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Effects of chronic intracerebroventricular infusion of neurosecretory protein GL on body mass and food and water intake in chicks

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ABSTRACT

Recently, we discovered a novel cDNA encoding the precursor of a small secretory protein, neurosecretory protein GL (NPGL), in the chicken mediobasal hypothalamus. In this study, immunohistochemical analysis revealed that NPGL was produced in the infundibular and medial mammillary nuclei of the mediobasal hypothalamus, with immunoreactive fibers also detected in the hypothalamus and the median eminence. As it is known that these regions are involved in feeding behavior in chicks, we surveyed the effects of chronic intracerebroventricular infusion of NPGL on feeding behavior and body mass for a period of two weeks. NPGL stimulated food and water intake, with a concomitant increase in body mass. However, NPGL did not influence mRNA expression of several hypothalamic ingestion-related neuropeptides. Our data suggest that NPGL may be a novel neuronal regulator involved in growth processes in chicks.

1. Introduction

Shortly after hatching, young chickens have relatively well-developed mechanisms that regulate feeding behavior, and these influence growth and lipid metabolism (Noy and Sklan, 2002; Richards et al., 2010). For example, newly-hatched broiler chicks which were food-restricted for two weeks had lower body and muscle masses and altered mRNA expression of growth-related genes in the muscle (Bigot et al., 2003; Li et al., 2007). It is likely that the feeding in chicks is regulated by similar orexigenic and anorexigenic factors to those found in mammalian systems, but these factors may operate differentially in birds and mammals. In mammals, neuropeptide Y (NPY), agouti-related protein (AgRP), orexin, melanin-concentrating hormone (MCH), ghrelin, and growth hormone releasing hormone (GHRH) are known to stimulate feeding behavior (Lawrence et al., 1999; Schwartz et al., 2000). On the contrary, α-melanocyte stimulating hormone (α-MSH), cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), corticotropin-releasing factor (CRF) and histamine are known to suppress feeding behavior in mammals (Lawrence et al., 1999; Schwartz et al., 2000). Unlike in mammals, orexin and MCH do not appear to affect feeding behavior in chicks, and ghrelin and GHRH can decrease food intake (Tachibana and Tsutsui, 2016). These findings suggest that feeding-related neuropeptides have differential effects in mammals and chickens. In addition, other, unknown, neuronal factors may be involved in the regulation of feeding behavior.

Recently, we identified a novel cDNA encoding the precursor of a neurosecretory small protein in the chicken mediobasal hypothalamus (Ukena et al., 2014). The precursor protein contained a signal peptide sequence, a mature protein sequence of 80 amino acid residues, 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). Following *in situ* hybridization (ISH), *NPGL* mRNA was found to be localized in the medial mammillary nucleus (MM) and the infundibular nucleus (IN) within the mediobasal hypothalamus (Ukena et al., 2014). The MM and IN in chicks are likely to be equivalent to the tuberomammillary nucleus (TMN) and the arcuate nucleus (Arc), regions known to be feeding centers in mammals, respectively. We also reported that mRNA levels of *NPGL* increased during post-hatching development

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(Ukena et al., 2014). Furthermore, we found that chronic subcutaneous infusion of NPGL increased body mass gain without affecting food intake in chicks (Ukena et al., 2014). These findings suggest that NPGL may participate in the development processes of chicks.

In the present study, we first attempted to detect, using immunohistochemistry (IHC), the production of the transcript of *NPGL* as a mature small protein. Subsequently, we elucidated the effect of chronic intracerebroventricular (i.c.v.) infusion of NPGL on body mass gain and feeding behavior in chicks.

2. Materials and methods

2.1. Animals

Male layer chicks (*Gallus domesticus*, one-day old) were purchased commercially (Nihon Layer, Gifu, Japan) and housed in a windowless room maintained at 28 °C on a 20-h light (4:00–24:00) and 4-h dark (0:00–4:00) cycle. The chicks were provided with 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. Production of chicken NPGL and antibodies

Chicken NPGL was synthesized with Fluorenylmethyloxycarbonyl (Fmoc) chemistry using a peptide synthesizer (Syro Wave; Biotage, Uppsala, Sweden) according to our previous method (Masuda et al., 2015). The small protein was cleaved from the resin with reagent K (trifluoloacetic acid: TFA 82.5%, phenol 5%, thioanisol 5%, H_2O 5%, and 1,2-ethanedithiol 2.5%) for 3 h. The crude small protein was purified by reverse-phase high-performance liquid chromatography (HPLC) using a C18 column (YMC-Pack ProC18, 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% TFA. The solvent was evaporated and lyophilized. The small protein was dissolved in dimethyl sulfoxide (DMSO) and then diluted to a final concentration of 0.5 mM glutathione disulfide, 5 mM glutathione, 50% acetonitrile, 1 mM EDTA, 10% DMSO, 0.4 M Tris-HCl (pH 8.5). The solvent was rotated for 2 days at room temperature, purified by HPLC and then lyophilized.

Guinea pig antisera were produced following our published method (Ukena et al., 2010) using synthetic chicken NPGL as the antigen. The antigen solution of 125 μg/animal was mixed with Freund's complete adjuvant and injected into four guinea pigs. After 3 booster injections, blood was collected from each guinea pig and the optimal serum with the highest titer was selected via a dot-blot analysis.

2.3. Immunohistochemistry

After chicks (eight-day-old) were sacrificed by decapitation, brains were extracted and fixed in 4% paraformaldehyde solution as described previously (Ukena et al., 2014). The brain was sectioned into 20 μm slices on a cryostat following cryoprotection and freezing. The sections were then mounted on slides. After blocking with 1% skim milk in PBS (10 mM, pH 7.4) containing 0.3% TritonX-100 for 1 h, the sections were incubated with the guinea pig *anti*-NPGL antibody (1: 1000 dilution) overnight at 4 °C. This primary immunoreaction was followed by incubation with Alexa Fluor 488-conjugated donkey *anti*-guinea pig IgG (1:1000 dilution, 706-545-148, Jackson ImmunoResearch, West Grove, PA). Preadsorption was performed as a control using the antigen, NPGL (100 μg/ml). The antigen and primary antibody were incubated overnight prior to running IHC in the same way as for the non-control slides.

2.4. Infusion of NPGL for 2 weeks

Eight-day-old chicks were i.c.v. infused with 0 (vehicle) or 15 nmol/day NPGL using an infusion cannula (model 328OP; Plastics One, Roanoke, VA) and an Alzet mini-osmotic pump (model 2001, delivery rate 1.0 μl/h; DURECT Corporation, Cupertino, CA). The dose was determined on the basis of a previous study (Rich et al., 2007). The infusion cannula tip was implanted into the lateral ventricle: 2.0 mm rostral to lambda, 1.0 mm lateral to midline, and 5.5 mm ventral to the skull surface. Osmotic mini-pumps containing vehicle or NPGL were implanted subcutaneously in the neck.

Body mass and food and water intake were measured daily for the duration of the experiment at the same time of day.

2.5. Real-time RT-PCR

After two weeks of i.c.v. NPGL infusion, chicks were sacrificed by decapitation between 13:00–15:00. The mediobasal hypothalamus which includes the IN, ventromedial hypothalamus, periventricular stratum and mammillary nucleus could be identified by the way it protruded from the brain and it was dissected out using fine forceps and small scissors and snap-frozen in liquid nitrogen for further RNA processing. RNA from the mediobasal hypothalamus was extracted using an RNAqueous-Micro kit (Ambion, Austin, TX) in accordance with the manufacturer's instructions. First-strand cDNA was synthesized from total RNA using a ReverTra Ace kit (TOYOBO, Osaka, Japan).

PCR amplifications were conducted with the THUNDERBIRD SYBR qPCR Mix (TOYOBO) using the following procedure: 95 °C for 20 s, followed by 40 cycles at 95 °C for 3 s, and at 60 °C for 30 s using a real-time thermal cycler (Step One; Applied Biosystems, Foster City, CA). *NPY*, *AgRP*, *proopiomelanocortin (POMC)*, *GLP-1*, *CCK*, *angiotensinogen (AGT)*, *angiotensin converting enzyme (ACE)* and *β-actin (ACTB)* amplifications were performed with the primer sets listed in Table 1. The relative quantification for each

Table 1
Sequences of PCR oligonucleotide primers used in this study.

Name	Sequence
for NPY	
Sense	5′-ACATGGCCAGATACTACTCG-3′
Antisense	5′-ACAAGAGGTCTGAGATCAGTG-3′
for AgRP	
Sense	5′-AGGCCAGACTTGGATCAGATG-3′
Antisense	5′-ACTCCAGGAGGCGGACAC-3′
for POMC	
Sense	5′-AGGAGTCGGCTGAGAGTTA-3′
Antisense	5′-TTCCTCTTCCTCCTCTTCTT-3′
for GLP-1	
Sense	5′-CGTCATTCACAAGGCACATTC-3′
Antisense	5′-GTCATTCTCTTTGTCCTCCTGTCC-3′
for CCK	
sense	5′-AGGTTCCACTGGGAGGTTCT-3′
antisense	5′-CGCCTGCTGTTCTTTAGGAG-3′
for AGT	
Sense	5′-AGCAGGTTTGAGAGGCAATGA-3′
Antisense	5′-GATTCCACCACTTCCCCAGG-3′
for ACE	
Sense	5′-GCCAAACTCAGGGAGGTGTT-3′
Antisense	5′-CCCCAGCGTCCATCTTATCC-3′
for ACTB	
Sense	5′-CCAGAGTCCATCACAATACC-3′
Antisense	5′-AGCCAACAGAGAGAAGATGA-3′

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