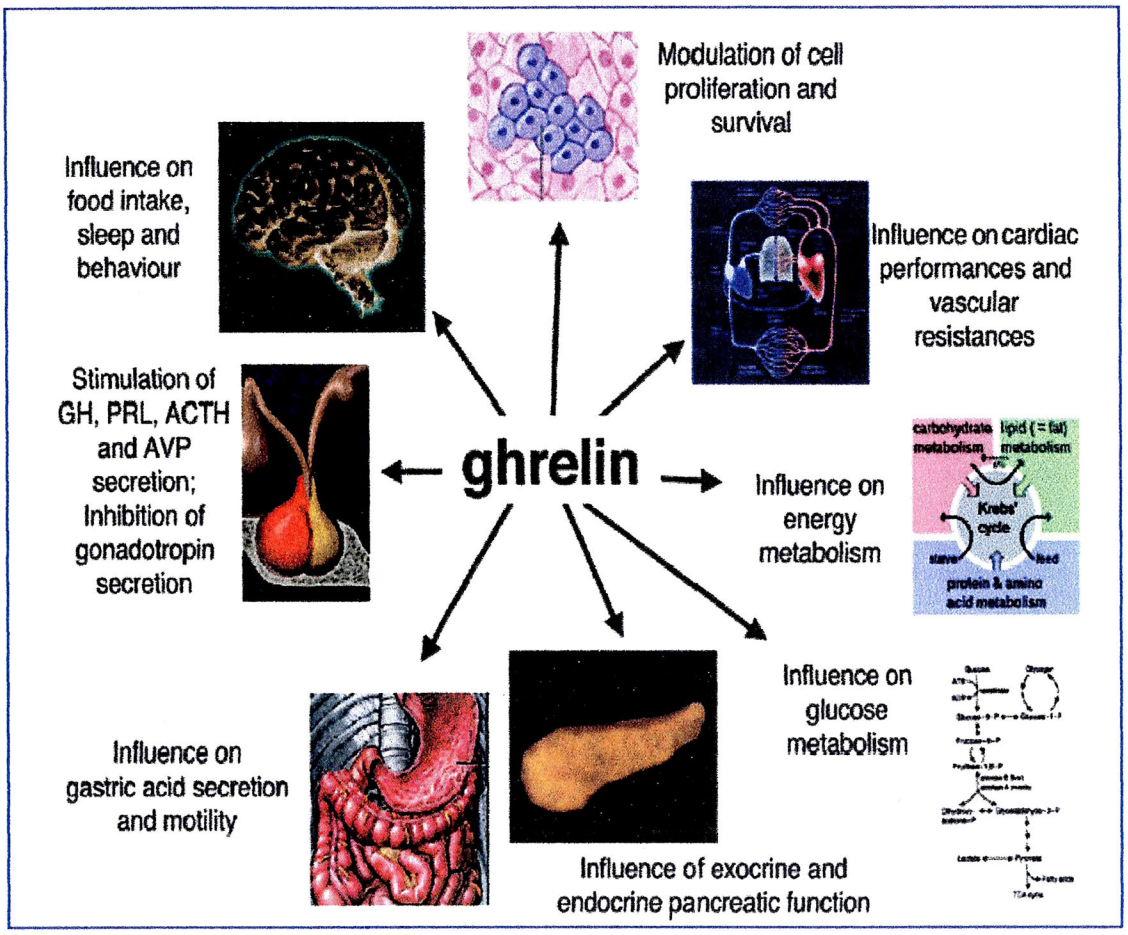

Figure 2. Schematic diagram shows some known biological activities of ghrelin. (van der Lely *et al.*, 2004).

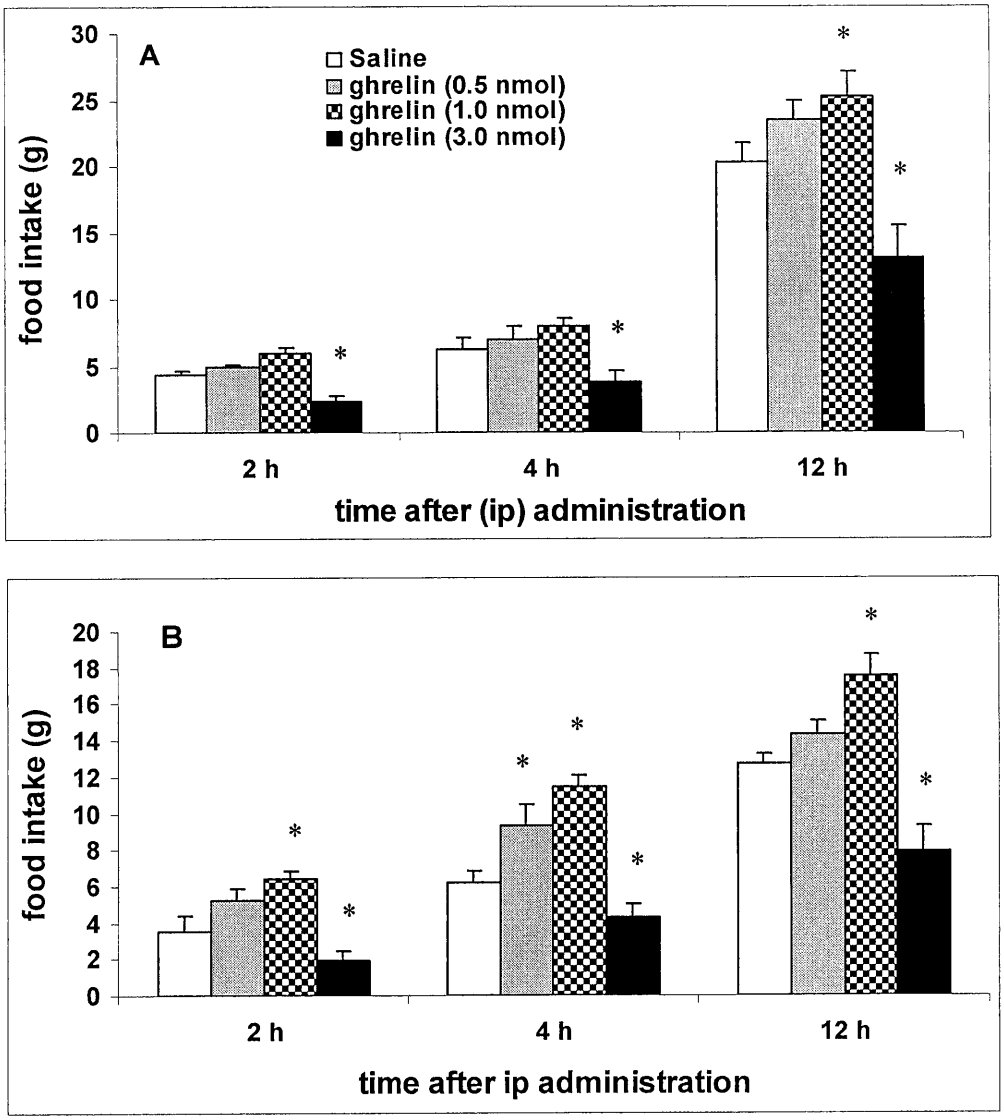

Figure 3. Effect of intraperitoneal (ip) administration of ghrelin on food intake in the Japanese quail. Saline (control), 0.5, 1.0 or 3.0 nmol ghrelin were injected ip at 0700 h (A) or 1900 h (B). Each bar and vertical line represents the mean ± SEM (n = 12). *Significantly different from the saline-treated group; $P<0.05$.

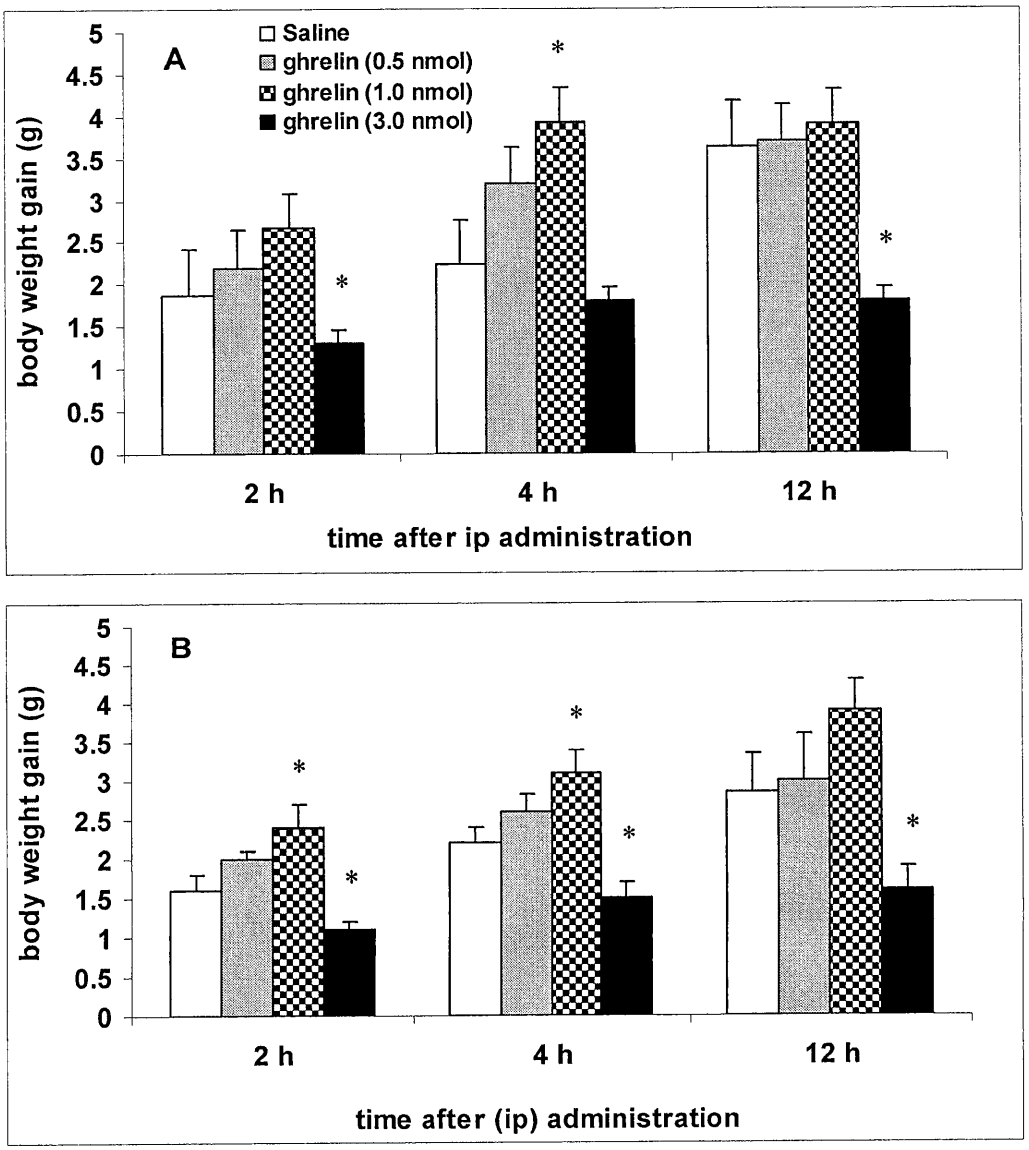

Figure 4. Effect of intraperitoneal (ip) administration of ghrelin on body weight change in the Japanese quail. Saline (control), 0.5, 1.0 or 3.0 nmol ghrelin were injected ip at 0700 h (A) or 1900 h (B). Each bar and vertical line represents the mean ± SEM (n = 12). *Significantly different from the saline-treated group; $P<0.05$.

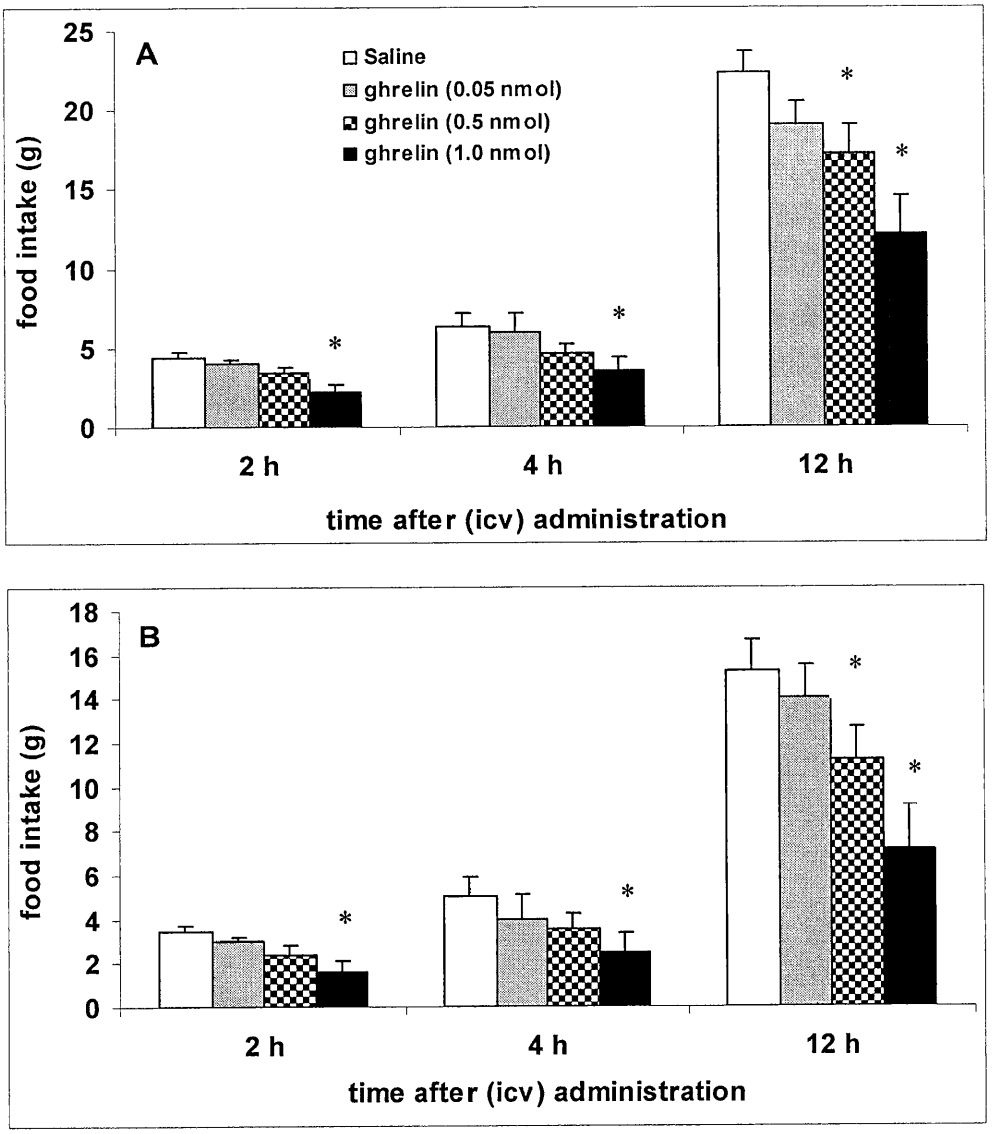

Figure 5. Effect of intracerebroventricular (icv) administration of ghrelin on food intake in the Japanese quail. Saline (control), 0.05, 0.5 or 1.0 nmol ghrelin were injected icv at 0700 h (A) or 1900 h (B). Each bar and vertical line represents the mean ± SEM (n = 12). *Significantly different from the saline-treated group; $P<0.05$.

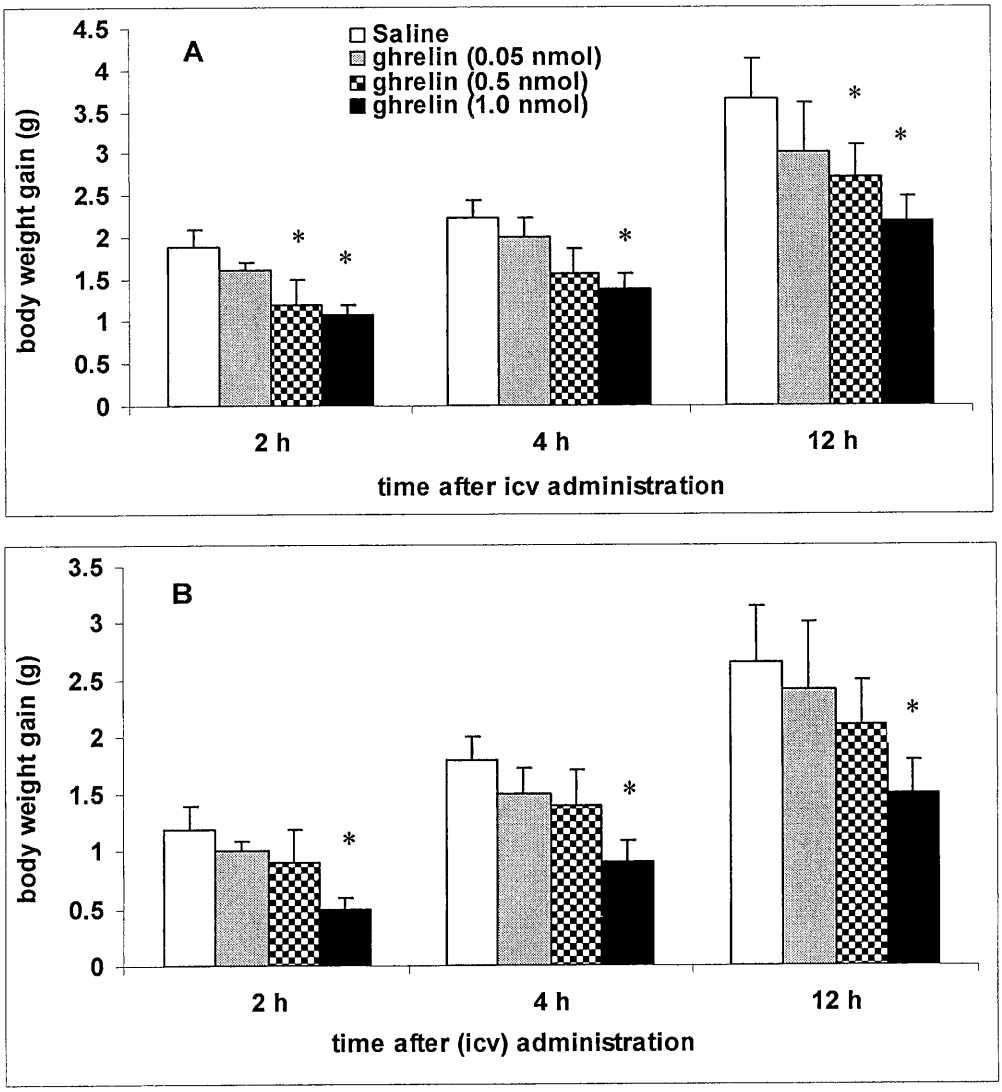

Figure 6. Effect of intracerebroventricular (icv) administration of ghrelin on body weight change in the Japanese quail. Saline (control), 0.05, 0.5 or 1.0 nmol ghrelin were injected icv at 0700 h (A) or 1900 h (B). Each bar and vertical line represents the mean ± SEM (n = 12). *Significantly different from the saline-treated group; $P<0.05$.

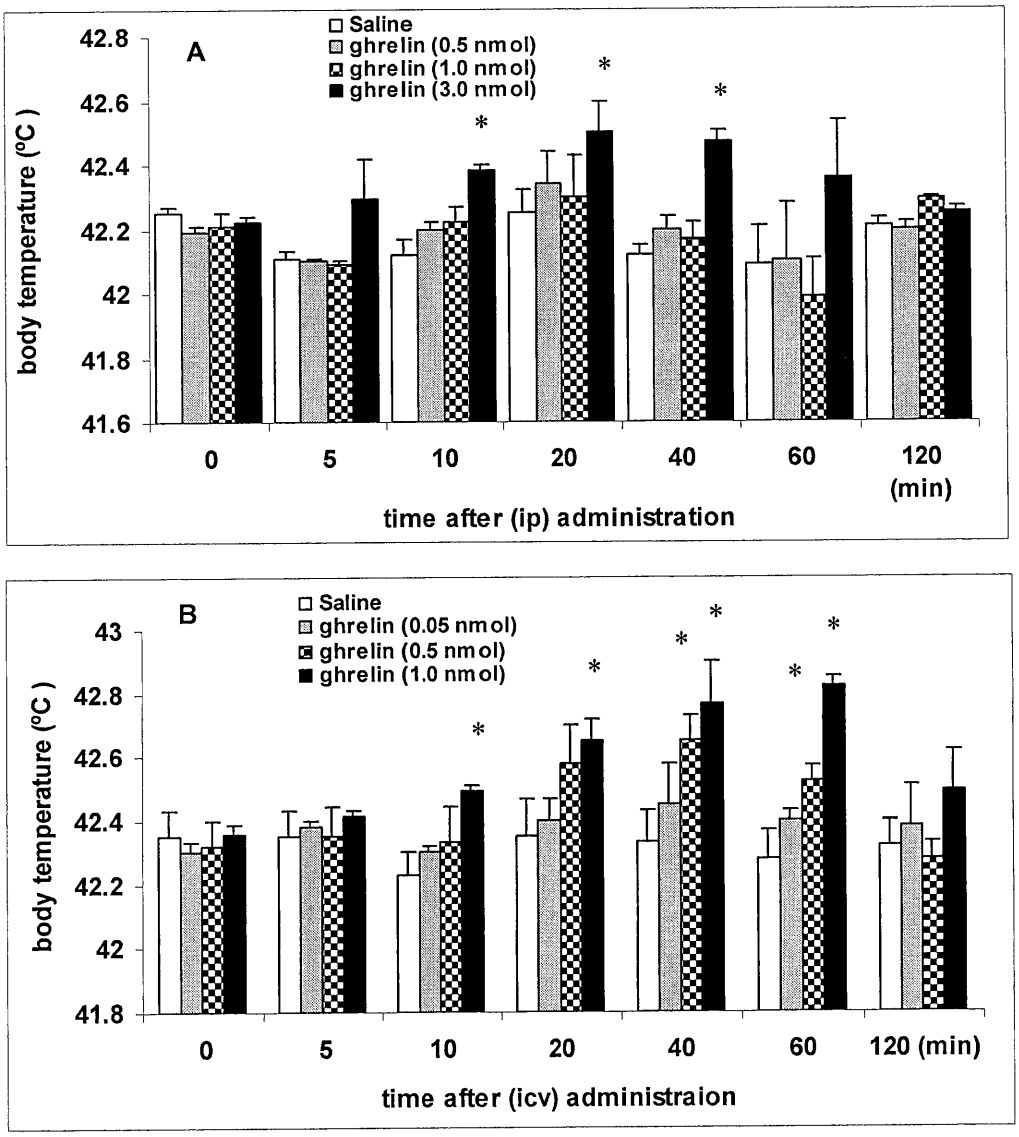

Figure 7. Effect of ip or icv administration of ghrelin on body temperature in the Japanese quail. Saline (control), 0.5, 1.0 or 3.0 nmol of ghrelin were injected ip (A), and saline (vehicle control), 0.05, 0.5 or 1.0 nmol ghrelin were injected icv (B) at 1000 h. Each bar and vertical line represents the mean ± SEM (n = 6). *Significantly different from the saline-treated group; $P<0.05$.

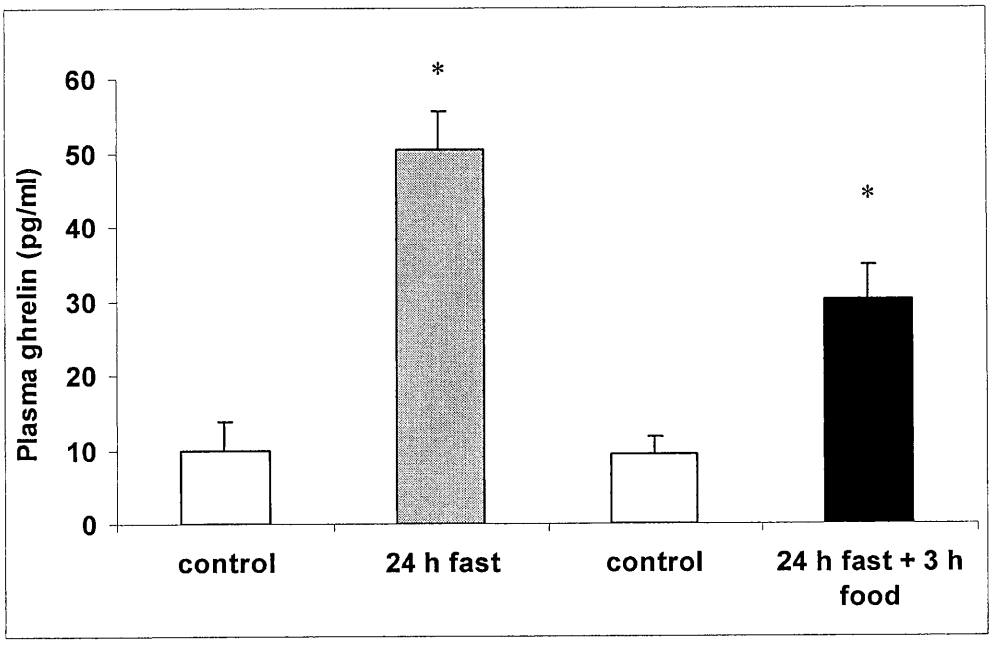

Figure 8. Plasma active ghrelin levels in free-feeding and 24-h fasted groups of Japanese quail. Sixteen Japanese quails were fasted for 24 h (from 1000 h), after which 8 were sacrificed (gray bar). The remaining 8 birds were supplied with food after the fast, then sacrificed 3 h later (black bar). Free-feeding animals were sacrificed at either 1000 h (left-hand white bar) or 1300 h (right-hand white bar) as controls. Each bar and vertical line represents the mean ± SEM (n = 8). *Significantly different from the saline-treated group; $P<0.05$.

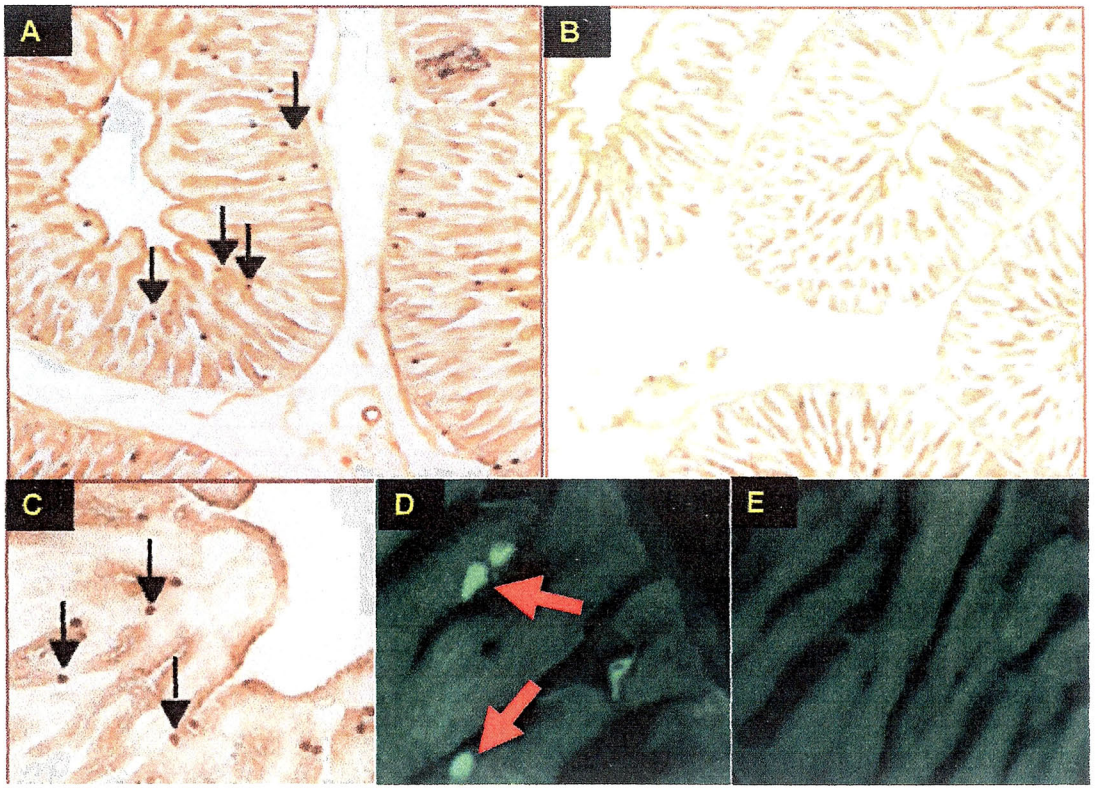

Figure 9. Localization of ghrelin-immunostained cells in the proventriculus of the Japanese quail. Ghrelin-immunopositive cells were observed in the mucosal layer of the proventriculus (A, C). Most of these cells (indicated by arrow), which are small and round (magnification: C), were found in the basal and middle regions of the mucosal layer. No immunostaining was observed in control sections (B), which were incubated with anti-ghrelin antisera that had been preadsorbed with 10 μg of synthesized rat ghrelin. Ghrelin-immunopositive cells by immunofluorescence staining (D). No ghrelin-immunopositive cells were observed by immunofluoresence in control section (E).

2.4. Discussion

The amino acid sequence of ghrelin in different species (Kaiya *et al.*, 2002) and the known biological activities (Van der Lely *et al.*, 2004) are illusterated in (Fig. 1 and Fig. 2), respectively. The finding that icv injection of rat ghrelin inhibits food intake in Japanese quails is in agreement with previous observations made in chickens as reported by (Furuse *et al.*, 2001; Saito *et al.*, 2002a). Since the amino acid sequence of rat ghrelin is quite different from that of the chicken hormone, except for the seven N-terminal residues (Kaiya *et al.*, 2002) (Fig. 1), rat ghrelin could possibly act as an antagonist of the chicken GHS-R, thus inhibiting the effect of endogenous chicken ghrelin. However, this inhibition might not be due to an antagonistic effect of rat ghrelin at the chick ghrelin receptor, since icv injections of chick ghrelin also inhibit food intake. Moreover, human or rat ghrelin can also cause an increase in GH secretion in the chicken. Therefore, avian ghrelin may act to inhibit food intake. However, if this is true, one would expect the pattern of plasma ghrelin secretion before and after feeding to be the reverse of that seen in rats. In the present study, contrary to the expectation, plasma ghrelin levels were increased 5-fold compared with those in free-feeding birds following a 24-h of fast. In addition, allowing the birds to feed for 3 h after the 24-h fast produced a decrease in their plasma ghrelin levels. These results suggest that increased peripheral ghrelin levels act as a hunger signal rather than a satiety signal. The present study also demonstrated that

ip administration of small doses (0.5 or 1.0 nmol) of ghrelin stimulates food intake; conversely, large doses (3.0 nmol) inhibit it (Fig. 3). However, icv ghrelin consistently decreased feeding. In agreement with this finding, previous studies have shown that neither rat nor chicken ghrelin has any transient stimulatory effect on food intake in neonatal chicks when administered centrally (Furuse *et al.*, 2001; Saito *et al.*, 2002a).

I do not know the reason for the discrepancy between the ip and icv effects of ghrelin on feeding regulation, or that between the effects of small and large ip doses of the peptide. However, there are at least two possible explanations. First, the mechanisms underlying the central and peripheral effects of ghrelin on feeding may differ, perhaps being anorexic and orexigenic, respectively. It has been shown that ghrelin can pass through the blood–brain barrier in rats (Banks *et al.*, 2002), so it may be that when the largest dose of ghrelin was injected ip in the present study, some of it reached central (arcuate nucleus) GHS-Rs through the blood–brain barrier, thereby inhibiting food intake. An analogous situation has been observed with neuropeptide YY (PYY) in rats; peripheral injections of PYY inhibited food intake, whereas icv injections increased it, suggesting that PYY acts both orexigenically and anorexically depending upon whether it is located centrally or peripherally (unpublished data). The existence of peripheral sensors for ghrelin is suggested by the finding that GHS-R mRNA is distributed in various peripheral organs, such as

the stomach and intestine, in the adult chicken (Tanaka *et al.*, 2003). Peripheral ghrelin acts via gastric vagal afferent nerves (Date *et al.*, 2002). The ghrelin signals from peripheral GHS-Rs may be converted to neurotransmitter-mediated signals in the nucleus of the solitary tract. This neurotransmitter could have the opposite effect to direct release of ghrelin in the hypothalamus. Second, ghrelin may act to down-regulate the GHS-R. GHS-Rs may become saturated following the administration (either ip or icv) of a large dose of ghrelin (Fig. 3 and Fig. 5). This might in turn cause a down-regulation of feeding stimuli, thus inhibiting food intake. The stimulatory effect of ghrelin on food intake was more marked during the dark period (resting phase) (Fig. 3B) than the light period (active phase) (Fig. 3A). The inverse was found following ip injections of 1.0 nmol ghrelin, which inhibited food intake over the 12-h light period but stimulated it during the 12-h dark period. This difference may be attributable to differences in the number of unsaturated receptors present when hungry (i.e., during the dark period) and sated (i.e., during the light period). It appears that relatively low levels of this peptide are sufficient to stimulate food intake in the Japanese quail, but further studies are required to confirm this. The number of ghrelin-immunostained cells in the Japanese quail was low compared with what has been observed previously for rat, cattle, pig, and horse (Hayashida *et al.*, 2001), and chicken (Wada *et al.*, 2003). It is

possible that relatively lower levels of this peptide are sufficient to stimulate food intake in the Japanese quail.

Ahmed and Harvey (2002) found that ghrelin immunoreactivity was present in the chicken hypothalamus, although not, as in rats, in the arcuate (infundibular) nucleus. Discrete parvocellular cells and neuronal fibers with ghrelin immunoreactivity were present in the anterior medial hypothalamus. This restriction of ghrelin immunoreactivity to the hypothalamus of chicks suggests that it evolved phylogenetically as a neuropeptide rather than as a gastrointestinal hormone. Since the parvocellular cells in the hypothalamus are thought to connect with portal blood vessels in the median eminence, the presence of ghrelin in the anterior medial hypothalamus suggests that it may be a hypophysiotropic releasing factor that stimulates pituitary GH release after secretion into the hypothalamo-hypophyseal circulation (Ramesh *et al.*, 2000).

Ghrelin injected either peripherally (Fig. 7A) or centrally (Fig. 7B) caused a transient, dose-dependent increase in body temperature, suggesting that it might affect energy expenditure in birds. After ip or icv administration of large doses of ghrelin, changes in feeding may produce a transient change in body temperature. The mechanism underlying this transient thermal change is unclear, but may involve changes in metabolism because the regulatory center for body temperature is located close to the feeding center in the hypothalamus. Daily 2-hour food restriction in rats has been found to induce

anticipatory increases in body temperature (Boulos & Terman, 1980), suggesting that the regulatory mechanisms for temperature and feeding may be linked. Further studies are required to confirm this possibility.

Earlier studies that reported the inhibitory effect of ghrelin on food intake in domestic chickens used the lateral ventricle as the route for central injections (Furuse *et al.*, 2001; Saito *et al.*, 2002a), whereas in the present study I used the third ventricle. I also observed an inhibitory effect of ghrelin on food intake. Therefore, although the route of injection was different from that used in the previous studies in chickens, the results for both chickens and Japanese quails were the same, and both were the opposite to those reported for mammals, suggesting that the site of injection does not influence the differences between birds and mammals.

In conclusion, the results of the present study suggest that an increase in peripheral ghrelin acts as a hunger signal to stimulate food intake in the Japanese quail.

CHAPTER 3

Effect of neuromedin S on feeding regulation in the Japanese quail

Abstract

Neuromedin S (NMS) was recently isolated from the brains of humans, mice and rats as an endogenous ligand for the orphan G protein-coupled receptors FM-3 and FM-4, which have been identified as neuromedin U (NMU) receptor 1 and 2, respectively. To investigate the role of NMS in avian species, I elucidated the effect of icv administration of rat NMS on food intake, body weight, body temperature and gross locomotor activity in adult Japanese quails. NMS significantly decreased food intake (and consequently body weight) in a time-dependent manner during 12 h light period, but increased both body temperature and gross locomotor activity. On the other hand, icv injection of rat NMU showed the reverse effects of NMS in Japanese quail. These results suggest that NMS may play an important role in regulating food intake and sympathetic nerve activity in the Japanese quail.

3.1. Introduction

Recently, a novel 36-amino acid residue neuropeptide was isolated from rat brain and was identified as an endogenous ligand for FM-3/GPR66 and FM-4/TGR-1 using a reverse-pharmacological technique (Mori *et al.*, 2005). FM-3/GPR66 and FM-4/TGR-1 have been already identified as neuromedin U (NMU) receptor type-1 (NMUR1) and NMU receptor type-2 (NMUR2), respectively (Howard *et al.*, 2000). The novel peptide was designated neuromedin S (NMS), because it is specifically expressed in the suprachiasmatic nucleus (SCN) (Mori *et al.*, 2005). Although NMS shares a C-terminal core structure (7 amino acid residues) with NMU, and activates recombinant NMU1R and NMU2R expressed in Chinese hamster ovary cells, it is not a splice variant of NMU because the genes for NMS and NMU have been mapped to discrete chromosomes (Mori *et al.*, 2005).

The physiological functions of NMU have recently been clarified. Its most marked effect is on feeding regulation (Howard *et al.*, 2000; Kojima *et al.*, 2000; Nakazato *et al.*, 2000). Intracerebroventricular (icv) administration of NMU decreases both the daily food intake during dark period and fasting- induced food intake in rats (Ivanov *et al.*, 2002; Wren *et al.*, 2002). Conversely, injection of anti-NMU IgG increases dark-phase feeding compared with preimmune serum IgG (Kojima *et al.*, 2000). Recently, it has also demonstrated that NMU-knockout mice become obese (Hanada *et al.*, 2004). These results

indicate that NMU is a potent endogenous anorexigenic peptide in rats. In addition to feeding regulation, NMU increases gross locomotor activity, body temperature and heat production in rats, suggesting that it is a catabolic signaling molecule (Nakazato *et al.*, 2000). I previously reported that synthetic Japanese quail NMU decreased food intake and increased both body temperature and gross locomotor activity in Japanese quails (Shousha *et al.*, 2005b), thus implying that avian NMU also plays important physiological roles.

As mentioned above, NMS shows homology of the C-terminal core structure with NMU and activates recombinant NMU1R and NMU2R expressed in Chinese hamster ovary cells. It may therefore also play important roles in feeding regulation, locomotor activity and body temperature. With this possibility in mind, I compared the effects of NMS with those of NMU in avian species.

3.2. Materials and Methods

3.2.1. Animals

Adult male Japanese quails (*Coturnix coturnix japonica*), weighing 110–120 g, were reared in individual net cages (W: 14 x L: 26 x H: 17 cm) in a room with a 12-h light (300 Lux) /12-h dark (dim light, 25 Lux) period (lights on at 700 h), at a temperature of 28 ± 1°C. The birds were given free access to food and water. Rat NMS or rat NMU (Peptide Institute, Osaka, Japan) was dissolved in 0.9% saline and several doses were administered icv to each of six free-feeding male birds in each experimental group. Each experiment was set for measurement only one parameter to avoid the effect of one parameter on the other. All the experiments were performed twice in order to confirm the results obtained in each experiment. I performed one week interval between the first and second time experiment to avoid the residual effects of repeated injection. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

3.2.2. Surgical procedures

For implantation of the icv cannula, each bird was anesthetized with 5% sodium pentobarbital (1.4 µl/g body weight) and placed in a stereotaxic frame. The method of cannulation has been reported in Chapter 1. The birds were returned to their individual cages and allowed to recover for at least 4 days. They were acclimatized to handling every day before the start of the experiments. The icv

injections were administered through the implanted guide cannulae without anesthesia or restraining of the birds. At the end of the experiments, proper placement of the cannulae was verified by administering Evans Blue dye (10 µl), followed by sacrifice and brain sectioning (20 µm intervals). Data for birds lacking dye in the third ventricle were excluded from the analysis.

3.2.3. Measurement of food intake

Before the feeding experiment, the birds were weighed and assigned to an experimental group based on their body weight. The average body weight (110–120 g) in each group was kept as uniform as possible. To examine the orexigenic or anorexic effect of NMS, rat NMS (0.1, 0.5 or 1.0 nmol / 10 µl saline) or saline (control) was administered icv at 0700 h. Food consumption was determined in the free-fed birds at 2, 4 and 12 h after administration by measuring the disappearance of food from a pre-weighed feeder placed in each individual cage. Care was taken to collect and weigh any spillage, thus making the determination of food intake as accurate as possible.

3.2.4. Measurement of body temperature

The quails' body temperature was measured at 0 min (before injection), then at 5, 10, 20, 40, 60 and 120 min after icv injection of rat NMS, rat NMU (each at doses of 0.1, 0.5 or 1.0 nmol / 10 µl saline) or saline vehicle (n = 6 in each group) at 1000 h using a previously reported method (Bayle *et al*; 1974). The method of body temperature measurement has been reported in Chapter 2.

3.2.5. Measurement of locomotor activity

Locomotor activity was measured in each bird under light/dark conditions for 1 week, and thereafter under constant dim light at an intensity of about 30 Lux. The method of measurement of locomotor activity has been reported in Chapter 1. Rat NMS, rat NMU (each at doses of 0.1, 0.5 or 1.0 nmol / 10 μL saline) or saline vehicle was administered icv at 1000 h (n = 8 per group). After the injections, the birds were immediately returned to their individual cages. Locomotor activity counts were made every 15 min and summed for the 2-h period following administration.

3.2.6. Statistical analysis

All results are expressed as mean ± SEM. The data were analyzed using analysis of variance and the *post hoc* Fisher's test.

3.3. Results

Icv administration of NMS 0.5 and 1.0 nmol significantly ($P<0.05$) decreased food intake in a time-dependent manner compared with saline alone (Fig. 2A). This anorexigenic action of NMS was apparent by 2 h and continued for 12 h after icv administration. The effect was no longer observable on the following day (data not shown). Concomitantly, a significant ($P<0.05$) decrease in body weight was observed at 2, 4 and 12 h after icv injection of NMS (Fig. 2B). The decrease in body weight was more pronounced than the decrease in food intake, and became quite considerable by 12 h after the injection. The effect of a smaller dose of NMS (0.01 nmol icv) was examined, but this dose effected no significant change in food intake ($n=6$; data not shown).

Icv injection of NMS also significantly ($P<0.05$) increased body temperature and locomotor activity (Fig. 3, 4). An increment of about 2 °C was observed in body temperature 40–60 min after icv injection of 1.0 nmol NMS. Although 0.1 nmol NMS also caused an increase in body temperature, the change was not significantly different from that seen with saline alone. Locomotor activity was increased 1.5-fold during the 2-h period following icv injection of 1.0 nmol NMS.

When the effects of icv injection of the same doses of rat NMU and rat NMS on food intake, body temperature and locomotor activity were compared in Japanese quails, opposite effects were observed. Fig. 5 shows that rat NMU produced an increase in food intake but

Fig. 6 and Fig. 7 show decreases in body temperature and locomotor activity, respectively.

Figure 1. Sequence comparison of NMS and NMU of some mammals (Human, Rat and Mouse) and Avian NMU. Conserved core sequences are indicated by a solid underline. (Mori *et al.*, 2005).

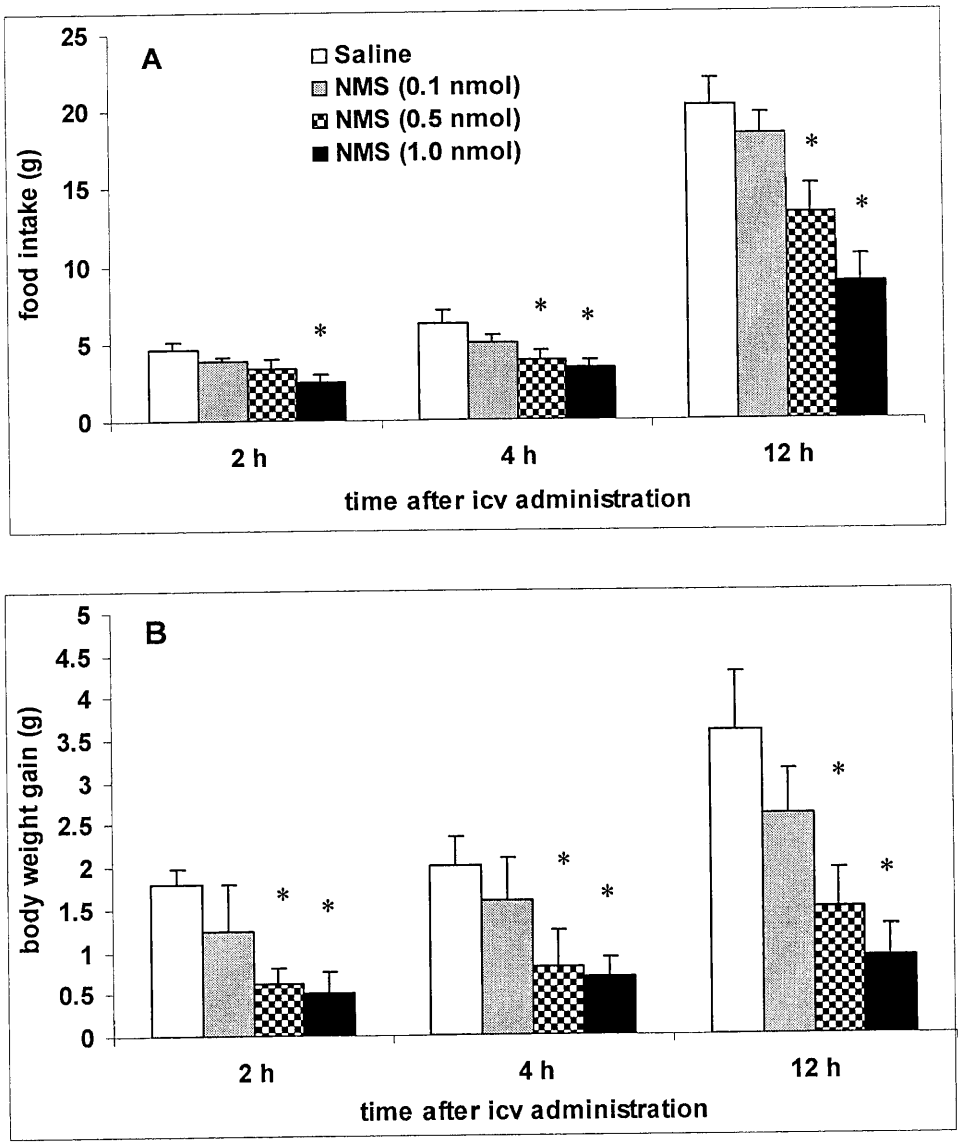

Figure 2. Effect of intracerebroventricular (icv) administration of rat NMS on (A) food intake and (B) body weight gain in the Japanese quail. Saline (vehicle control) or NMS (0.1, 0.5 or 1.0 nmol) was injected icv at 0700 h. Each bar and vertical line represents the mean ± SEM (n = 12). *Significantly different from the saline-treated group; P<0.05.

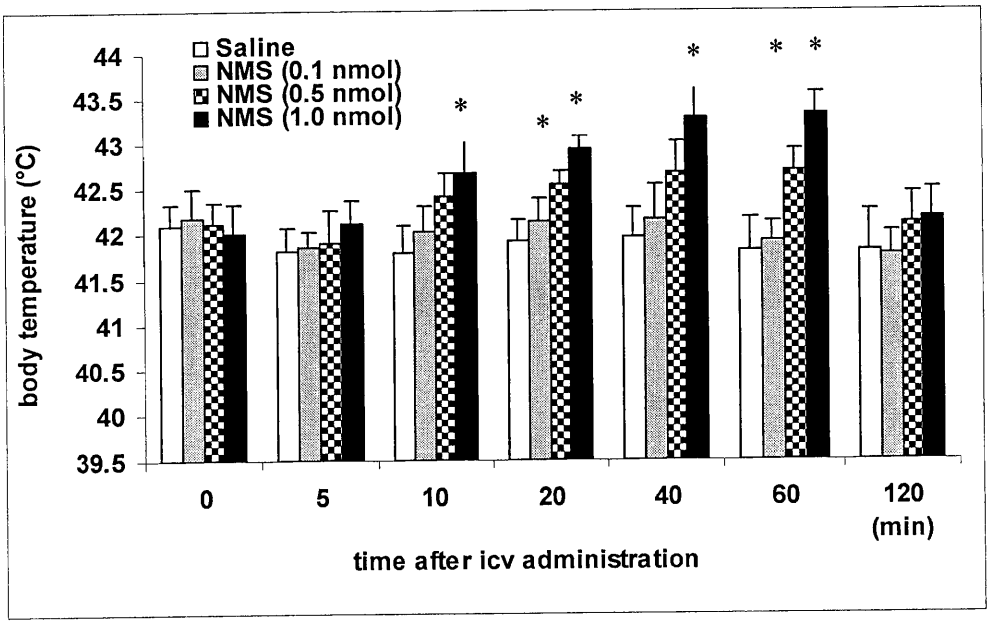

Figure 3. Effect of intracerebroventricular (icv) administration of rat NMS on body temperature in the Japanese quail. Saline (control) or NMS (0.1, 0.5 or 1.0 nmol) was injected icv at 1000 h. Each bar and vertical line represents the mean ± SEM (n = 6). *Significantly different from the saline-treated group; $P<0.05$.

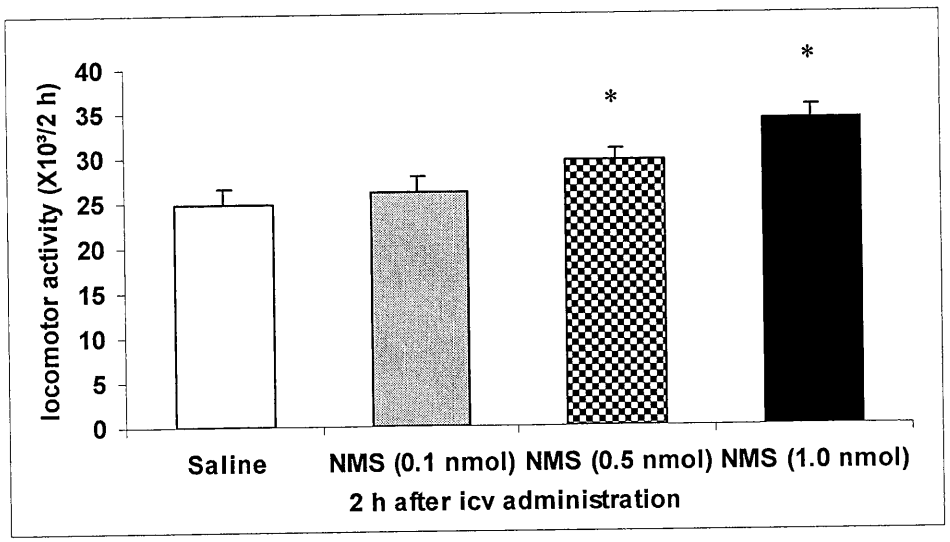

Figure 4. Effect of intracerebroventricular (icv) administration of rat NMS on gross locomotor activity in the Japanese quail. Saline (control) or NMS (0.1, 0.5 or 1.0 nmol) was injected icv at 1000 h. Each bar and vertical line represents the mean ± SEM (n = 8). *Significantly different from the saline-treated group; $P<0.05$.

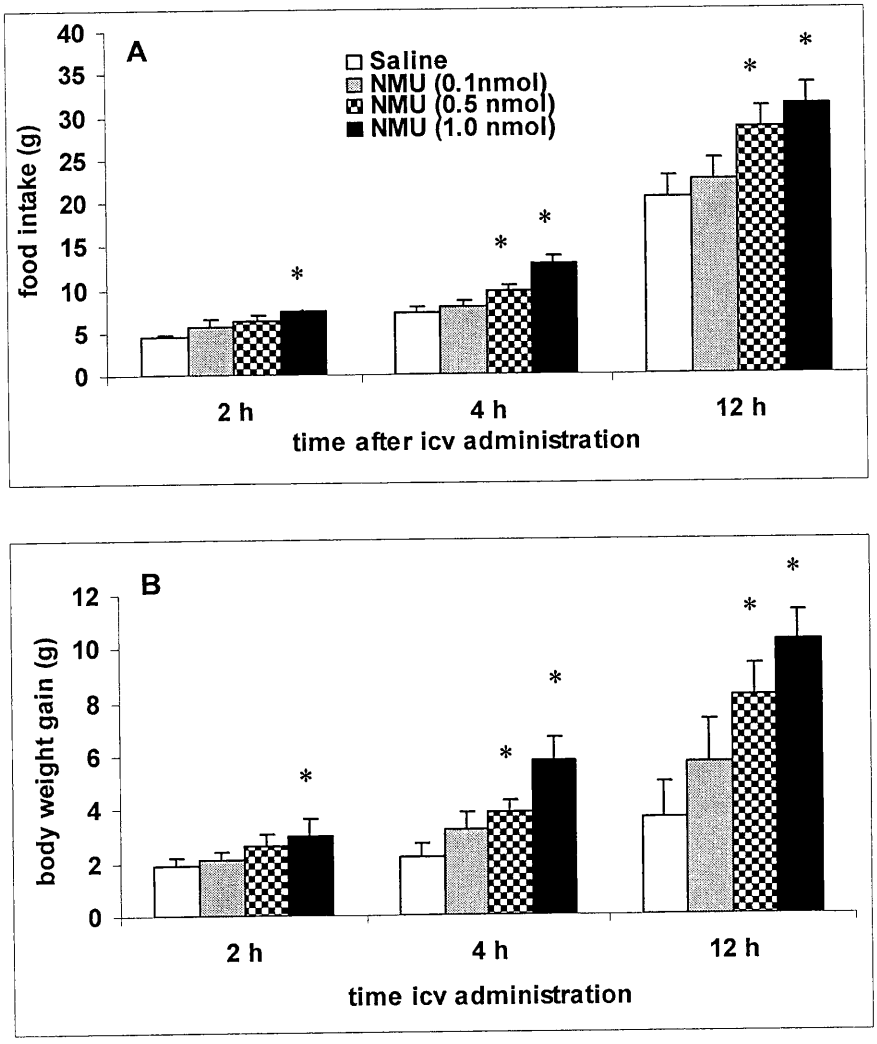

Figure 5. Effect of icv administration of rat NMU on food intake (A), and body weight gain (B) in the Japanese quail. Saline (control) or NMU (0.1, 0.5 or 1.0 nmol) was injected icv at 0700 h. Each bar and vertical line represents the mean ± SEM. (n = 12). *Significantly different from the saline treated group; $P<0.05$.

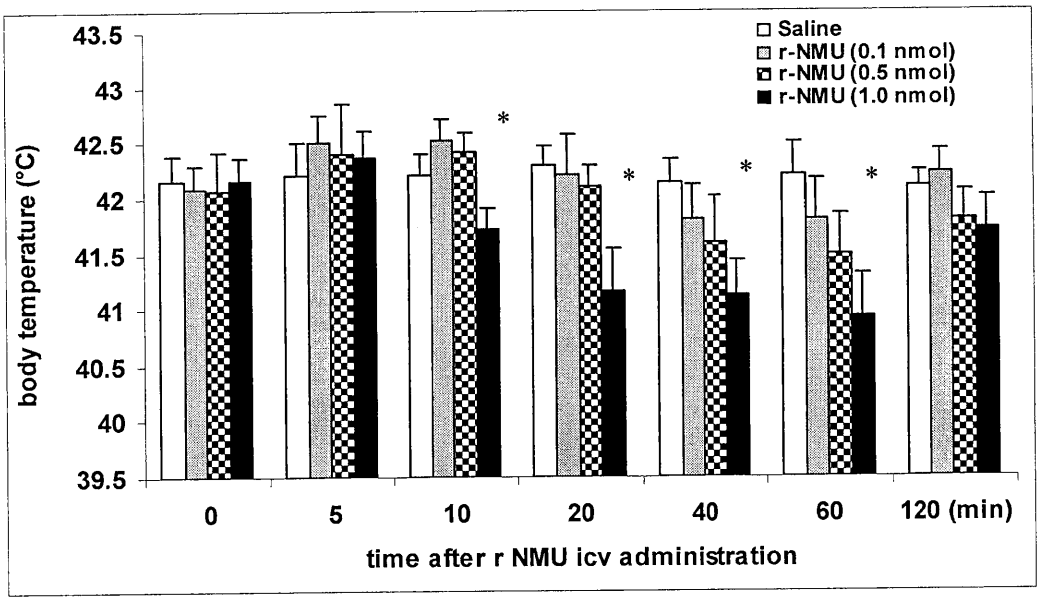

Figure 6. Effect of icv administration of rat NMU on body temperature in the Japanese quail. Saline (control) or NMU (0.1, 0.5 or 1.0 nmol) was injected icv at 1000 h. Each bar and vertical line represents the mean ± SEM. (n = 6). *Significantly different from the saline treated group; $P<0.05$.

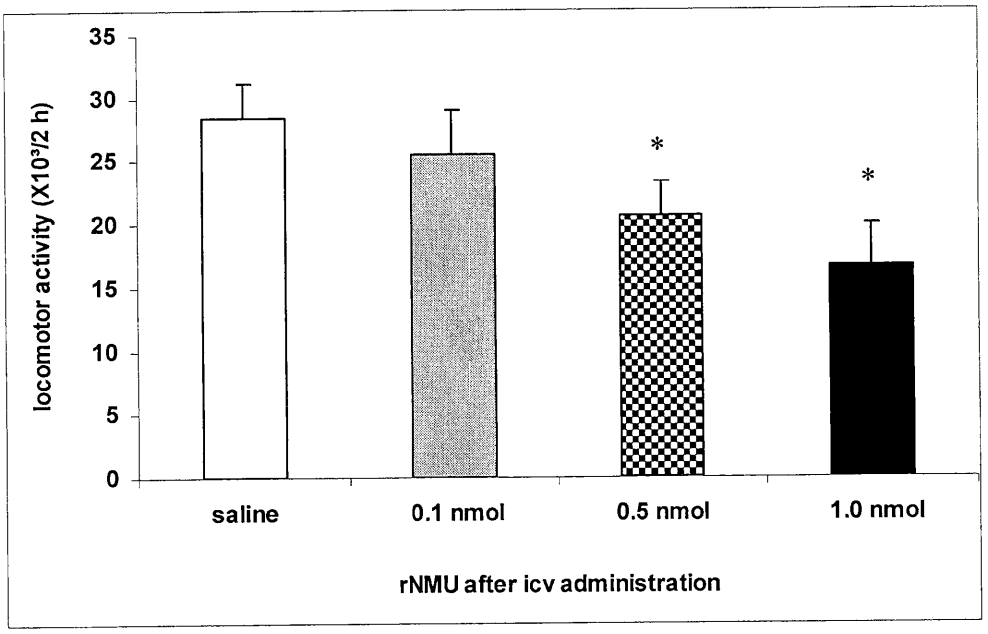

Figure 7. Effect of icv administration of rat NMU on gross locomotor activity in the Japanese quail. Saline (control) or NMU (0.1, 0.5 or 1.0 nmol) was injected icv at 1000 h. Each bar and vertical line represents the mean ± SEM. (n = 8). *Significantly different from the saline treated group; $P<0.05$.

3.4. Discussion

The amino acid sequences of NMU and NMS in some mammalian and avian species are illusterated in Fig. 1. The present study demonstrates that rat NMS suppresses food intake but promotes locomotor activity and increases body temperature in avian species. The suppression of feeding is unlikely to be due to any side-effect of NMS, since the quails in the treated group did not show any abnormal behavior. The noticeable decrease in body weight after icv injection of NMS may be due to both a decrease in food intake and an increase in energy expenditure. These results therefore suggest that central NMS may play important roles in the regulation of feeding and the sympathetic nervous system in avian species.

In a previous study, the distribution of NMS mRNA in various rat tissues was investigated using a quantitative reverse-transcriptase polymerase chain reaction technique (Mori *et al.*, 2005). NMS mRNA was expressed mainly in the hypothalamus, spleen and testis. Within the hypothalamus, NMS mRNA was expressed predominantly in the SCN; there was only very slight expression in other brain regions, such as the paraventricular nucleus (PVN) and arcuate nucleus (Arc) (Mori *et al.*, 2005). *In situ* hybridization histochemistry also showed that NMS mRNA expression was restricted to the SCN. No hybridization signal was observed in any other brain region (Mori *et al.*, 2005). In the case of NMS, therefore, its action on the PVN and Arc through the NMS projection from the SCN may be important.

Recently, it is observed that icv NMS also suppressed food intake in rats (Ida *et al.*, 2005). In that case, cFos expression was detected in preopiomelanocortin (POMC) -neuron in the arcuate nucleus and corticotropin-releasing hormone (CRH) -secreting cells in the paraventricular nucleus. This suggests that neuron containing POMC (a precursor of α-melanocyte-stimulating hormone; α-MSH) and CRH may be the targets for suppression of food intake by NMS, because CRH and α-MSH are known to be anorexigenic hormones in chicken (Kawakami *et al.*, 2000a; Zhang *et al.*, 2001). However, further study is required to elucidate the mechanism of action of NMS in avian species. Because NMS contains the active core C-terminus of NMU and binds to the same receptors (NMU1R and NMU2R), rat NMS and rat NMU would be expected to have very similar actions on food intake, locomotor activity and body temperature in Japanese quails. However, opposite effects were observed. Previously, we reported that Japanese quail NMU, but not rat NMU, suppressed food intake in Japanese quails, and that pretreatment with rat NMU inhibited the Japanese quail NMU-induced suppression of food intake (Shousha *et al.*, 2005b). Rat NMU therefore appears to have an antagonistic action on Japanese quail NMU, possibly through competition for NMU receptors. If this is so, why did rat NMS not show similar antagonism? The reason for the discrepancy is unclear from the present study; however, the following considerations may provide possible explanations. First, the structure of avian NMS may be close to that of

rat NMS. If this is so, rat NMS may not act antagonistically at NMU receptors, and may be able to have same physiological function as avian NMS. Although we tried cloning Japanese quail NMS using essentially the same method as that used for cloning Japanese quail NMU (Shousha *et al.*, 2005b), we were unsuccessful and could not therefore perform direct experiments with Japanese quail NMS. Second, there may be a specific receptor for NMS other than the NMU1R and NMU2R, and NMS may act on feeding and locomotion through it.

In conclusion, NMS, a novel peptide, appears to play important roles in the regulation of feeding, locomotor activity and body temperature in avian species. As this is the first paper to describe the actions of NMS in avian species, further research will be required to elucidate the exact mechanisms of action of NMS and any further physiological functions that it may have.

CHAPTER 4

Effect of glucagon-like peptide-1 and -2 on feeding regulation in the Japanese quail

Abstract

To investigate the physiological roles of glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) in avian species, we elucidated the effect of ip and icv administration of GLP-1 and GLP-2 on food intake, body temperature and gross locomotor activity in adult Japanese quail. Both ip and icv administration of GLP-1 suppressed food intake at 2, 4, and 12 h after administration. Moreover, both ip and icv administration of GLP-1 significantly decreased both body temperature and gross locomotor activity 2 h after administration. On the other hand, both ip and icv administration of GLP-2 had no effect on food intake, body temperature or gross locomotor activity. These results suggest that GLP-1 may have an important role in the regulation of food intake, body temperature and locomotor activity while GLP-2 may have no apparent effect on feeding regulation in adult Japanese quail.

4.1. Introduction

Glucagon-like peptide-1 (GLP-1) and -2 (GLP-2) are produced in endocrine L-cells which are located mainly in the mucosa of the ileum and colon, and arise as the result of proteolytic cleavage of proglucagon in the gut (Kieffer & Habener, 1999). Both peptides are released in equimolar amounts after food intake (Holst, 1997). Interestingly, the amino acid sequences of GLP-1 and GLP-2 have been highly conserved throughout the evolution, suggesting that both peptides are physiologically important (Orskov, 1992). The proglucagon-derived peptides have been shown to be involved in a variety of both peripheral and central functions in both humans and rodents, and several studies have demonstrated that they have potent metabolic (insulinotropic) activities both *in vitro* (Drucker *et al*; 1987), and *in vivo* (Mojsov *et al*; 1987). GLP-1 not only stimulates glucose-dependent insulin secretion, but also increases somatostatin (D'Alessio *et al*; 1989) and inhibits glucagon secretion (Komatsu *et al*; 1989), gastric emptying (Willms *et al*; 1996) and gastric acid secretion (Schjoldager *et al*; 1989). In addition, GLP-1 reduces food intake and core body temperature (O'Shea *et al*; 1996) after both intracerebroventricular (icv) (Turton *et al*; 1996) and peripheral administration (Flint *et al*; 1998). On the other hand, with respect to GLP-2, the main focus has been on its trophic effects on the mucosa of the gut (Tsai *et al*; 1997), although it also up-regulates intestinal glucose transporter activity and inhibits gastric acid secretion and

gastric motility (Lovshin & Drucker, 2000). Recent studies have also demonstrated that icv-injected GLP-2 decreases food intake in rats (Tang-Christensen *et al*; 2000). However, peripheral administration of GLP-2 to rats failed to demonstrate any effect on body weight (Scott *et al*; 1998).

In avian species, the physiological role of GLP-1 and GLP-2 has not yet been clarified. There are no published data regarding the peripheral or central effects of GLP-1 and GLP-2 in adult avian species, as previous studies to date were conducted using neonatal chicks (Bungo *et al*; 1999; Furuse *et al*; 1997a; 1997b). I recently examined the physiological roles of some novel peptides, such as ghrelin, neuromedin U and S, in avian species using adult Japanese quail and found that the regulations of feeding, body temperature and locomotor activity by such peptides are different from those in mammals (Shousha *et al*; 2005a; 2005b; 2006). In the present study, therefore, I estimated the effects of peripherally and centrally administered GLP-1 and GLP-2 on food intake, body temperature and gross locomotor activity in adult Japanese quails.

4.2. Materials and Methods

4.2.1. Animals

Adult male Japanese quails (*Coturnix coturnix japonica*) were housed in individual net cages (W: 14 x L: 26 x H: 17 cm) in a room with a 12-h light (300 lux) /12-h dark (dim light, 25 lux) regime (lights on at 0700 h) and a temperature of 28 ± 1 °C, and given free access to food and water. Before the feeding experiment, the birds were weighed and assigned to an experimental group (6 birds in each group) based on their body weight. The average body weight (110-120 g) in each group was kept as uniform as possible. To examine the effect of glucagon-like peptide-1 and -2, rat GLP-1 and GLP-2 (Peptide Institute, Osaka, Japan) or 0.9% saline (vehicle control) was administered ip or icv at 0700 h. The doses of GLP-1 injected ip were 0.1 (0.33 μg), 0.5 (1.68 μg), and 1.0 (3.3 μg) nmol/200 μl saline, whereas the doses injected icv were 0.01 (0.033 μg), 0.1 (0.33 μg) and 1.0 (3.3 μg) nmol/10 μl saline; the doses of GLP-2 injected ip were 0.5 (1.898 μg), 2.5 (9.49 μg), and 5.0 (18.98 μg) nmol/200 μl saline; whereas the doses injected icv were 0.01 (0.0379 μg), 0.1 (0.379 μg) and 1.0 (3.79 μg) nmol/10 μl saline. The doses of GLP-1 were decided as referred to the earlier studies in chicks and my preliminary experiments. All the experiments were performed twice. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

4.2.2. Surgical procedures

For implantation of the icv cannula, each bird was anesthetized with 5% sodium pentobarbital (1.4 µl/g body weight) and placed in a stereotaxic frame. The method of cannulation had been reported in Chapter 1. The birds were returned to their individual cages and allowed to recover for at least 4 days. They were acclimated to handling every day before the start of the experiments. The icv injections were administered through the implanted guide cannulae without anesthesia or restraint. At the end of the experiments, proper placement of the cannula was verified by administering Evans blue dye (10 µl), followed by sacrifice and brain sectioning at 20-µm intervals. Data for any birds that lacked dye staining in the third ventricle were excluded from the analysis.

4.2.3. Measurement of food intake

Food consumption was determined 2, 4 and 12 h after administration of GLP-1, GLP-2, or saline by measuring the disappearance of food from the pre-weighed feeder. Any spillage was also collected and weighed.

4.2.4. Measurement of body temperature

The body temperature of the quails was measured 0 (before injection), 5, 10, 20, 40, 60 and 120 min after administering GLP-1, GLP-2 or saline at the doses stated above (n = 6 in each group) at 1000 h, using a method reported previously (Bayle *et al*; 1974). The method of body temperature measurement had been reported in

Chapter 2.

4.2.5. Measurement of locomotor activity

Locomotor activity was measured by method (Marumato *et al*; 1996) as mentioned in Chapter 1. Rat GLP-1, GLP-2 or saline was injected at the doses stated above (n = 8 in each group) at 1000 h, using a method reported previously (Bayle *et al*; 1974). Immediately after injection, the birds were returned to their individual cages. Locomotor activity counts were made every 15 min and summed for 2 h after administration.

4.2. 6. Statistical analysis

Data were analyzed by analysis of variance and *post hoc* Fisher's test, and the results are expressed as mean ± SEM.

4.3. Results

Both ip and icv administration of GLP-1 to quails resulted in a significant ($P < 0.05$) dose-dependent decrease in food intake in comparison with saline injection (Fig. 3A and 4A). This reduction of food intake was apparent at 2 h after injection and continued for 12 h after either both ip injection of 0.5 and 1.0 nmol {saline, 20.3 ± 1.64; GLP-1 (1.0 nmol), 13.88 ± 1.1 g} or icv injection of 0.1 nmol and 1.0 nmol {saline, 20.3 ± 1.65; GLP-1 (1.0 nmol), 10.13 ± 1.17 g} ($P < 0.05$ vs saline). Also, body weight gain decreased significantly ($P < 0.05$ vs saline) in a dose-dependent manner after both ip and icv administration of GLP-1 (Fig. 3B and 4B). This decrease in body weight gain was apparent at 2 h after injection and continued for 12 h after either ip or icv injection. I examined the effect of GLP-1 on the body temperature of Japanese quail. Body temperature decreased significantly ($P < 0.05$) after both ip and icv injection of 0.5 and 1.0 nmol of GLP-1 (Fig. 5A and 5B). This effect was apparent sooner after icv injection (Fig. 5B) than after ip injection (Fig. 5A). Body temperature decreased by 2.1 °C 1 h after icv injection of 1.0 nmol GLP-1 and by 1.7 °C after ip injection of 1.0 nmol GLP-1. I also examined the effect of GLP-1 injection on gross locomotor activity (Fig. 6). Both ip (Fig. 6A) and icv (Fig. 6B) administration of GLP-1 resulted in a significant ($P < 0.05$) decrease of gross locomotor activity (52.7 % in case of icv injection of 1.0 nmol and 47.83 % in case of ip injection of 1.0 nmol). It is clear from (Fig. 2 and Fig. 3)

that the effect of GLP-1 injected icv at the level of 0.1 nmol (0.33 μg) was more stronger than the effect of GLP-1 injected ip at a higher level 0.5 nmol (1.68 μg). Also the effect of GLP-1 injected icv at the level of 1.0 nmol (3.3 μg) was more stronger than the effect of GLP-1 injected ip at the same level even both were effective.

No significant difference was observed between the saline group and the 0.01 nmol icv GLP-1 -treated group (Fig. 2B, 3B, 3D). Also no significant difference was observed between the saline group and the 0.1 nmol ip GLP-1 -treated group (Fig. 2A, 3A, 3C).

The effects of smaller doses of GLP-1 (0.001 nmol icv and 0.01 nmol ip) were examined, but these doses effected no significant change in food intake (n = 6; data not shown). Besides the experiments using GLP-1, I measured the effect of GLP-2 on food intake, body temperature, and gross locomotor activity in Japanese quail. The results revealed no effect of rat GLP-2 on food regulation, body temperature or locomotor activity as shown in (Fig. 7, 8, 9 and 10).

A

Human	**HAEGT FTSDY SSYLE GQAAK EFIAW LVKGR**
RAT	**HAEGT FTSDY SSYLE GQAAK EFIAW LVKGR**
Chicken (87%)	**HAEGT YTSD I TSYLE GQAAK EFIAW LVNGR**

B

Human	**HA DGS FSDEM NTILD NLAAR DFI NW LIQTK ITD**
Rat (97%)	**HA DGS FSDEM NTILD NLATR DFI NW LIQTK ITD**
Chicken (58%)	**HA DGS FTSD I NKILD DMAAK EFLKW LINTK VTQ**

Figure 1. shows the amino acid sequences of GLP-1 (A) and GLP-2 (B) in human , rat and chicken. Letters with solid underline show homology between chicken and mammalian peptides.

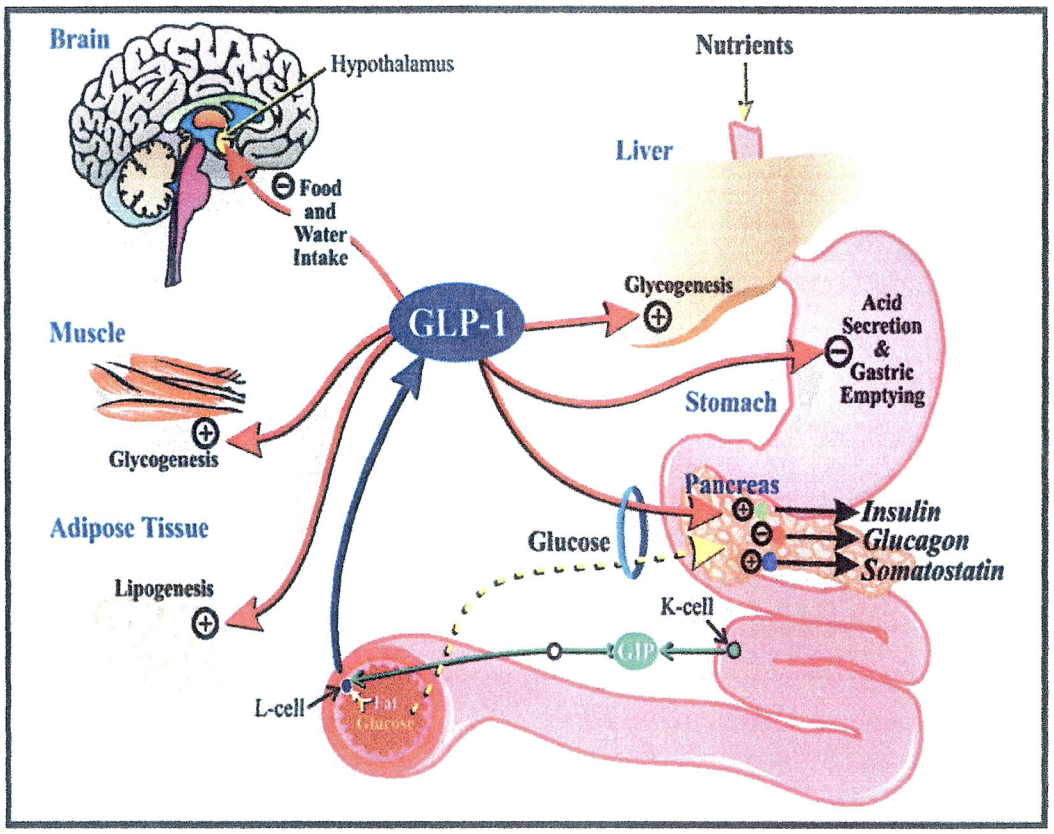

Figure 2. Summary of GLP-1 actions. The *diagram* summarizes the currently understood targets of GLP-1 actions.

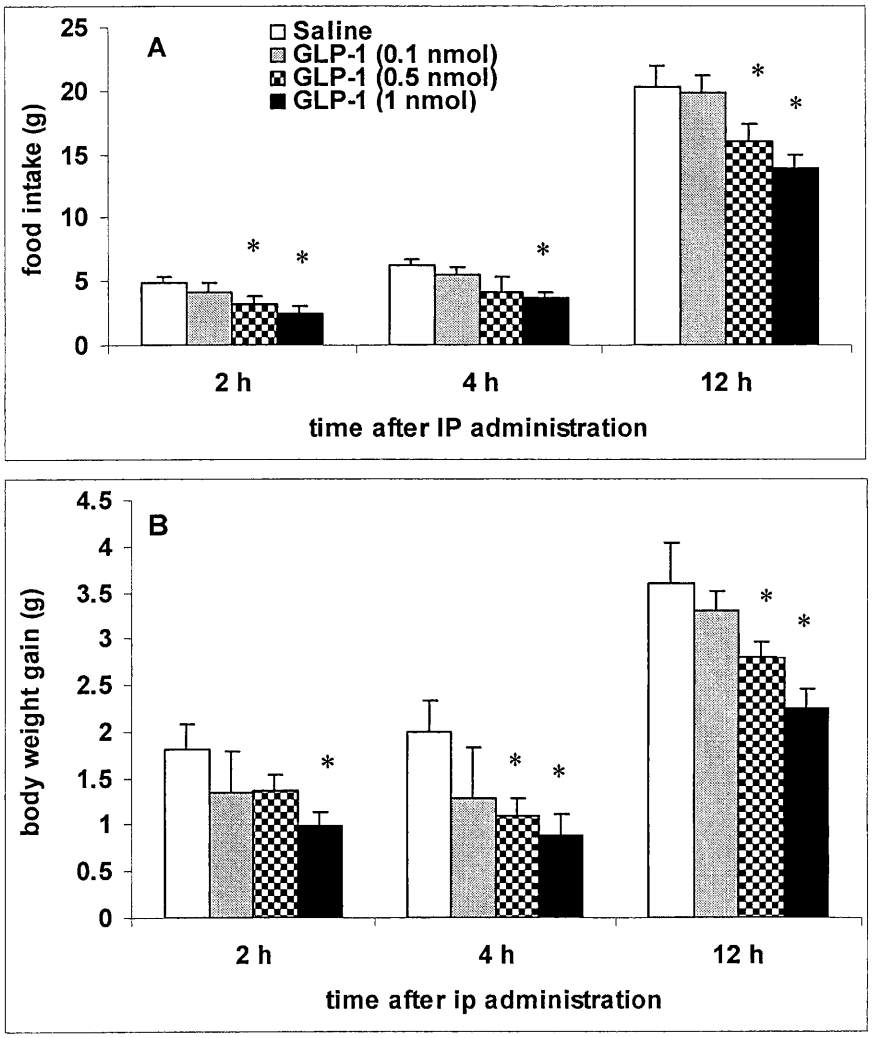

Figure 3. Effect of ip administration of rat GLP-1 on (A) food intake and (B) body weight gain in the Japanese quail. Saline (control), 0.1, 0.5, and 1.0 nmol of GLP-1 were injected ip at 0700 h. Each bar and vertical line represents the mean ± SEM (n = 12). *Significantly different from the saline-treated group; $P < 0.05$.

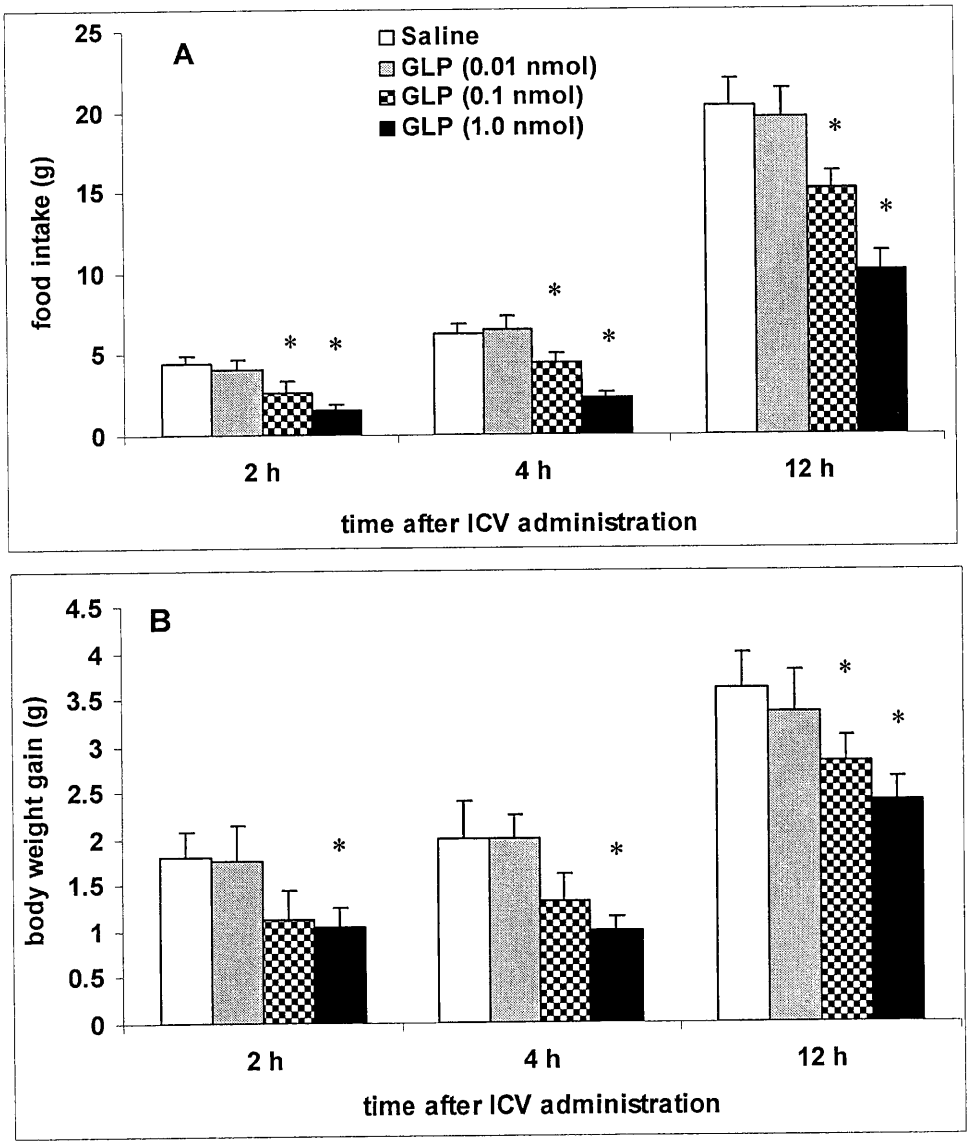

Figure 4. Effect of icv administration of rat GLP-1 on (A) food intake and (B) body weight gain in the Japanese quail. Saline (control), 0.01, 0.1, and 1.0 nmol of GLP-1 were injected ip at 0700 h. Each bar and vertical line represents the mean ± SEM (n = 12). *Significantly different from the saline-treated group; $P < 0.05$.

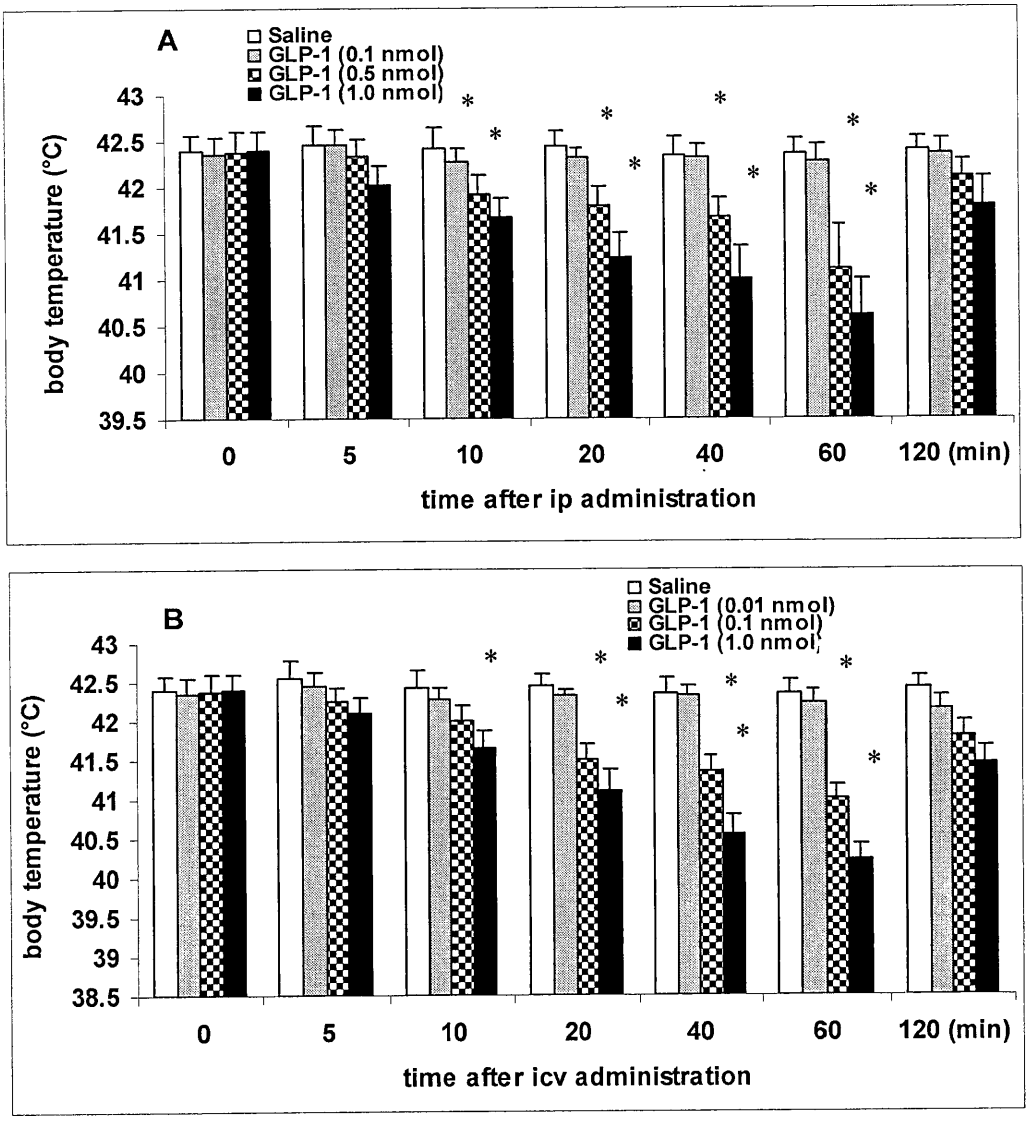

Figure 5. Effect of ip (A) and icv (B) administration of rat GLP-1 on body temperature in the Japanese quail. Saline (control), 0.1, 0.5 and 1.0 nmol of GLP-1 were injected ip or saline (control), 0.01, 0.1, and 1.0 nmol of GLP-1 were injected icv at 1000 h. Each bar and vertical line represents the mean ± SEM (n = 6). *Significantly different from the saline treated group; $P < 0.05$.

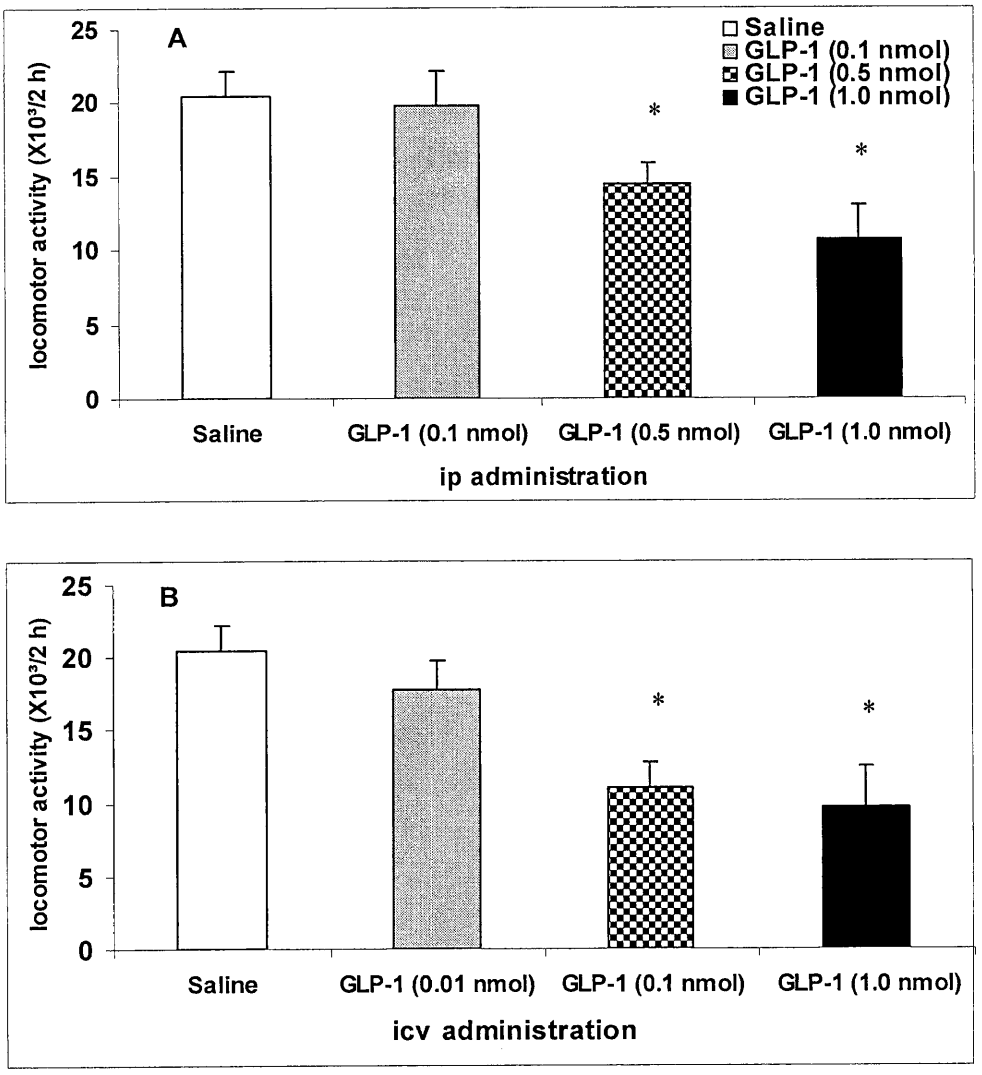

Figure 6. Effect of ip (A) and icv (B) administration of GLP-1 on gross locomotor activity in the Japanese quail. Saline (control), 0.1, 0.5 and 1.0 nmol of GLP-1 were injected ip and Saline (control), 0.01, 0.1 and 1.0 nmol of GLP-1 were injected icv at 1000 h. Each bar and vertical line represents the mean ± SEM. (n = 8). *Significantly different from the saline treated group; $P < 0.05$.

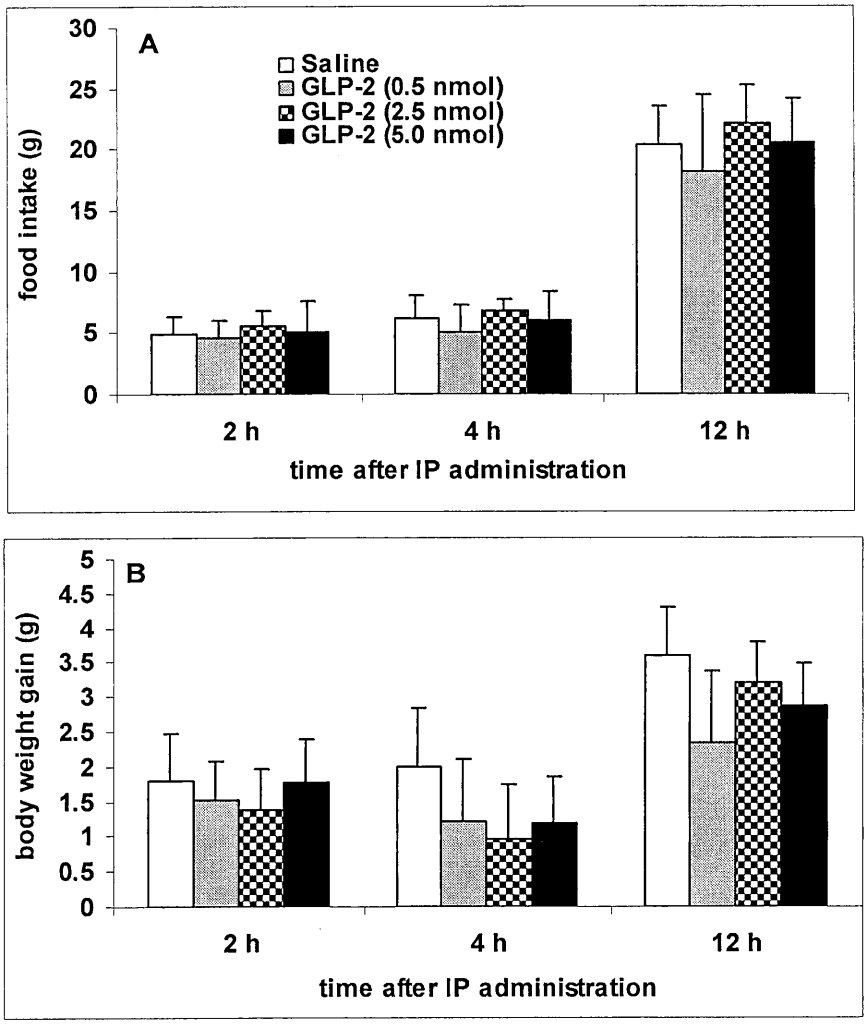

Figure 7. Effect of ip administration of rat GLP-2 on food intake (A) and body weight gain (B) in the Japanese quail. Saline (control), 0.5, 2.5, and 5.0 nmol of GLP-2 were injected ip at 0700 h. Each bar and vertical line represents the mean ± SEM (n = 12). *Significantly different from the saline-treated group; $P < 0.05$.

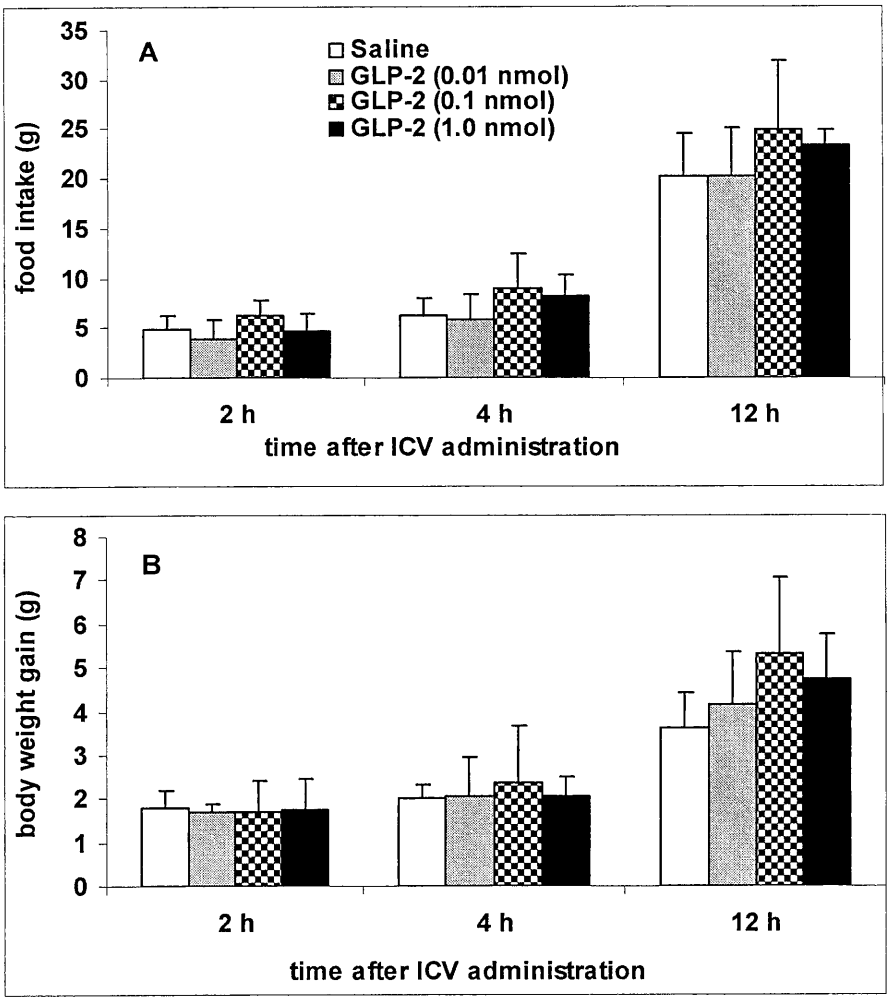

Figure 8. Effect of icv administration of rat GLP-2 on food intake (A) and body weight gain (B) in the Japanese quail. Saline (control), 0.01, 0.1, and 1.0 nmol of GLP-2 were injected icv at 0700 h. Each bar and vertical line represents the mean ± SEM (n = 12). *Significantly different from the saline-treated group; $P < 0.05$.

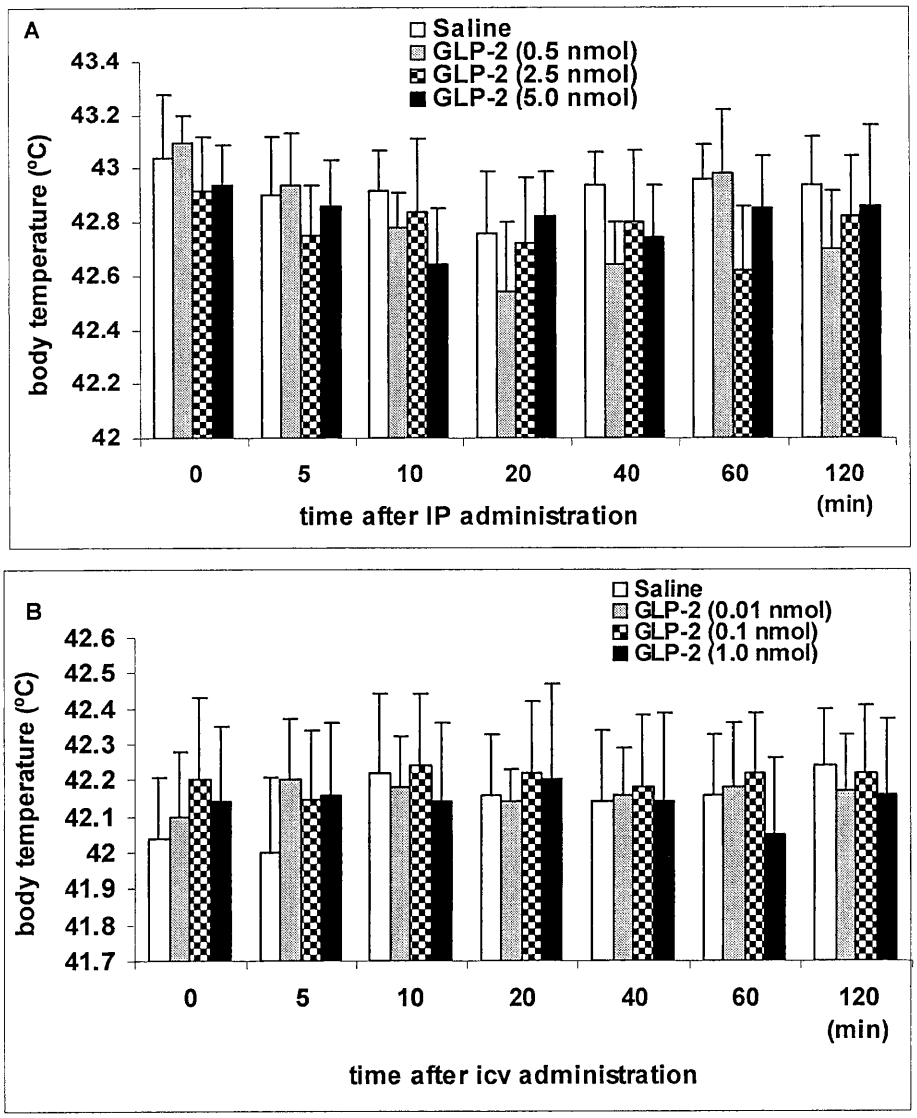

Figure 9. Effect of ip and icv administration of rat GLP-2 on body temperature in the Japanese quail. Saline (control), 0.5, 2.5 and 5.0 nmol of GLP-2 were injected ip (A) or saline (control), 0.01, 0.1, and 1.0 nmol of GLP-2 were injected icv (B) at 1000 h. Each bar and vertical line represents the mean ± SEM (n = 6). *Significantly different from the saline treated group; $P < 0.05$.

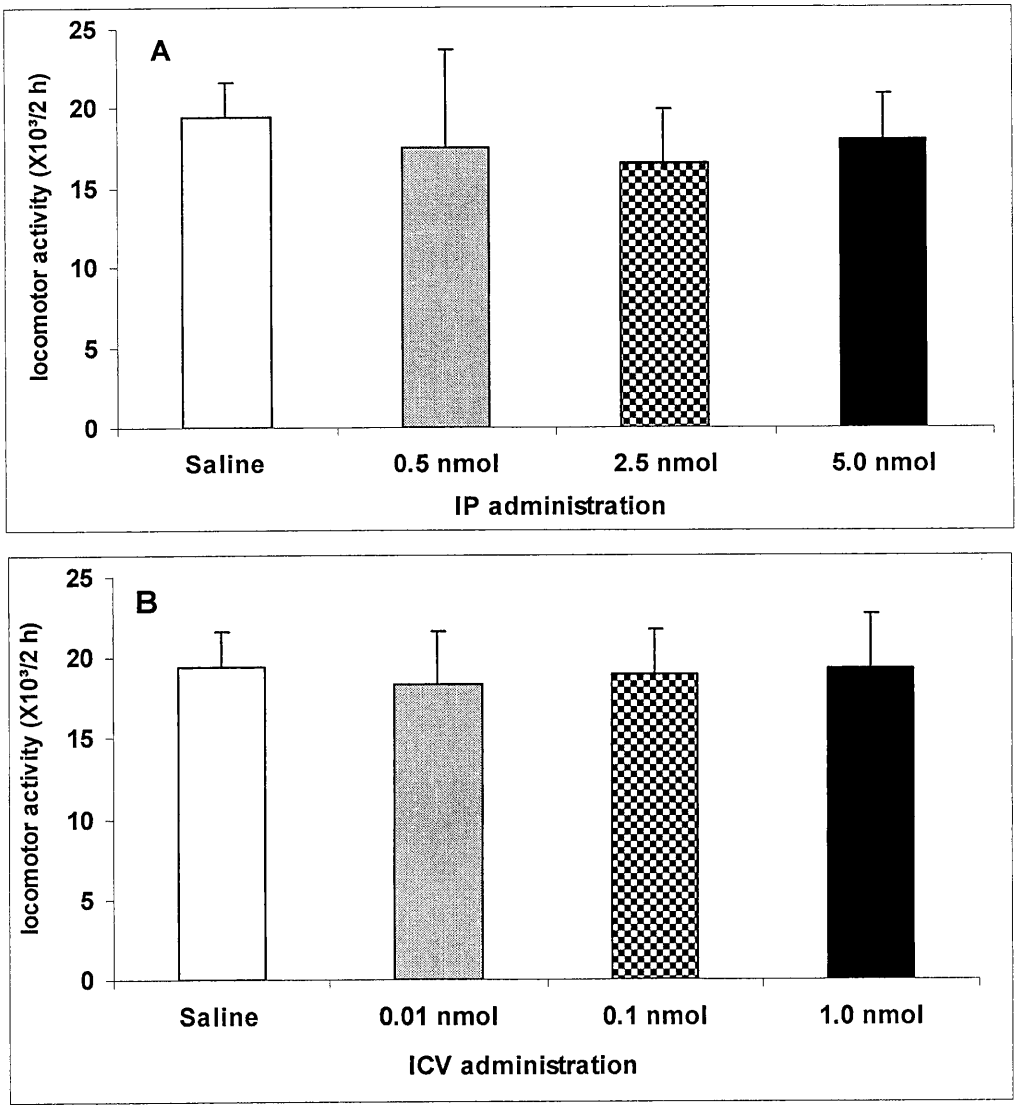

Figure 10. Effect of ip (A) and icv (B) administration of GLP-2 on gross locomotor activity in the Japanese quail. Saline (control), 0.5, 2.5 and 5.0 nmol of GLP-2 were injected ip and Saline (control), 0.01, 0.1 and 1.0 nmol of GLP-2 were injected icv at 1000 h. Each bar and vertical line represents the mean ± SEM. (n = 8). *Significantly different from the saline treated group; $P < 0.05$.

4.4. Discussion

The amino acid sequences of GLP-1 and GLP-2 in avian and mammalian species are illusterated in (Fig. 1). Summary of the currently understood targets of GLP-1 actions is illusterated in (Fig. 2).

Although GLP-1 binding sites and receptors are widely distributed in the central nervous system (Shughrue *et al.*, 1996), icv of GLP-1 exerted no significant effect at small doses. It was expected that GLP-1 given icv at a dose less than 0.1 nmol would be effective. There are some possibilities that might explain why GLP-1 given icv can induce its anorexigenic effect only at high doses. First, GLP-1 may act preferentially mainly through GLP-1 receptors located outside central nervous system as in pancreatic islets, stomach, lung, heart, kidney, small intestines (Bullock *et al.*, 1996) and therefore high central dose is required for central effect. Second, mammalian GLP-1 maybe have low affinity to bind with GLP-1 binding sites in central nervous system in Japanese quail and therefore small dose is not enough to induce effect. Third, Japanese quails maybe have few binding sites for GLP-1 in the central nervous system and therefore high central dose was needed. The difference in the dose response between Japanese quail in our study and neonatal chicks in the earlier studies maybe due to that the sensitivity of GLP-1 may be altered by aging, as we used adult quails, while the earlier studies used neonatal chicks that were being more sensitive. Another important factor maybe species differences in amino acid sequences of GLP-1. We think the central and peripheral

distribution of GLP-1 mRNA and GLP-1 receptors could explain any differences in the results. Further studies are required to know the GLP-1 binding sites, receptors structure and its tissue distribution in Japanese quail.

I found that GLP-1 administered both icv and ip decreased food intake and consequently body weight gain, and also decreased both body temperature and gross locomotor activity in the adult Japanese quail. Such suppression of food intake was similar to that observed previously in rats (Turton *et al*; 1996), since the amino acid sequence of mammalian GLP-1 shows high sequence homology (87 %) and differs from that of avian species at four positions only (Hasegawa *et al.*, 1990). Therefore these results suggest that central GLP-1 may play important roles in the regulation of feeding in adult avian species.

The actions of GLP-1 in rats are mediated by the glucagon-like peptide-1 receptor (GLP-1R), which is a member of the G protein coupled receptor (GPCR) family (Thorens, 1992) expressed on islet β cells (Thorens, 1992). GLP-1 receptor mRNA (Alvarez *et al*; 1996; Merchenthaler *et al*; 1999) and GLP-1 binding sites (Turton *et al*; 1996) are distributed in the rat hypothalamus. Additionally, icv injection of GLP-1 induces the expression of Fos-like-immunoreactive (FLI) cells in the hypothalamus, especially in the paraventricular nucleus (PVN) in rats (Turton *et al*; 1996). These observations suggest that the anorexic effect of centrally administered GLP-1 is mediated by the hypothalamus, especially the PVN. It is possible that the avian PVN

mediates the anorexic effect of GLP-1, in a manner similar to that in rats, since this brain region plays an important role in feeding regulation in avian species. Many reports have investigated the involvement of the hypothalamus in feeding regulation in chickens (Sugahara *et al*; 1999). Icv injection of GLP-1 into the lateral hypothalamic area (LHA) and ventromedial hypothalamic nucleus (VMN) has been reported to inhibit feeding in chicks and to stimulate the expression of Fos-like immunoreactive (FLI) cells in the ventromedial hypothalamic nucleus (VMN), suggesting that the hypothalamic nuclei are involved in the anorexic effect of GLP-1 in the chicken and that the VMN and LHA could possibly mediate the effect of GLP-1 (Tachibana *et al*; 2004). How peripherally secreted or injected GLP-1 can evoke central effects, although most of the GLP-1 binding sites in the hypothalamic and extrahypothalamic nuclei are separated from the circulation by the blood-brain barrier. There are at least two possible explanations for the effect of peripherally injected GLP-1 on central regulation of feeding. First, it is conceivable that transport into the central nervous system via specific carriers or endothelial leaks, making peripheral GLP-1 accessible to central binding sites. Evidence for such transport mechanisms has been reported by identifying the peripherally injected ^{125}I-GLP-1 in the subfornical organ and the periphery of the area postrema (Orskov *et al.*, 1996). Therefore, it is possible that GLP-1 injected peripherally exerts its effects on feeding behaviour via binding sites in these areas of the

brain stem. Second, I think that peripherally injected GLP-1 could stimulate peripheral vagal afferent nerve fibers that project to central nuclei in the hypothalamus. It is demonstrated that in vagally denervated rats, no suppression of feeding behaviour was observed after either peripheral or intracerebroventricular injection of GLP-1 (Imeryuz *et al.*, 1997). In birds, it is reported that the dorsal motor nucleus of the vagus (MnX) has been shown to send efferent fibers to and innervate visceral organs (Katz & Karten, 1985). Moreover, it has been demonstrated that there are GLP-1-like immunoreactive (GLI) cells in the chick proventriculus, which corresponds to the mammalian stomach, suggesting some physiological role of peripheral GLP-1 in the chicken (Martinez *et al.*, 1991). These facts, together with observations in mammals (Tolessa *et al.*, 1998), suggest that central and/or peripheral GLP-1 participates in the regulation of visceral function in the chicken and Japanese quail, as well as in rats.

Although the half-life of mammalian GLP-1 is short as it is rapidly degraded by enzyme dipeptidyl peptidase IV (DPP IV), it is reported that anorexigenic effect of GLP-1 lasted for at least 2 h in rats (Donahey *et al.*, 1998). However in avian species, it is reported that both mammalian and chicken GLP-1 inhibited food intake over 2 h in neonatal chick (Furuse *et al.*, 1997a). Also it is reported that GLP-1 0.01 nmol (0.03 μg)/10 μl injected by the icv route strongly inhibited food intake for over 4 h after injection in neonatal chicks (Furuse *et al.*, 1997c). Moreover the half-life of GLP-1 in avian species has not been

determined and maybe differences among avian species are also existed and this could explain the longer anorexigenic effect of GLP-1 in Japanese quail. Further studies will be beneficial to investigate the half-life of GLP-1 in avian species.

GLP-1 induces sleep-like behavior and reduces locomotion (Bungo *et al*; 1999). In the chicken brain, GLP-1 has different effects on behavior such as vocalization and locomotion. In the present study, GLP-1 appeared to act within the central nervous system of quails to reduce food intake, body temperature, and locomotor activity. The effect of GLP-1 on body temperature that observed in this study is in agreement with a previous study that demonstrated a transient reduction of core body temperature after central and peripheral injection of GLP-1 into rats (O'Shea *et al*; 1996). The actual reason was not known for the shorter effect of GLP-1 on body temperature than its effect on food intake. GLP-1 injected either peripherally or centrally caused a transient, dose-dependent decrease in body temperature, suggesting that it might affect energy expenditure in birds. After ip or icv administration of GLP-1, changes in feeding may produce a transient change in body temperature. The mechanism underlying this transient thermal change is unclear, but may involve changes in metabolism because the regulatory center for body temperature is located close to the feeding center in the hypothalamus. In addition, daily 2-hour food restriction in rats has been found to induce anticipatory increases in body temperature (Boulos & Terman,

1980), suggesting that the regulatory mechanisms for temperature and feeding may be linked. Further studies are required to confirm this possibility. GLP-1 also appears to reduce locomotor activity, as has also been reported in neonatal chicks, which became very calm and moved less (Furuse *et al*; 1997b), exhibiting sleep like behavior and reduced locomotion within 15 min after icv injection of GLP-1 (Bungo *et al*; 1999). On the basis these previous data for neonatal chickens, and our present results, it is highly likely that GLP-1 influences both energy intake and energy expenditure.

Parallel to the experiments involving GLP-1, the same experiments were conducted using rat GLP-2. Neither peripheral nor central administration of GLP-2 affected food intake, body temperature or locomotor activity. The amino acid sequence of mammalian GLP-2 shows low homology with that of avian GLP-2 (58 %) (Burrin *et al.*, 2003), suggesting a possible ineffectivity of mammalian GLP-2 in quail. In conclusion, GLP-1 may play an important role in the regulation of feeding in Japanese quail however; GLP-2 may be ineffective as there were no apparent effects of GLP-2 in this avian species in the present study.

GENERAL DISCUSSION

Appetite is controlled by a complicated system with hunger and satiety signals interacting in complex pathways both peripherally and centrally. Food intake behavior and appetite are the most important physiological functions that maintain life. It has been reported that there are many discrepancies in feeding regulation between mammals and avian species; for examples; orexin-A, orexin-B (Szekely *et al.*, 2002), motilin (Garthwaite, 1985; Rosenfeld and Garthwaite, 1987), melanin-concentrating hormone (Qu *et al.*, 1996; Rossi *et al.*, 1997), ghrelin (Nakazato *et al.*, 2001), and galanin (Akabayashi *et al.*, 1994) have been reported to stimulate food intake in rats, these peptides failed to either stimulate or inhibit feeding in the neonatal chick (Ando *et al.*, 2000; Furuse *et al.*, 1999a, 2001). Since then, many reports about feeding behavior in neonatal chicks were published, I turned my attention to feeding regulation using some novel peptides (neuromedin U & S), ghrelin, GLP-1 and GLP-2) in adult avian species using Japanese quails.

In the first chapter, I elucidated the peripheral and central effects of NMU on food intake, body temperature and gross locomotor activity in the Japanese quail. Then I compared the effect of rat NMU with that of Japanese quail NMU after determining its amino acid sequence. I demonstrated that rat and Japanese quail NMU have opposing effects on feeding and body temperature in the Japanese quail. Therefore, the biologically active region of this peptide may not

necessarily have the same activity in different species even it is conserved. However the results obtained after injection of quail NMU in Japanese quail are consistent with those obtained in rats following injections of rat NMU indicating that NMU acts both anorectically and catabolically in avian species as well as in mammals. Why rat NMU has opposite effect to quail NMU in Japanese quail, I presume that there is competitive antagonism between rat NMU and endogenous Japanese quail NMU receptors and therefore pretreatment with either rat or quail NMU blocked the effect of subsequent treatment with either quail or rat NMU, respectively, on food intake.

In the second chapter, I demonstrated that ip administration of small doses of ghrelin stimulates food intake; conversely, large doses inhibit it while icv ghrelin consistently decreased feeding in Japanese quail. On the other hand, the pattern of plasma ghrelin secretion before and after feeding is the same as seen in rats suggesting that increased peripheral ghrelin levels act as a hunger signal rather than a satiety signal. In agreement with this finding, previous studies have shown that neither rat nor chicken ghrelin has any transient stimulatory effect on food intake in neonatal chicks when administered centrally (Furuse *et al.*, 2001; Saito *et al.*, 2002a). I think that the mechanisms underlying the central and peripheral effects of ghrelin on feeding may differ, perhaps being anorexic and orexigenic, respectively. It may be probable that ghrelin can pass through the blood–brain barrier reaching central (arcuate nucleus) GHS-Rs,

thereby inhibiting food intake in Japanese quail as has been reported in rats (Banks *et al.*, 2002). The existence of peripheral sensors for ghrelin (GHS-R mRNA) in various peripheral organs in adult chicken is reported (Tanaka *et al.*, 2003). Peripheral ghrelin acts via gastric vagal afferent nerves (Date *et al.*, 2002). The ghrelin signals from peripheral GHS-Rs may be converted to neurotransmitter-mediated signals in the nucleus of the solitary tract. This neurotransmitter could have the opposite effect to direct release of ghrelin in the hypothalamus. Another suggestion is that ghrelin may act to down-regulate the GHS-R.

In the third chapter, I demonstrated that rat NMS suppresses food intake but promotes locomotor activity and increases body temperature in Japanese quails. The decrease in body weight after icv injection of NMS may be due to both a decrease in food intake and an increase in energy expenditure. Recently, it is observed that icv NMS also suppressed food intake in rats (Ida *et al.*, 2005) and C-Fos expression was detected in preopiomelanocortin (POMC) -neuron in the arcuate nucleus and corticotropin-releasing hormone (CRH) -secreting cells in the paraventricular nucleus. This suggests that neurons containing POMC (a precursor of α-melanocyte-stimulating hormone; α-MSH) and CRH may be the targets for suppression of food intake by NMS, because CRH and α-MSH are known to be anorexigenic hormones in chicken (Kawakami *et al.*, 2000a; Zhang *et al.*, 2001). I suggest that there is may be a specific receptor in avian

species for NMS other than the NMU1R and NMU2R, and NMS may act on feeding and locomotion through it.

In the fourth chapter, I found that GLP-1 administered both icv and ip decreased food intake and consequently body weight gain, and also decreased both body temperature and gross locomotor activity in the adult Japanese quail. Such suppression of food intake was similar to that observed previously in rats (Turton *et al.*, 1996) and this may be due to high amino acid sequence homology of GLP-1 between mammalian and avian species. Therefore, these results suggest that central GLP-1 may play important roles in the regulation of feeding in adult avian species. The results suggest that the avian PVN mediates the anorexic effect of GLP-1, in a manner similar to that in rats, since this brain region plays an important role in feeding regulation in avian species. It is reported that the hypothalamic nuclei are involved in the anorexic effect of GLP-1 in the chicken and that the VMN and LHA could possibly mediate the effect of GLP-1 (Tachibana *et al.*, 2004). Peripherally injected GLP-1 could stimulate peripheral vagal afferent nerve fibers that project to central nuclei in the hypothalamus as it is demonstrated in vagally denervated rats that showed no feeding suppression after either peripheral or central injection of GLP-1 (Imeryuz *et al.*, 1997). Moreover, it has been demonstrated that there are GLP-1-like immunoreactive (GLI) cells in the chick proventriculus, which corresponds to the mammalian stomach, suggesting some physiological role of peripheral GLP-1 in the

chicken (Martinez *et al.*, 1991). These facts, together with observations in mammals (Tolessa *et al.*, 1998), suggest that central and/or peripheral GLP-1 participates in the regulation of visceral function in the chicken and Japanese quail, as well as in rats.

In conclusion, the present study reveals that avian feeding has been regulated by many central and peripheral peptides, as is the case for mammals as well. However, the regulatory mechanisms are partially different between mammals and avian species, such as the central ghrelin has opposite effect on feeding between them. In the near future, it might be possible to control food intake or body weight gain in avian species either in breeders or broilers by regulation of endogenous peptides related to feeding.

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Summary

Food intake regulation is a fundamental concern of poultry producers. Recently, many novel peptides have been discovered as an endogenous ligand for the G-protein coupled orphan receptor. Some of them have been shown to stimulate or inhibit food intake in mammals. This study was conducted to investigate the central and peripheral regulation of food intake in avian species using the novel peptides related to feeding regulation. In this study, I used ghrelin and neuromedin (U and S) as stimulatory and inhibitory peptides for food intake, respectively. For avian species, adult Japanese quail were selected because of the stability of their body weight, as well as the possibility of intracerebroventricular injection (icv).

I. Effect of neuromedin U on the feeding regulation in the Japanese quail

Neuromedin U (NMU) has been identified as an endogenous ligand for the orphan receptor FM3/FM4 in 2000 and has been shown to inhibit food intake in mammals. I found that the amino acid sequence of Japanese quail NMU has high homology with that of chicken, with the exception of two amino acids, and low homology with that of the rat, with the exception of the C-terminal biologically active region by gene cloning analysis. RT-PCR analysis revealed that NMU mRNA was expressed in a wide range of various peripheral and central tissues. Both ip and icv administration of synthetic Japanese quail NMU resulted in a significant reduction in food intake at 2, 4, and 12 hours and an increase in both body temperature and gross locomotor activity within 2 hours after administration in Japanese quails. Conversely, both ip and icv administration of rat NMU into Japanese quail resulted in the opposite effects on food intake, body temperature and gross locomotor activity. These opposing results suggest that rat NMU might act as an antagonist toward the Japanese quail NMU receptor. Accordingly, the results of the

current research suggest that endogenous NMU plays an important role in the regulation of food intake and body temperature.

II. Effect of peripheral and central ghrelin on regulation of food intake in the Japanese quail

Ghrelin was identified as an endogenous ligand for orphan receptor GHSR in 1999 and has been shown to stimulate food intake in mammals. The molecular structure of ghrelin has been well conserved between vertebrates with high homology. An immunohistochemical study, performed with an antibody that recognizes the N-terminal region of ghrelin, revealed that immunostained cells were located in the stomach, but they were few in number compared with what is usually observed in rats and other animals. In control quails, plasma ghrelin levels were significantly increased in the fasting state; subsequent feeding produced a reduction, suggesting that ghrelin may act as an orexigenic signal in Japanese quails as well as mammals. Food intake was stimulated by ip (but not icv) injections of small doses of ghrelin, whereas large doses of both ip and icv injections inhibited feeding. A large dose of ghrelin also increased body temperature. These results suggest that an increase in peripheral ghrelin may act as a hunger signal to induce food intake through stimulation of the afferent vagal nerve in the Japanese quail, as in rats, whereas central ghrelin may inhibit feeding differently in the two species.

III. Effect of neuromedin S on feeding regulation in the Japanese quail

Neuromedin S (NMS) has been identified as an endogenous ligand for orphan receptor FM3/FM4 as was NMU in 2005 and has been shown to inhibit food intake in mammals. I elucidated the effect of icv administration of rat NMS on food intake, body weight, body temperature and gross locomotor activity in adult Japanese quails and compared its effect with that of rat NMU. NMS significantly decreased food intake (and consequently body weight) in a time-dependent manner

during 12 h light period, but increased both body temperature and gross locomotor activity. On the other hand, icv injection of rat NMU showed the reverse effects of NMS in Japanese quail. These results suggest that NMS may play an important role in regulating food intake and sympathetic nerve activity in the Japanese quail.

IV. Effect of glucagon-like peptide-1 and -2 on feeding regulation in the Japanese quail

GLP-1 and -2 (GLP-2) are members of the glucagon super family and have been shown to inhibit food intake in mammals. I elucidated the effect of ip and icv administration of GLP-1 and GLP-2 on food intake, body temperature and gross locomotor activity in adult Japanese quails. Both ip and icv administration of GLP-1 suppressed food intake at 2, 4, and 12 h after administration. Moreover, both ip and icv administration of GLP-1 resulted in a significant decrease in both body temperature and gross locomotor activity 2 h after administration. On the other hand, both ip and icv administration of GLP-2 had no effect. These results suggest that GLP-1 may play an important role in the regulation of feeding in Japanese quail however; GLP-2 may have no apparent effect on feeding regulation in adult Japanese quail.

In conclusion, the present study reveals that avian feeding is regulated by many central and peripheral peptides, as is the case for mammals as well. However, the regulatory mechanisms are partially different between mammals and avian species, such as the central ghrelin has the opposite effect on feeding between them. In the near future, it might be possible to control the food intake or body weight gain through regulation of the endogenous peptides related to feeding in avian species.

Curriculum Vitae

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