Biochemical and Biophysical Research Communications 446 (2014) 298–303

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of a cDNA encoding a novel small secretory protein, neurosecretory protein GL, in the chicken hypothalamic infundibulum

Kazuyoshi Ukena [a,*], Eiko Iwakoshi-Ukena [a], Shusuke Taniuchi [a], Yuki Bessho [a], Sho Maejima [a], Keiko Masuda [a], Kenshiro Shikano [a], Kunihiro Kondo [a], Megumi Furumitsu [a], Tetsuya Tachibana [b]

[a] Section of Behavioral Sciences, Graduate School of Integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima 739-8521, Japan
[b] Department of Agrobiological Science, Faculty of Agriculture, Ehime University, Matsuyama 790-8566, Japan

ARTICLE INFO

Article history:
Received 11 February 2014
Available online 28 February 2014

Keywords:
Neurosecretory protein
Chicken
Brain
Hypothalamus
Body weight
Growth

ABSTRACT

To find novel neuropeptide and/or peptide hormone precursors in the avian brain, we performed a cDNA subtractive screen of the chicken hypothalamic infundibulum, which contains one of the feeding and neuroendocrine centers. After sequencing 596 clones, we identified a novel cDNA encoding a previously unknown protein. The deduced precursor protein consisted of 182 amino acid residues, including one putative small secretory protein of 80 amino acid residues. This small protein was flanked at the N-terminus by a signal peptide and at the C-terminus by a glycine amidation signal and a dibasic amino acid cleavage site. Because the predicted C-terminal amino acids of the small protein were Gly-Leu-NH_2, the small protein was named neurosecretory protein GL (NPGL). Quantitative RT-PCR analysis demonstrated specific expression of the NPGL precursor mRNA in the hypothalamic infundibulum. Furthermore, the mRNA levels in the hypothalamic infundibulum increased during post-hatching development. *In situ* hybridization analysis showed that the cells containing the NPGL precursor mRNA were localized in the medial mammillary nucleus and infundibular nucleus within the hypothalamic infundibulum of 8- and 15-day-old chicks. Subcutaneous infusion of NPGL in chicks increased body weight gain without affecting food intake. To our knowledge, this is the first report to describe the identification and localization of the NPGL precursor mRNA and the function of its translated product in animals. Our findings indicate that NPGL may participate in the growth process in chicks.

1. Introduction

Neuropeptides and peptide hormones are bioactive peptides produced in the central and peripheral nervous systems. They act as neurotransmitters, neuromodulators, and/or neurosecretory hormones [1] and contribute to various physiological processes, such as neuroendocrine, behavioral, sensory, autonomic, emotional, and higher cognitive processes [2]. The identification of novel bioactive peptides can help elucidate the molecular mechanisms that underlie brain functions. Since the mid 1990s, DNA database queries have identified many types of orphan G protein-coupled receptors (GPCRs) that bind unknown ligands. Identifying the natural ligands of orphan GPCRs is referred to as the "orphan receptor strategy" or "reverse pharmaceutical research" [3]. Examples of GPCR deorphanization include the discoveries of orexin/hypocretin [4], ghrelin [5], and kisspeptin [6]. These bioactive peptides participate in narcolepsy, metabolism, and reproduction, respectively. However, because of the difficulty in matching ligands and GPCRs, there have been few recent reports describing the discovery of novel bioactive peptides, although it is estimated that over 50 orphan GPCRs for peptidergic signaling molecules await deorphanization [7]. During the last decade, some bioactive peptides that act not only in peripheral tissues but also in the brain have been identified through various approaches. These peptides include adrenomedullin 2/intermedin [8], neuropeptide S [9], neuromedin S [10], nesfatin-1 [11], and neuroendocrine regulatory peptide (NERP) [12]. To date, the cognate receptors for nesfatin-1 and NERP have not yet been identified.

We recently identified and characterized novel LPXRFamide peptides, including gonadotropin-inhibitory hormone (GnIH) [13] and 26RFa/QRFP [14], in the avian brain. Because we are interested in avian feeding and growth control processes, we sought to identify previously unknown neuropeptides or peptide hormones in the chicken hypothalamus that affect feeding behavior and/or the neuroendocrine control of growth. Food intake, energy expenditure, and body weight gain are closely related to development and growth in animals. In the mammalian brain, the arcuate

* Corresponding author. Fax: +81 82 424 0759.
E-mail address: ukena@hiroshima-u.ac.jp (K. Ukena).

http://dx.doi.org/10.1016/j.bbrc.2014.02.090
0006-291X/

nucleus (Arc) of the hypothalamus is one of the regulatory centers that controls feeding and body weight. The Arc produces neuropeptide Y (NPY) and α-melanocyte-stimulating hormone (α-MSH), which are potent orexigenic and anorexigenic factors, respectively [15]. In addition, the mediobasal hypothalamic region, including the Arc, contains many cells that produce hypothalamic hypophysiotropic hormones; these neurosecretory hormones are released through the median eminence (ME), located on the lower side of the Arc, into the pituitary gland [16]. Therefore, we chose to study the chicken hypothalamic infundibulum of the mediobasal hypothalamic region, which includes the infundibular nucleus (IN; corresponds to the mammalian Arc) and the ME. To find novel neuropeptides or peptide hormones, we used a subtractive screening method and searched for novel cDNAs that encoded precursors of bioactive peptides. Typically, the precursor protein of a secretory peptide consists of an N-terminal signal peptide sequence, a bioactive peptide sequence, and a dibasic amino acid sequence (Lys/Arg–Lys/Arg) in the internal sequence. In addition, approximately half of all bioactive peptides possess C-terminal amidation, which is considered necessary for receptor binding and/or protection against degradative enzymes [17]. For C-terminal amidation, the Gly residue after the bioactive peptide sequence serves as the amidation donor. The purpose of the present study was to discover a cDNA that encodes a novel precursor protein with these structural features.

In the present study, we identified a cDNA synthesized from chicken hypothalamic infundibular mRNA that encoded a novel small secretory protein. The brain regions expressing the small protein precursor mRNA were investigated using quantitative RT-PCR and *in situ* hybridization. We also assessed the effects of the small protein on growth and feeding behavior in chicks.

2. Materials and methods

2.1. Animals

Male layer chicks (*Gallus domesticus*, 1–15 days old) were purchased from a commercial company (Nihon Layer, Gifu, Japan) and housed in a windowless room maintained at 28 °C, with constant lighting. The chicks were provided food and tap water *ad libitum*. The experimental protocols were in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Hiroshima University (Higashi-Hiroshima, Japan).

2.2. RNA and cDNA preparation

Chicks were sacrificed by decapitation. The telencephalon, diencephalon, mesencephalon, cerebellum, and hypothalamic infundibulum were dissected and snap-frozen in liquid nitrogen for further RNA processing. RNA from chicken brain tissues was extracted using TRIzol reagent or an RNAqueous-Micro kit (Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instructions.

First-strand cDNA synthesis was performed with RNA isolated from each brain region. We used a PCR-Select cDNA Subtraction kit (Clontech Laboratories, Mountain View, CA, USA) for cDNA synthesis in accordance with the manufacturer's instructions.

2.3. Subtractive cDNA screening

The method used was similar to that described in a previous report [18]. We used PCR-Select cDNA Subtraction and PCR-Select Differential Screening kits (Clontech Laboratories) for subtractive screening in accordance with the manufacturer's instructions.

The subtracted DNA inserts of 596 clones were amplified by PCR using universal M13 primers. Nucleotide sequences were determined using an ABI PRISM BigDye Terminator Ready Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA), an ABI PRISM 310 Genetic Analyzer (Applied Biosystems), and DNASIS Pro software (Hitachi Software Engineering, Kanagawa, Japan). The nucleotide sequences were further analyzed with a chicken nucleotide BLAST search, and previously characterized genes were omitted.

2.4. Real-time PCR

The differential expression of the unknown nucleotide sequences was assessed in a second screen by real-time PCR analysis of cDNA from different brain regions (telencephalon, diencephalon, mesencephalon, cerebellum, and hypothalamic infundibulum). A ReverTra Ace kit (Toyobo, Osaka, Japan) was used for real-time PCR analyses. One unknown gene was specifically amplified in the hypothalamic infundibulum. The open reading frame of the unknown gene was sequenced using the 3′-rapid amplification of cDNA ends (RACE) method with a 3′-Full RACE Core Set kit (TaKaRa Bio, Shiga, Japan) as described previously [19].

Subsequently, the expression of the unknown gene was examined in different regions of the brain in 1-day-old chicks ($n = 5$) and during development in the hypothalamic infundibulum in 1-, 8-, and 15-day-old chicks ($n = 5$) by amplifying cDNA. The following primers were used: sense primer, 5′-CTAGGAAAAAGACAGCTTGC-3′ and antisense primer, 5′-CTTTCTTCGTCAGAACTGGT-3′. PCR amplifications were carried out with the THUNDERBIRD SYBR qPCR Mix (Toyobo) using the following program: 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. The PCR products in each cycle were monitored using a StepOne Real-Time Thermal Cycler (Applied Biosystems). mRNA levels in the brain were quantified relative to the level of β-actin (ACTB) mRNA, which was used as an internal control.

2.5. In situ hybridization

To study the localization of cells that contained mRNA encoding the small protein precursor, 1-, 8-, and 15-day-old chicks ($n = 5$) were sacrificed, and their brains were fixed in 4% paraformaldehyde solution overnight at 4 °C. After fixation, the brains were placed in a refrigerated sucrose solution (30% sucrose in 10 mM phosphate-buffer, pH 7.4) until they sank. The tissues were embedded in Tissue-Tek OCT Compound (Sakura Finetek, Tokyo, Japan) and sectioned coronally at a thickness of 14 μm with a cryostat at −20 °C. The sections were then mounted on slides.

The procedure for *in situ* hybridization was similar to that described previously [19]. Digoxigenin (DIG)-labeled antisense and sense RNA probes were produced from a section of the small protein precursor cDNA sequence using a DIG RNA Labeling kit with SP6 and T7 polymerases (Roche Diagnostics, Basel, Switzerland). The DIG-labeled sense RNA probe, which was complementary to the antisense probe sequence, was used as a control to verify specificity.

2.6. Production of the small protein

The 80-amino acid small protein was synthesized with Fmoc chemistry using a peptide synthesizer (Syro Wave; Biotage, Uppsala, Sweden). After synthesis, the small protein was cleaved from the resin with reagent K (82.5% trifluoroacetic acid, 5% phenol, 5% thioanisole, 5% H_2O, and 2.5% 1,2-ethanedithiol) for 3 h. The small protein was then purified by reverse-phase high-performance liquid chromatography (HPLC) using a C18 column (YMC-Pack Pro

C18, 10 × 150 mm; YMC, Kyoto, Japan) at a flow rate of 1.0 mL/min for 40 min with a linear gradient of 40–60% acetonitrile containing 0.1% trifluoroacetic acid. The solvent was evaporated and lyophilized. Intramolecular disulfide bond formation was induced by incubation in oxidizing solution (0.5 mM glutathione disulfide, 5 mM glutathione, 50% acetonitrile, 1 mM EDTA, 10% DMSO, 0.4 M Tris–HCl, pH 8.5) for 2 days. The mature small protein was further purified by HPLC and then lyophilized.

2.7. Subcutaneous infusion of the small protein

Chicks were placed in individual cages before the experiments. They were weighed and divided into experimental treatment groups as uniformly as possible. Eight-day-old chicks ($n = 8$) were infused subcutaneously with the small protein (6 nmol/day) dissolved in 30% propylene glycol or with vehicle using an Alzet mini-osmotic pump (model 2001; delivery rate 1.0 μL/h; DURECT Corporation, Cupertino, CA, USA) as previously described [20]. The body weight and food weight in the feeder were measured between 9:00 and 10:00 every day.

2.8. Statistical analysis

Data were analyzed with Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test as a post hoc test. The significance level was set at $P < 0.05$. All results are expressed as the mean ± SEM.

3. Results

3.1. Identification of cDNA encoding a novel protein

Differential screening after subtractive hybridization combined with suppression PCR yielded 596 cDNA clones that were highly expressed in the hypothalamic infundibulum. The nucleotide sequences were determined by DNA sequencing. Subsequently, 20 unknown nucleotide sequences were further analyzed in a second screen using real-time PCR. Finally, one cDNA highly expressed in the hypothalamic infundibulum was selected for further study. The nucleotide sequence of the open reading frame was read by the RACE method. The open reading frame was 549 bp, and the deduced protein consisted of 182 amino acid residues (Fig. 1). The nucleotide sequence has been deposited in the DDBJ/EMBL/GenBank databases (accession no. AB909129).

Analysis of the N-terminal sequence of the deduced protein with the SignalP program (www.cbs.dtu.dk/services/SignalP/) revealed the presence of a typical 32-amino acid signal peptide (Fig. 1). The predicted 80-amino acid residue small protein is flanked at the C-terminus by a dibasic Lys–Arg motif, which constitutes a potential proteolytic processing site (Fig. 1). The precursor protein also possesses a Gly residue at its C-terminal end that contributes to amidation (Fig. 1). Because the predicted C-terminal amino acid sequence of the small protein is Gly-Leu-NH_2, the small protein was named neurosecretory protein GL (NPGL). NPGL contains two Cys residues, suggesting intramolecular disulfide bond formation (Fig. 1).

Subsequently, we performed a TBLASTN search on the genome database (Ensembl Genome Browser; www.ensembl.org) using the amino acid sequence of the NPGL precursor. We identified orthologous genes in human and rat and paralogous genes in chicken, human, and rat. The alignment of the precursor protein sequences deduced from the orthologous and paralogous genes in chicken, human, and rat is shown in the Supplementary data. Each of the precursor proteins consists of a signal peptide sequence at the N-terminus, a mature small protein sequence, a Gly amidation donor and a dibasic cleavage site (Lys/Arg–Arg) at the C-terminus of the small protein, and an extended C-terminal sequence. In addition, two Cys residues were conserved. The alignment revealed high sequence homology between the predicted mature small proteins. Because the C-terminal amino acid sequence of the mature small proteins derived from the paralogous genes is Gly-Met-NH_2 rather than Gly-Leu-NH_2, we named the paralogous small protein neurosecretory protein GM (NPGM).

3.2. Expression of the NPGL precursor mRNA

The NPGL precursor mRNA levels within different regions of the brain, such as the telencephalon, diencephalon, mesencephalon,

```
ATGGATTTTGGTAACAGAGGGAGAATCCACTACAACATGAGGCTCACCTACTCTCTGCTA 60
M  D  F  G  N  R  G  R  I  H  Y  N  M  R  L  T  Y  S  L  L    20
GTCATGGGAGTGTTCTGTGTGACACCTTCCCTCTGCCATAGCCAGATTGATCCACTGGCT 120
V  M  G  V  F  C  V  T  P  S  L  C  H  S  Q  I  D  P  L  A    40
CTTGGGCGAGCAGACCCTCAGTGCTGGGAATCCTCCTCAGCTGTATTACTGGAGATGAGG 180
L  G  R  A  D  P  Q  C  W  E  S  S  S  A  V  L  L  E  M  R    60
AAGCCTCGCATTTCTGACTCTGTCAGTGGCTTTTGGGACTTCATGATCTTCCTGAAATCA 240
K  P  R  I  S  D  S  V  S  G  F  W  D  F  M  I  F  L  K  S    80
TCAGAAAACTTGAAACATGGGGCTCTGTTCTGGGACCTGGCTCAGCTATTCTGGGATATC 300
S  E  N  L  K  H  G  A  L  F  W  D  L  A  Q  L  F  W  D  I   100
TATGTGGACTGTGTGCTCTCCAGAACCCATGGCCTAGGAAAAAGACAGCTTGCAAAAGCT 360
Y  V  D  C  V  L  S  R  T  H  G  L  G  K  R  Q  L  A  K  A   120
CAACAGAGGATCACTACTCTACCTTCTCAGTTCACAGGGAGAAACCAAGGGATGTTCACT 420
Q  Q  R  I  T  T  L  P  S  Q  F  T  G  R  N  Q  G  M  F  T   140
CATATTCAGAGGTCACCAGTTCTGACGAAGAAAGACTTCTTTGAAGATTTAATAAAAAAC 480
H  I  Q  R  S  P  V  L  T  K  K  D  F  F  E  D  L  I  K  N   160
CACAAGCATAAGAGTAGATCTACGTTACTTGGAAGAATCACTGGAGAGCTAGGGAAAAAG 540
H  K  H  K  S  R  S  T  L  L  G  R  I  T  G  E  L  G  K  K   180
AGGAAATGA                                                     549
R  K  *                                                       182
```

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the neurosecretory protein Gly-Leu (NPGL) precursor. The predicted signal peptide is denoted by a wavy line. The sequence of the predicted 80-amino acid residue mature small protein is presented in boldface. The Gly (G) C-terminal amidation signal and the Lys (K)–Arg (R) dibasic processing site are underlined. Two Cys (C) residues are boxed. The asterisk indicates the stop codon (TGA).

cerebellum, and hypothalamic infundibulum, were examined in 1-day-old chicks by real-time PCR. The NPGL precursor mRNA was exclusively expressed in the hypothalamic infundibulum; its expression in other regions of the brain was near background levels (Fig. 2A).

Furthermore, we measured the NPGL precursor mRNA levels in the hypothalamic infundibulum during post-hatching development in 1-, 8-, and 15-day-old chicks. Expression in 8- and 15-day-old chicks was higher than that in 1-day-old chicks (Fig. 2B). The expression in 8- and 15-day-old chicks did not differ significantly.

The localization of cells containing the NPGL precursor mRNA was further analyzed by *in situ* hybridization (Fig. 3). Cells expressing the NPGL precursor mRNA were distributed in the medial mammillary nucleus (MM) of the hypothalamic infundibulum in 1-day-old chicks (Fig. 3C), but not in the IN (Fig. 3D). On the other hand, in 8- and 15-day-old chicks, cells expressing the NPGL precursor mRNA were localized in the MM and IN of the hypothalamic infundibulum (Fig. 3E and F). Positive signals were observed with the antisense probe, but not with the sense probe (data not shown).

3.3. Effect of subcutaneous small protein infusion

To elucidate the biological actions of NPGL in chicks, we tested the effects of NPGL infusion on feeding behavior and body weight gain. NPGL infusion significantly increased body weight gain in chicks until 3 days after surgery (Fig. 4A). On the other hand, NPGL did not affect food intake at any of the time points examined, although there was a trend toward increased food intake (1 day after surgery: $P = 0.077$; 3 days after surgery: $P = 0.057$) (Fig. 4B).

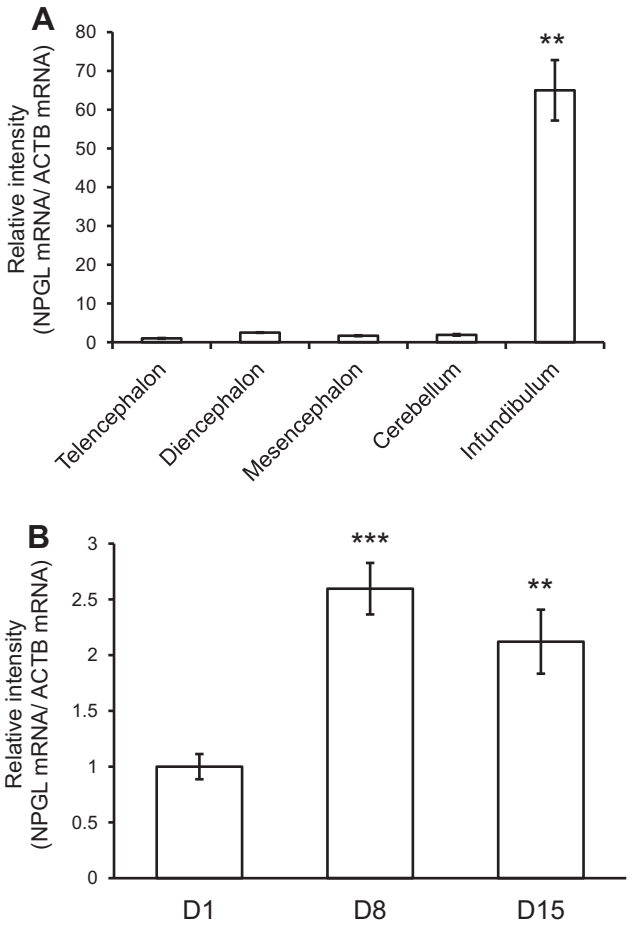

Fig. 2. Real-time PCR analyses of the NPGL precursor mRNA concentrations in different brain regions (A) and in the hypothalamic infundibulum during development in 1-, 8-, and 15-day-old chicks (B). The NPGL precursor mRNA levels were quantified relative to the level of β-actin (ACTB) mRNA. Each value represents the mean ± SEM ($n = 5$). Asterisks indicate statistically significant differences ($^{**}P < 0.01$, $^{***}P < 0.001$).

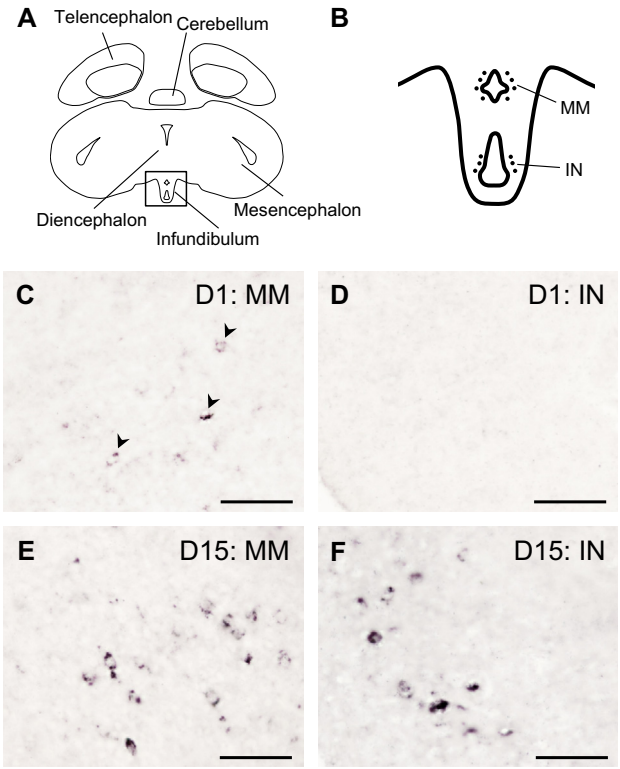

Fig. 3. Schematic representation and photomicrographs illustrating the distribution of cells expressing the NPGL precursor mRNA in the hypothalamic infundibulum. The location of the hypothalamic infundibulum is shown in the coronal brain illustration (A). Cells expressing the NPGL precursor mRNA in the medial mammillary nucleus (MM) and infundibular nucleus (IN) within the hypothalamic infundibulum are represented by dots in the illustration (B). Photomicrographs of cells expressing the NPGL precursor mRNA in the MM (C and E) and IN (D and F) were obtained after performing *in situ* hybridization with coronal brain sections from 1- (C and D) and 15-day-old (E and F) chicks. The arrowheads indicate positive cells. Scale bars = 50 μm.

4. Discussion

In the present study, we identified a cDNA encoding a novel 80-amio acid residue protein in the hypothalamic infundibulum. This small neurosecretory protein, which does not belong in any of the known protein families in animals, was designated NPGL after the last two C-terminal amino acids. The precursor protein contains an N-terminal signal peptide and the NPGL protein followed by a Gly amidation signal and a dibasic amino acid cleavage site, as well as the precursors of other bioactive peptides [4–6,8–14]. This structure suggests that NPGL is a secretory signaling molecule. After a database search, we identified two paralogous genes encoding NPGL and NPGM in birds and mammals (Supplementary data). The amino acid sequences of the predicted mature NPGL and NPGM molecules are highly conserved, whereas the sequences after the dibasic cleavage site are very different. Furthermore, NPGL possesses two Cys residues, which likely form an intramolecular disulfide bond. The positions of the Cys residues are the same in NPGL and NPGM. Therefore, these two genes may have derived

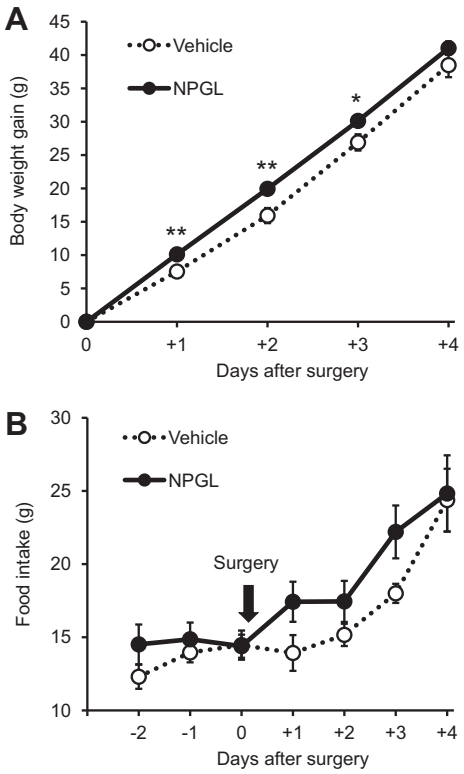

Fig. 4. Effects of subcutaneous infusion of vehicle or NPGL (6 nmol/day) on body weight gain (A) and food intake (B). Body weight gain after infusion was calculated using the pre-surgical body weight as the baseline (A). The 24-h food intake is shown as one data point every day (B). Data are expressed as the mean ± SEM ($n = 8$). Asterisks indicate statistically significant differences ($^{*}P < 0.05$, $^{**}P < 0.01$).

from a common ancestral gene. Future studies will investigate the biological actions of NPGL and NPGM in various animals.

The NPGL precursor mRNA was exclusively expressed in the hypothalamic infundibulum. The expression level was low just after hatching and increased until the chicks were 8 days old. These data are consistent with the morphological observations from *in situ* hybridization studies. Some cells expressing the NPGL precursor mRNA were scattered in the MM within the hypothalamic infundibulum in 1-day-old chicks. On the other hand, in 8- and 15-day-old chicks, intense signals were detected in both the MM and IN. These results suggest that NPGL is necessary for post-hatching development and growth. Although the functional significance of the MM in the avian brain is not well understood, the function of the avian IN appears to be identical to that of the mammalian Arc, a brain center that controls feeding and body weight [21]. We speculated that NPGL participates in feeding behavior. Therefore, NPGL was administered to chicks by acute intracerebroventricular injection. However, we did not observe a significant effect on food intake (data not shown). Given that the avian hypothalamic infundibulum in the mediobasal hypothalamic region appears to contain many hypothalamic hypophysiotropic hormones [22], it is possible that NPGL is released into the pituitary gland via the ME to act as a hypothalamic hypophysiotropic hormone. Therefore, we investigated the effect of subcutaneous chronic infusion of NPGL on development and growth. NPGL increased body weight gain when compared with vehicle. However, NPGL did not significantly affect daily food intake. Taken together, these findings suggest that NPGL acts on the pituitary gland, particularly on somatotropic cells, through the blood, promotes the release of growth hormone, and accelerates somatic growth in chicks. In future studies, we will investigate the effect of NPGL on growth hormone release *in vitro* and *in vivo*. In addition, the effects of NPGL on energy expenditure and the weight of visceral tissues, including fat and muscle, remain to be investigated.

The effect of NPGL on body weight gain lasted for 3 days after the beginning of infusion. The effect disappeared 4 days after infusion, when body weight gain was similar to that in vehicle-treated chicks. The transient effect of NPGL may be attributable to the gradual denaturation of NPGL in the osmotic minipump. In the present study, we used disulfide-bonded NPGL because the reduced form of NPGL became insoluble. Thus, disulfide bonding may be necessary for the solubility, stability, and activity of NPGL. Future studies should focus on phenotypic analysis of NPGL overexpression and/or knockdown in chicks to determine the exact physiological functions of NPGL.

In summary, we identified a novel secretory molecule, NPGL, in the chicken hypothalamus that may have a physiological role in growth regulation. Identification of the cognate receptor for NPGL will help elucidate the mechanisms of NPGL activity in the chicken brain. The receptor for NPGL may be present in the orphan GPCR libraries [7]. We are searching for the cognate NPGL receptor.

Disclosure statement

The authors declare that there are no conflicts of interest.

Acknowledgments

We are grateful to Dr. Yojiro Muneoka (Professor Emeritus at Hiroshima University, Japan), Dr. Yumiko Saito and Dr. Yasuo Furukawa (Hiroshima University, Japan), Dr. Noboru Murakami (University of Miyazaki, Japan) for their valuable discussion and encouragement. This work was supported by MEXT/JSPS KAKENHI Grants (22687004 to K.U. and 25440171 to E.I.-U.), the Toray Science Foundation (K.U.), the Kieikai Research Foundation (K.U.), and the Program for Promotion of Basic and Applied Research for Innovations in Bio-oriented Industry (K.U.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.090.

References

[1] E. Marder, Neuromodulation of neuronal circuits: back to the future, Neuron 76 (2012) 1–11.
[2] T. Hökfelt, C. Broberger, Z.-Q.D. Xu, V. Sergeyev, R. Ubink, M. Diez, Neuropeptides – an overview, Neuropharmacology 39 (2000) 1337–1356.
[3] O. Civelli, GPCR deorphanizations: the novel, the known and the unexpected transmitters, Trends Pharmacol. Sci. 26 (2005) 15–19.
[4] T. Sakurai, A. Amemiya, M. Ishii, I. Matsuzaki, R.M. Chemelli, H. Tanaka, S.C. Williams, J.A. Richardson, G.P. Kozlowski, S. Wilson, J.R. Arch, R.E. Buckingham, A.C. Haynes, S.A. Carr, R.S. Annan, D.E. McNulty, W.S. Liu, J.A. Terrett, N.A. Elshourbagy, D.J. Bergsma, M. Yanagisawa, Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior, Cell 92 (1998) 573–585.
[5] M. Kojima, H. Hosoda, Y. Date, M. Nakazato, H. Matsuo, K. Kangawa, Ghrelin is a growth-hormone-releasing acylated peptide from stomach, Nature 402 (1999) 656–660.
[6] M. Kotani, M. Detheux, A. Vandenbogaerde, D. Communi, J.M. Vanderwinden, E. Le Poul, S. Brézillon, R. Tyldesley, N. Suarez-Huerta, F. Vandeput, C. Blanpain, S.N. Schiffmann, G. Vassart, M. Parmentier, The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54, J. Biol. Chem. 276 (2001) 34631–34636.
[7] O. Civelli, R.K. Reinscheid, Y. Zhang, Z. Wang, R. Fredriksson, H.B. Schiöth, G protein-coupled receptor deorphanizations, Annu. Rev. Pharmacol. Toxicol. 53 (2013) 127–146.
[8] Y. Takei, K. Inoue, M. Ogoshi, T. Kawahara, H. Bannai, S. Miyano, Identification of novel adrenomedullin in mammals: a potent cardiovascular and renal regulator, FEBS Lett. 556 (2004) 53–58.
[9] Y.L. Xu, R.K. Reinscheid, S. Huitron-Resendiz, S.D. Clark, Z. Wang, S.H. Lin, F.A. Brucher, J. Zeng, N.K. Ly, S.J. Henriksen, L. de Lecea, O. Civelli, Neuropeptide S: a

neuropeptide promoting arousal and anxiolytic-like effects, Neuron 43 (2004) 487–497.
[10] K. Mori, M. Miyazato, T. Ida, N. Murakami, R. Serino, Y. Ueta, M. Kojima, K. Kangawa, Identification of neuromedin S and its possible role in the mammalian circadian oscillator system, EMBO J. 24 (2005) 325–335.
[11] S. Oh-I, H. Shimizu, T. Satoh, S. Okada, S. Adachi, K. Inoue, H. Eguchi, M. Yamamoto, T. Imaki, K. Hashimoto, T. Tsuchiya, T. Monden, K. Horiguchi, M. Yamada, M. Mori, Identification of nesfatin-1 as a satiety molecule in the hypothalamus, Nature 443 (2006) 709–712.
[12] H. Yamaguchi, K. Sasaki, Y. Satomi, T. Shimbara, H. Kageyama, M.S. Mondal, K. Toshinai, Y. Date, L.J. González, S. Shioda, T. Takao, M. Nakazato, N. Minamino, Peptidomic identification and biological validation of neuroendocrine regulatory peptide-1 and -2, J. Biol. Chem. 282 (2007) 26354–26360.
[13] K. Ukena, K. Tsutsui, A new member of the hypothalamic RF-amide peptide family, LPXRF-amide peptides: structure, localization, and function, Mass Spectrom. Rev. 24 (2005) 469–486.
[14] K. Ukena, T. Tachibana, Y. Tobari, J. Leprince, H. Vaudry, K. Tsutsui, Identification, localization and function of a novel neuropeptide, 26RFa, and its cognate receptor, GPR103, in the avian hypothalamus, Gen. Comp. Endocrinol. 190 (2013) 42–46.
[15] G.J. Morton, D.E. Cummings, D.G. Baskin, G.S. Barsh, M.W. Schwartz, Central nervous system control of food intake and body weight, Nature 443 (2006) 289–295.
[16] E. Szarek, P.S. Cheah, J. Schwartz, P. Thomas, Molecular genetics of the developing neuroendocrine hypothalamus, Mol. Cell. Endocrinol. 323 (2010) 115–123.
[17] K.-H. Kim, B.L. Seong, Peptide amidation: production of peptide hormones *in vivo* and *in vitro*, Biotechnol. Bioprocess Eng. 6 (2001) 244–251.
[18] L. Diatchenko, S. Lukyanov, Y.F. Lau, P.D. Siebert, Suppression subtractive hybridization: a versatile method for identifying differentially expressed genes, Methods Enzymol. 303 (1999) 349–380.
[19] K. Ukena, T. Tachibana, E. Iwakoshi-Ukena, Y. Saito, H. Minakata, R. Kawaguchi, T. Osugi, Y. Tobari, J. Leprince, H. Vaudry, K. Tsutsui, Identification, localization, and function of a novel avian hypothalamic neuropeptide, 26RFa, and its cognate receptor, G protein-coupled receptor-103, Endocrinology 2151 (2010) 2255–2264.
[20] R. Moriya, H. Sano, T. Umeda, M. Ito, Y. Takahashi, M. Matsuda, A. Ishihara, A. Kanatani, H. Iwaasa, RFamide peptide QRFP43 causes obesity with hyperphagia and reduced thermogenesis in mice, Endocrinology 147 (2006) 2916–2922.
[21] W.J. Kuenzel, M.M. Beck, R. Teruyama, Neural sites and pathways regulating food intake in birds: a comparative analysis to mammalian systems, J. Exp. Zool. 283 (1999) 348–364.
[22] S. Mikami, S. Yamada, Immunohistochemistry of the hypothalamic neuropeptides and anterior pituitary cells in the Japanese quail, J. Exp. Zool. 232 (1984) 405–417.