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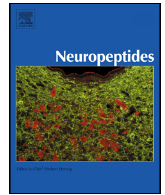

Gonadotropin-inhibitory hormone-stimulation of food intake is mediated by hypothalamic effects in chicks

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Gonadotropin-inhibitory hormone (GnIH), a 12 amino acid peptide, is expressed in the avian brain and inhibits luteinizing hormone secretion. Additionally, exogenous injection of GnIH causes increased food intake of chicks although the central mechanism mediating this response is poorly understood. Hence, the purpose of our study was to elucidate the central mechanism of the GnIH orexigenic response using 12 day post hatch layer-type chicks as models. Firstly, via mass spectrometry we deduced the chicken GnIH amino acid sequence: SIRPSAYLPLRFamide. Following this we used chicken GnIH to demonstrate that intracerebroventricular (ICV) injection of 2.6 and 7.8 nmol causes increased food intake up to 150 min following injection with no effect on water intake. The number of c-Fos immunoreactive cells was quantified in appetite-associated hypothalamic nuclei following ICV GnIH and only the lateral hypothalamic area (LHA) had an increase of c-Fos positive neurons. From whole hypothalamus samples following ICV GnIH injection abundance of several appetite-associated mRNA was quantified which demonstrated that mRNA for neuropeptide Y (NPY) was increased while mRNA for proopiomelanocortin (POMC) was decreased. This was not the case for mRNA abundance in isolated LHA where NPY and POMC were not affected but melanin-concentrating hormone (MCH) mRNA was increased. A comprehensive behavior analysis was conducted after ICV GnIH injection which demonstrated a variety of behaviors unrelated to appetite were affected. In sum, these results implicate activation of the LHA in the GnIH orexigenic response and NPY, POMC and MCH are likely also involved.

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1. Introduction

The dodecapeptide gonadotropin-inhibitory hormone (GnIH) was first isolated from the quail brain and was found to inhibit luteinizing hormone secretion from the anterior pituitary (Tsutsui et al., 2000). This was the first report of hypothalamic inhibition of gonadotropin release in any vertebrate species, and there has since been an extensive amount of research focused on the reproductive aspects of GnIH in the avian class (Tsutsui et al., 2012).

In birds, GnIH is found throughout the diencephalon and mesencephalon (Bentley et al., 2003; Ubuka et al., 2008; Ukena et al., 2003), with the most dense concentration of GnIH-immunoreactive cell bodies in the paraventricular nucleus (PVN) of the hypothalamus (Tsutsui et al., 2000). The receptor for GnIH was identified in Japanese quail and is distributed throughout the diencephalon (Yin et al., 2005). GnIH is a member of the RFamide neuropeptide family, a vast group of peptides that all have terminal arginine (R) and amidated phenylalanine (F) amino acids (Bechtold and Luckman, 2007). This family of peptides is associated with a range of physiological effects across a spectrum of species, from invertebrates to humans. Members of the RFamide family have received considerable attention for their effect on appetite-associated processes. Indeed, we reported that exogenous RFamides affect chick food intake (Cline and Mathews, 2008; Cline and Sliwa, 2009; Cline et al., 2007, 2008, 2009, 2010; Newmyer and Cline, 2009). Because GnIH is found in the PVN, a major appetite center, and is also an RFamide, it is not surprising that it also affects appetite.

The appetite-associated effect of quail GnIH and its related peptides (GnIH-RP1 and GnIH-RP2) was first documented in the avian

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class using 6 day old layer chicks (Tachibana et al., 2005). Central injection of quail GnIH increased food intake at 60 and 120 min following injection in *ad libitum* fed chicks whereas anti-GnIH antiserum reduced food intake at 30 and 60 min following injection in fasted chicks. Through opioid receptor antagonism it was demonstrated that the orexigenic effect of GnIH was mediated via mu receptors, but not delta or kappa type receptors in chicks (Tachibana et al., 2008).

Because quail rather than chicken GnIH has been used in all past chicken reports associated with appetite, we designed the study reported herein to identify chicken GnIH and further investigate the central mechanism of GnIH's orexigenic effect in chicks, with a focus on the hypothalamus. We measured individual appetite-associated hypothalamic nuclei activation after central GnIH injection, determined the effect of GnIH on mRNA abundance of appetite-associated neurotransmitters, and also conducted a comprehensive behavior analysis.

2. Methods

2.1. Animals

For the identification of chicken GnIH, Julia male layer chicks were obtained from a local hatchery and housed in groups in metal cages at a constant temperature of 30 ± 1 °C under continuous light until 14 days of age. Diet (commercial starter diet (energy: 3050 kcal ME/kg and 24% CP); Toyohashi Feed and Mills Co. Ltd., Aichi, Japan) and water were available *ad libitum*. For the functional analysis of identified chicken GnIH, Leghorn chicks, hatched at Virginia Tech, were used. Chicks were group caged for 1 d, then individually in a room at 30 ± 2 °C and 50 ± 5% relative humidity where they had *ad libitum* access to a mash diet (20% crude protein, 2,685 kcal ME/kg) and tap water. The individual cages allowed visual and auditory contact with other chicks. Chicks were handled twice daily to adapt to handling. All trials were conducted between 08:00 and 13:00 h using 14 d post hatch chicks, an aged based upon our previous report (Chowdhury et al., 2012). Experimental procedures for chicken GnIH identification were performed according to the Faculty of Agriculture of Kyushu University and the Law (No. 105) and Notification (No. 6) of the Japanese Government. All other procedures were performed according to the National Research Council publication, Guide for Care and Use of Laboratory Animals and were approved by the Virginia Tech Animal Care and Use Committee.

2.2. Experiment 1: identification of chicken GnIH by immunoaffinity purification and mass spectrometry

To identify endogenous mature peptides in the chicken brain, we carried out affinity purification and immunoassay with the antiserum raised against quail GnIH. Diencephalon including mesencephalon (n = 40) were boiled for 7 min and homogenized in 5% acetic acid, as described previously (Chowdhury et al., 2011; Osugi et al., 2006; Tsutsui et al., 2000; Ubuka et al., 2008). The homogenate was centrifuged at 15,000 g for 20 min at 4 °C and the supernatant was collected. After precipitation with 75% acetone, the supernatant was passed through a disposable C-18 cartridge column (Mega Bond-Elut; Varian, Harbor, CA, USA) and the retained material (RM) eluted with 60% methanol was loaded onto an immunoaffinity column. The affinity chromatography was carried out as described elsewhere (Chowdhury et al., 2011; Osugi et al., 2006; Sawada et al., 2002; Ubuka et al., 2008). The antibodies against GnIH were conjugated to CNBr-activated Sepharose 4B as an affinity ligand. The brain extract was applied to the immunoaffinity column at 4 °C and the adsorbed materials were eluted with 0.3 M acetic acid containing 0.1% 2-mercaptoethanol. The eluted fractions were concentrated and subjected to a reversed-phase HPLC column (ODS-80TM; Tosoh, Tokyo, Japan) with gradients of acetonitrile (10–50%/100 min) containing 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min, and fractioned every 2 min for 4–104 min. The immunoreactive fractions were assayed by a dot immunoblot assay, and the molecular mass of the materials was analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) (AXIMA-CFR plus; Shimadzu, Kyoto, Japan). The predicted chicken GnIH (SIRPSAYLPLRFamide) was custom synthesized by MBL (Molecular and Biological Laboratories Co. Ltd., Nagoya Japan) and molecular behavior of the synthetic peptide was further confirmed using MALDI-TOF MS.

2.3. Intracerebroventricular (ICV) injection procedure

Chicks were injected using a method adapted from Davis et al. (1979) that does not appear to induce physiological stress (Furuse et al., 1999). The head of the chick was briefly inserted into a restraining device that left the cranium exposed and allowed for free-hand injection. Injection coordinates were 3 mm anterior to the coronal suture, 1 mm lateral from the sagittal suture, and 2 mm deep targeting the left lateral ventricle. Anatomical landmarks were determined visually and by palpation. Injection depth was controlled by placing a plastic tubing sheath over the needle. The needle remained at injection depth in the un-anesthetized chick for 10 s post injection to reduce backflow. Chicks were assigned to treatments at random. Chicken GnIH (MBL, Co. Ltd., Nagoya, Japan) was dissolved in artificial cerebrospinal fluid (Anderson and Heisley, 1972) as a vehicle for a total injection volume of 5 µl with 0.1% Evans Blue dye to facilitate injection site localization. After data collection, the chick was decapitated and its head sectioned along the frontal plane to determine the site of injection. Any chick without dye present in the lateral ventricle system was eliminated from analysis. Sex of chicks was determined visually by dissection.

2.4. Experiment 2: food and water intake

Chicks were randomly assigned to receive either 0 (vehicle only), 0.9, 2.6 or 7.8 nmol chicken GnIH by ICV injection. After injection, chicks were returned to their individual home cages and given *ad libitum* access to both food and water. Food and water intake were monitored (0.01 g) every 30 min for 180 min post injection. Water weight (g) was converted to volume (ml; 1 g = 1 ml). Data were analyzed using analysis of variance (ANOVA) within each time point. The initial ANOVA included the main effects of sex and GnIH dose and their interaction. Neither sex nor the interaction was significant, thus a second ANOVA was conducted within each time point with the statistical model including only the main effect of dose. When dose effects were significant, Tukey's method of multiple comparisons was used to separate the means within each time point. Statistical significance was set at $P < 0.05$ for all experiments.

2.5. Experiment 3: c-Fos immunohistochemistry

Chicks were randomly assigned to receive either vehicle or 2.6 nmol chicken GnIH by ICV injection. Chicks were allowed *ad libitum* access to food and water until injection, after which food was withheld to prevent c-Fos immunoreactivity associated with food consumption. Sixty min post injection as this is the time expected for the most robust c-Fos expression (Muller et al., 1984), chicks were deeply anesthetized with sodium pentobarbital via cardiopuncture, then perfused via the carotid artery with ice-cold 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) containing 0.2% picric acid at pH 7.4. Brains were removed from skulls and post-fixed for 60 min in the same solution, after which they were blocked and placed through a series of graded sucrose incubations, consisting of 20, 30 and 40% in 0.1 M PB, until

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