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The central anorexigenic mechanism of amylin in Japanese quail (*Coturnix japonica*) involves pro-opiomelanocortin, calcitonin receptor, and the arcuate nucleus of the hypothalamus

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ABSTRACT

Amylin is a 37-amino acid peptide hormone that exerts anorexigenic effects in humans and animals. We demonstrated that central injection of amylin into chicks affected feeding and related behaviors via the hypothalamus and brainstem, although the molecular mechanisms remained elusive. Thus, the objective of this study was to investigate the molecular mechanisms underlying anorexigenic effects of amylin in 7 day-old Japanese quail. Food but not water intake was reduced after intracerebroventricular amylin injection, and the behavior analysis indicated that this was associated with decreased food pecks and preening. Whole hypothalamus and hypothalamic nuclei including the arcuate nucleus (ARC), paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), dorsomedial nucleus (DMN) and lateral hypothalamic area (LH) were extracted from quail at 1 h post-injection for total RNA isolation. Real time PCR was performed to quantify mRNA abundance of amylin receptors, appetite-associated neuropeptides and monoamine-synthesis-related enzymes. Central amylin injection increased the mRNA abundance of calcitonin receptor (CALCR), receptor activity modifying protein 1 (RAMP1), pro-opiomelanocortin (POMC), and aromatic L-amino acid decarboxylase (AADC) in the hypothalamus and individual hypothalamic nuclei. Relative quantities of CALCR and POMC mRNA were greater in the ARC of the amylin- than vehicle-treated group. Thus, amylin-mediated effects on food intake may involve POMC, monoamine synthesis, and amylin receptor 1 (a complex of CALCR and RAMP1) in the ARC. Together, these data provide novel insights on the hypothalamic-specific molecular mechanisms of amylin-induced food intake.

1. Introduction

Amylin, also known as islet amyloid polypeptide (IAPP), is a 37-amino acid peptide hormone that is secreted with insulin by pancreatic beta cells in response to feeding. IAPP is also expressed in the central nervous system and plays a role in maternal behavior (Dobolyi, 2009) and food intake regulation (Li et al., 2015). The physiological roles ascribed to amylin are thought to be mediated by amylin receptors which consist of heterodimerized complexes of the calcitonin receptor (CALCR) interacting with one of the receptor activity-modifying proteins (RAMPs) (Muff et al., 1999; Poyner et al., 2002). Amylin receptors are implicated in maternal physiological functions (Dobolyi, 2009), Alzheimer's disease, and diabetes mellitus (Fu et al., 2013). The most intensely investigated functions of amylin receptors in the brain relate to energy balance (Hay et al., 2015).

Amylin contributes to the short- and long-term regulation of food intake via the peripheral and central circulation, as reviewed by (Lutz, 2005) and (Mietlicki-Baase and Hayes, 2014), which documented the multiple roles of amylin as a peptide hormone and neuropeptide. Amylin-induced feeding may involve the hypothalamus (Lutz, 2012; Potes et al., 2010), within which the feeding-related nuclei sense and integrate multiple peripheral signals such as leptin and insulin to regulate pathways controlling food intake and energy expenditure in the central nervous system (CNS) (Simpson et al., 2009). On the basis that receptor-binding sites for amylin are present in the hypothalamus (Paxinos et al., 2004; Sexton et al., 1994), amylin crosses the blood-brain barrier (Banks and Kastin, 1998), and that IAAP is expressed in the brain (Fan et al., 1994), it is plausible that amylin mediates energy

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balance effects via the hypothalamus and its nuclei. Third-ventricular amylin infusion led to decreased food intake (Rushing et al., 2002), and inhibition of central amylin signaling increased food intake in rats (Rushing et al., 2001). Similarly, we demonstrated that central and peripheral injection of amylin into chicks led to anorexigenic effects via the hypothalamus (Cline et al., 2008).

Compared to the intense artificial selection for certain traits in the broiler chicken (breast yield, rapid growth, feed efficiency etc.) since the beginning of the last century, the Japanese quail represents a domesticated species from the same family Phasianidae that has not been subjected to as intense genetic selection for specific growth-related traits (Narinc et al., 2014). Thus, it represents a unique model to understand appetite regulation in avians and the effects of genetic selection on feeding behavior. Moreover, central molecular mechanisms of amylin-induced food intake are poorly understood, which also suggests that further exploration into amylin-mediated control of feeding via the hypothalamus is warranted.

In the present study, four experiments were conducted to investigate the effect of amylin on food intake behaviors using quail as a model. In particular, we evaluated the expression profile of hypothalamic genes involved in the amylin pathway and control of energy balance, and also expression profiles of candidate genes in discrete hypothalamic nuclei, with the objective of elucidating the central molecular mechanisms responsible for exogenous amylin-induced food intake in quail.

2. Materials and methods

2.1. Animals

All protocols were approved by the Institutional Animal Care and Use Committee at Virginia Tech. Japanese quail (*Coturnix japonica*) were obtained from a random mating population at Virginia Tech. Chicks were raised in the brooder until day 4 post-hatch when forty-eight chicks weighing 11–14 g were individually caged in a room at a constant temperature of 33 ± 2 °C and 50 ± 5% relative humidity with ad libitum access to a mash corn and soybean meal-based diet (24% crude protein, 2900 kcal ME/kg) formulated to meet the requirements for the starter phase of Japanese quail (NRC, 1994) and tap water under 14 L: 10D light until 7 days of age. Before the experiment, chicks were conditioned once a day to acclimate them to handling and thereby reduce the stress of injection.

2.2. Intracerebroventricular (ICV) injection procedure

On day 7 post-hatch at 1300, chicks were intracerebroventricularly (ICV) injected using a method adapted (Davis et al., 1979) which does not appear to induce physiological stress (McConn et al., 2015). The head of the quail 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 in vivo in the un-anesthetized quail for 5 s to reduce backflow. Chicks were assigned to treatments at random. Rat amylin (American Peptide, Sunnyvale, CA, USA) was dissolved in avian artificial cerebrospinal fluid as a vehicle for a total injection volume of 5 μL with 0.1% Evans Blue dye to facilitate injection site localization (Anderson and Heisley, 1972). After data collection, the quail was decapitated and its head sectioned coronally to verify the injection site. Data from chicks without dye present in the lateral ventricle system were eliminated from further statistical analysis. Sex was determined visually by dissection.

2.3. Experiment 1: food and water intake in 7 day-old quail

Prior to injection, quail were fasted for 180 min, and then randomly assigned to receive either 0 (vehicle only), 57, 170 or 510 pmol amylin by ICV injection. After injection, quail 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 until 180 min post-injection. Water weight (g) was converted to volume (mL; 1 g = 1 mL). Data were analyzed using analysis of variance (ANOVA) in SAS (SAS Institute Inc., Cary, NC) within each time point with the statistical model including 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. For all experiments, data are presented as means ± standard error (SE) and statistical significance set at $P < 0.05$.

2.4. Experiment 2: hypothalamic mRNA abundance in 7 day-old quail

Quail (n = 12 per group), fasted for 180 min, were randomly assigned to receive 0 (vehicle only) or 510 pmol amylin by ICV injection. Sixty minutes following ICV injection, chicks were deeply anesthetized with sodium pentobarbital via cardiopuncture, decapitated, and brains removed. The inverted brain was submerged in liquid nitrogen such that the most ventral aspect of the optic lobe was level with the surface of the liquid nitrogen for 11 s. Perpendicular to the midline suture, a cut was made at the septopallio-mesencephalic tract and at the third cranial nerves. At 2.0 mm parallel to the midline, two cuts were made. The final cut was made from the anterior commissure to 1.0 mm ventral to the posterior commissure. This block comprising the hypothalamus was stored in RNAlater (Qiagen, Valencia, CA, USA). Hypothalamus was homogenized using 5 mm stainless steel beads (Qiagen), Isol-RNA Lysis reagent (5-Prime, Gaithersburg, MD, USA) and a Tissue Lyser II (Qiagen). The RNeasy Mini Kit (Qiagen) and RNase-free DNase I (Qiagen) were then used for total RNA isolation. The integrity of total RNA samples was evaluated by agarose-formaldehyde gel electrophoresis, and concentration and purity assessed by spectrophotometry at 260/280/230 nm with a Nanophotometer Pearl (IMPLEN, WestlakeVillage, CA, USA).

First-strand cDNA was synthesized in 20 μL reactions from 200 ng of total RNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA), following the manufacturer's instructions. Reactions were performed under the following conditions: 25 °C for 10 min, 37 °C for 2 h and 85 °C for 5 min. Primers for real time PCR, listed in Table 1, were designed with Primer Express 3.0 software (Applied Biosystems) and validated for amplification efficiency (95–105%) before use. Real time PCR was performed in 10 μL reactions containing 5 μL Fast SYBR Green Master Mix (Applied Biosystems), 0.25 μL each of 5 μM primers, 1.5 μL nuclease free water, and 3 μL 10-fold diluted cDNA using a 7500 Fast Real-Time PCR System (Applied Biosystems). The reactions were performed under the following conditions: 95 °C for 20 s and 40 cycles of 90 °C for 3 s plus 60 °C for 30 s. A dissociation step consisting of 5 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 s was performed at the end of each PCR reaction to ensure amplicon specificity.

Data were analyzed using the ΔΔCT method with β-actin as the reference gene and the average of the vehicle-treated chicks as the calibrator sample. Relative quantity values calculated as $2^{-\Delta\Delta CT}$ were used for statistical analysis. Data were analyzed by pairwise Student's *t*-tests with R software. Genes included receptor activity modifying proteins 1, 2, and 3 (RAMP1, 2, and 3, respectively) and calcitonin receptor (CALCR), and genes encoding enzymes that participate in the biosynthesis of serotonin and dopamine including aromatic L-amino acid decarboxylase (AADC), dopamine beta-hydroxylase (DBH) and tryptophan hydroxylase 2 (TPH2), and appetite-associated factors including neuropeptide Y (NPY), agouti-related peptide (AgRP), pro-melanin-concentrating hormone (MCH), pro-opiomelanocortin