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Short communication

Exogenous prolactin-releasing peptide's orexigenic effect is associated with hypothalamic neuropeptide Y in chicks

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ABSTRACT

Exogenous administration of prolactin-releasing peptide (PrRP) exerts anorexigenic effects in rats while causing orexigenic effects in chicks. While the central mechanism mediating PrRP's effect on food intake in rodents is somewhat understood, in chicks information is lacking. Therefore, this study was designed to elucidate the hypothalamic mechanism of PrRP induction of hunger perception in chicks. Chicks that received intracerebroventricular (ICV) injections of PrRP dose-dependently increased their food intake with no effect on water intake or whole blood glucose concentration. The threshold of food intake stimulation was as low as 3 pmol, thus as compared to other neuropeptides PrRP is exceptionally potent. The mRNA abundance of several appetite-associated neuropeptide genes was quantified and hypothalamic neuropeptide Y (NPY) mRNA was increased in PrRP-injected chicks. Therefore, the orexigenic effects of PrRP may be associated with increased NPY-ergic tone. These results provide insight into the evolutionary aspects of appetite regulation during the course of divergent evolution of mammals and birds.

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1. Introduction

Prolactin-releasing peptide (PrRP) was first isolated from the bovine hypothalamus and was so named for its effect on prolactin release (Hinuma et al., 1998). However, since that time PrRP has been demonstrated to have other functions unrelated to prolactin release, for example, PrRP mediates stress responses through the release of adrenocorticotropic hormone (ACTH), regulates reproduction by increasing plasma luteinizing hormone and follicle stimulating hormone concentrations in rats (Lawrence et al., 2000; Maruyama et al., 2001; Seal et al., 2000), and affects growth (Iijima et al., 2001), pain (Kalliomaki et al., 2004) and cardiovascular function (Samson et al., 2000). Additionally, centrally injected PrRP decreases food intake in rats and goldfish but in birds it stimulates hunger (Kelly and Peter, 2006; Lawrence et al., 2000; Tachibana et al., 2005).

The mechanism of PrRP's anorexigenic effect in rodents is partly understood. ICV administration of PrRP induced marked c-Fos-positive neuronal profiles in the paraventricular nucleus (PVN) in the hypothalamus (Bechtold and Luckman, 2006; Lawrence et al., 2002), and it was suggested that hypothalamic oxytocin and corticotrophin releasing factor (CRF) may mediate the effects of PrRP on food intake (Bechtold and Luckman, 2006; Ellacott et al., 2002; Matsumoto et al., 2000). In rats, PrRP mRNA was detected in the caudal portion of the ventromedial (VMH) and dorsomedial nucleus (DMN) of the hypothalamus (Iijima et al., 1999; Minami et al., 1999), and PrRP receptor expression was greatest in the DMN and PVN in the hypothalamus (Roland et al., 1999).

Unlike mammals, in which only a single PrRP receptor (PrRPR) has been identified, two forms of chick PrRP receptors (cPrRPR1 and cPrRPR2) have been identified (Wang et al., 2012) and their function has been characterized. Based on a co-injection (neuropeptide Y [NPY] + PrRP) experiment it was concluded that PrRP-induced hunger was independent of NPY because dual injection did not have an additive effect on food intake (Tachibana et al., 2004). Moreover, involvement of adrenergic alpha-2 receptors in PrRP-regulation of food intake is unlikely because yohimbine had no effect on the magnitude of PrRP-induced food intake stimulation (Tachibana et al., 2009). To our knowledge, this is the extent of what is known regarding PrRP's orexigenic effect in chicks. Thus, in the present study we measured effects of ICV PrRP injection on food and water intake, blood glucose concentration, and the number of c-Fos immunopositive cells across the whole hypothalamus. We also measured mRNA abundance of several appetite-associated factors as a means to further elucidate the hypothalamic mechanism of action.

2. Materials and methods

2.1. Animals

Unsexed Hubbard × Cobb-500 broiler chicks (*Gallus gallus*) from breeders 42 weeks of age were obtained from a commercial hatchery on the morning of hatch. They were caged individually in a room at

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30 ± 2 °C and 50 ± 5% relative humidity with access to a mash diet (20% crude protein and 2685 kcal ME/kg) and tap water. In all experiments, chicks were injected at day 4 post-hatch, and each experiment was conducted using chicks from separate hatches. Experiments were conducted sequentially in the order described below. All experimental 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 Institutional Animal Care and Use committee.

2.2. Intracerebroventricular (ICV) injection procedure

Chicks were ICV injected using a method adapted from Davis et al. (1979) that does not appear to induce physiological stress (Furuse et al., 1999; Saito et al., 2005). 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 in vivo in the un-anesthetized chick for 5 s to reduce backflow. Chicks were assigned to treatments at random. Prolactin-releasing peptide (PrRP, 3594.0 molecular weight, American Peptide, Sunnyvale, CA, USA) was dissolved in avian artificial cerebrospinal fluid as a vehicle for a total injection volume of 5 μL with 0.06% Evans Blue dye to facilitate injection site localization (Anderson and Heisley, 1972). After data collection, the chick 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 statistical analysis. Sex was determined visually by dissection.

2.3. Experiment 1: effect on food and water intake with high doses

In Experiment 1, chicks were randomly assigned to receive 0 (vehicle only), 12, 47, or 188 pmol PrRP. Following ICV injection, chicks were returned to their individual cages and given ad libitum access to food and water. Food intake and water intake were measured (to 0.01 g) every 30 min for 180 min post-injection. Data were analyzed using analysis of variance (ANOVA) at each time point using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC). The model included PrRP dose, sex and the interaction of sex with PrRP dose. Sex and the interaction of sex and PrRP dose were not significant and were eliminated from the model (and the effect of sex was not tested in subsequent experiments). When significant treatment effects were found, Tukey's method of multiple comparisons was used to separate the means at each time period. For this and all proceeding experiments, statistical significance was set at $P < 0.05$.

Trunk blood was collected from chicks immediately after the 180 min food and water intake reading. Whole blood glucose concentration was determined in duplicate using the One Touch Basic glucose measurement system (Lifescan, Milpitas, CA, USA). Blood glucose data were analyzed using ANOVA via the GLM procedure of SAS. Tukey's method of multiple comparisons was used to separate the means.

2.4. Experiment 2: effect on food and water intake with lower doses

Procedures were identical to those described in Section 2.3 except that the doses used were 0 (vehicle only), 0.75, 3, or 12 pmol.

2.5. Experiment 3: hypothalamic expression of appetite-associated factor mRNA

Chicks were randomly assigned to receive vehicle or 188 pmol PrRP via ICV injection. Following injection food was withheld to prevent effects associated with food consumption. Sixty minutes following injection, chicks were deeply anesthetized with sodium pentobarbital via cardiopuncture and decapitated, and brains were removed. The whole upside-down brain was lowered into liquid nitrogen to the point where the most ventral aspect of the optic lobe was level with the surface of the liquid nitrogen. The brain was left in this position for 11 s. This procedure resulted in brain regions around the hypothalamus freezing and providing firmness necessary to make precise cuts for hypothalamus extraction. The hypothalamus was dissected visually based on the following anatomical landmarks: anterior cut made at the corticoseptomesencephalic tract, posterior cut at the third cranial nerves, laterally cut 1.5 mm parallel to the midline on both sides of the brain and finally the dorsal cut will be made from the anterior commissure to 1.0 mm ventral to the posterior commissure (Puelles et al., 2007). It was collected in RNAlater (Qiagen) and homogenized using 5 mm stainless steel beads and 1 mL Isol Lysis reagent (5-Prime, USA) for 2 × 2 min at 20 Hz (Tissue Lyser II; Qiagen). After centrifugation for 10 min at 12,000 ×*g* at 4 °C, the supernatant was removed and total RNA separated, following the manufacturer's instructions (5-Prime). Following the step of addition to 70% ethanol, mixtures were transferred to spin columns and total RNA purified using the RNeasy Mini Kit (Qiagen, USA), including the optional on-column RNase-free DNase I step (Qiagen, USA). The eluted total RNA samples were evaluated for integrity by agarose-formaldehyde gel electrophoresis and concentration and purity assessed by spectrophotometry at 260/280/230 nm.

Single-strand cDNA was synthesized from 200 ng total RNA in 20 μL reactions with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), following the manufacturer's instructions. Reactions were performed under the following conditions: 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min. Primers for real time PCR are listed in Table 1 and amplification efficiency was validated for all primer pairs before use (95–100% efficiency). Real-time PCR reactions were performed in duplicate with Fast SYBR Green Master Mix (Applied Biosystems, USA) and 10-fold diluted cDNA. PCR was 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 95 °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.

3. Results

3.1. Experiment 1: effect on food and water intake with higher doses

Chicks that received PrRP increased food intake (Fig. 1). All dosed tested were effective at stimulating food intake up to 120 min following

Table 1
Primers used for real time PCR.

Gene[a]	Accession ID	Sequence 5′ to 3′ (forward/reverse)
β-Actin	NM_205518.1	GTCCACCGCAAATGCTTCTAA/TGCGCATTT ATGGGTTTTGTT
NPY	M87294.1	CATGCAGGGCACCATGAG/CAGCGACAA GGCGAAAGTC
AgRP	AB029443.1	GGTTCTTCAACGCCTTCTGCTA/TTCTTGCC ACATGGGAAGGT
PrRP	NM_001082419.1	GAGCGCTCCATGGAAATCAG/ATGCCA CGCCGGTGTAC
Orexin	NM_204185.2	CCAGGAGCACGCTGAGAAG/CCCATCTC AGTAAAAGCTCTTTGC
Oxytocin	XM_001231491.3	TGGCTCTCTCCTCAGCTTGTTAT/GGCA CGGCACGCTTACC
CRF	NM_001123031.1	TCAGCACCAGAGCCATCACA/GCTCTATAA AAATAAAGAGGTGACATCAGA
Galanin	NM_001159678.1	CGAATTTCTGACTTACTTGCATCTTAA/A AAGGTTTGTTTCCTCTGGTGAAG
PC2	XM_004940215.1	TGGGAAGGCAAGGCAATG/CCTGACTGTT TGCAATGCACTT

[a] NPY: neuropeptide Y; AgRP: agouti-related peptide; CRF: corticotropin-releasing factor; PC2: prohormone convertase 2.