Central mechanisms of prolactin-releasing peptide's orexigenic effect in chickens

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ABSTRACT

Prolactin-releasing peptide (PrRP) is an endogenous hypothalamic neuropeptide that when exogenously injected increases food intake in chickens, but decreases it in rodents and goldfish. We designed three sets of experiments to elucidate the mechanisms of PrRP's orexigenic effect in chicks. In experiment one, food and water intake were evaluated in chicks after receiving intracerebroventricular (ICV) injection of the vehicle, 0.75, 3, 12, 47 or 188 pmol PrRP. The administration of 12 and 47 pmol PrRP increased food intake for up to 120 min after injection, and 188 pmol increased it for up to 180 min. The lowest effective dose was 3 pmol, which increased food intake for up to 60 min after injection. Water intake was not affected. To investigate the molecular mechanisms, c-Fos immunohistochemistry was performed and mRNA expression of some appetite-associated neurotransmitters was measured in chicks that received either vehicle or 188 pmol of PrRP. The rostral paraventricular nucleus (PVN) was activated which coincided with increased neuropeptide Y (NPY) mRNA expression in the whole hypothalamus. In experiment two, food and water intake were evaluated in chicks fed a high carbohydrate (HC), high fat (HF) or high protein (HP) diet after ICV injection of vehicle, 3 or 188 pmol PrRP. Chicks fed the HP diet increased food intake at a lower dose than chicks fed HF and HP diets after ICV PrRP injection. In addition, ICV injection of vehicle, 3 and 188 pmol PrRP were performed in chicks fed all three diets, and ICV PrRP injection

induced preferential intake of the HP diet over HC and HF diets. The expression of some appetite-associated neuropeptides in the hypothalamus was also measured in chicks fed the HC, HF or HP diet after ICV injection of vehicle or 188 pmol PrRP. There was a diet effect on mRNA abundance of all appetite-associated genes measured ($P < 0.05$), with greater expression in chicks fed the HF or HP than HC diet. While neuropeptide Y (*NPY*) mRNA abundance was similar between vehicle and PrRP-injected chicks that consumed HP or HF diets, expression was greater ($P < 0.05$) in PrRP- than vehicle-injected chicks that consumed the HC. In experiment three, the orexigenic effect of PrRP was tested in chicks selected for low (LWS) and high (HWS) body weight after central administration of vehicle, 24, 94 and 375 pmol PrRP. The LWS chicks had a lower threshold and higher magnitude of food intake increase in response to PrRP injection.

Results demonstrate that PrRP is a potent orexigenic factor in chickens and that effects are likely mediated through the hypothalamus. The orexigenic effect of PrRP was influenced by dietary macronutrient composition, and diet in turn influenced the food intake response to PrRP. These results may contribute to a novel understanding of appetite regulation.

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Chapter I: Introduction

Broiler chickens have been intensively selected for meat production and there has been a 300% increase in growth rates (a daily increase from 25 g to 100 g) (Knowles et al., 2008). The rapid growth rate is accompanied by increased body fat deposition, higher incidence of metabolic diseases, higher mortality and more skeletal disorders (Zubair and Leeson, 1996). These diseases not only result in economic losses to producers, but also affect the well-being of broilers (Leeson et al., 1991). Broiler chickens are likely to have a dietary energy intake three or four times of their maintenance needs when fed ad libitum (Boekholt et al., 1994). Therefore, early feed restriction is practiced to reduce these problems and compensatory growth enables producers to produce carcasses with similar weight to control groups.

The increased fat deposition in broiler chickens raises economical concerns because fat represents an uneconomical and undesirable product (Yu and Robinson, 1992; Urdaneta-Rincon and Leeson, 2002). In the broiler industry, excessive fat is currently one of the major concerns, not only because it lowers feed efficiency and carcass yields, but also increases difficulties in processing and leads to rejection of the meat product by consumers (Sahraei, 2012). In order to produce leaner carcasses and reduce the negative effects of fat on human health, it is a goal of the poultry industry to reduce fat deposition.

Even though feed restriction has been relatively effective at reducing the negative effects of chronic positive energy balance, it is associated with welfare concerns related to hunger and may cause aggressive pecking, which is increasingly prevalent in commercial flocks of broiler breeders (Hocking, 1993; Hocking et al., 2004). In addition,

such practice increases management cost (Renema and Robinson, 2004). Food intake is a critical factor that affects body weight gain and feed efficiency in broiler chickens. Thus, understanding the regulation of food intake and energy balance in chickens will be important to develop strategies to better manage poultry production.

Like mammals, birds have complicated physiological mechanisms regulating food intake. The regulation of food intake involves not only sites within the central nervous system (CNS) but also sites outside of the CNS, with signals integrated within the CNS. Peripheral sites include the gastrointestinal tract, liver, adipose tissue, pancreas, as well as neural inputs (e.g., vagal afferents). In the CNS, the hypothalamus is the major site for the regulation of food intake (Hussain and Bloom, 2013). The hypothalamus integrates signals that are received from the gut, liver, pancreas, adipose tissue and other parts of the brain (Richards and Proszkowiec-Weglarz, 2007). Neuropeptides are among the signaling molecules that are produced by the CNS and peripheral tissues in response to nutritional and environmental changes, relaying information about the status of whole-body nutrition.

Chapter II: Literature Review

Appetite regulation in rodents

The neural systems regulating energy homeostasis are complex and organized hierarchically. Within the CNS, the hypothalamus is able to detect various metabolic signals from the peripheral system, and sequentially generate signals into brain regions that are responsible for coordinating feeding behavior and energy expenditure (Jeong et al., 2014).

There are several nuclei which play important roles in regulating food intake. Arcuate (ARC) neurons, located at the bottom of the hypothalamus around the third ventricle, are known as first order neurons since they have direct contact with peripheral satiety factors. The blood brain barrier (BBB) is not existent in the median eminence (ME) which overlies the ARC, thus ARC axon terminals are in direct contact with the bloodstream, while ARC neuronal cell bodies are protected by the BBB and are not in direct contact with the blood stream (Peruzzo et al., 2000). The ARC contains neurons that express neuropeptide Y (NPY) and agouti-related peptide (AgRP), which are both associated with stimulating food intake in chickens (Furuse et al., 1997; Tachibana et al., 2001), and melanocortin precursor proopiomelanocortin (POMC) neurons, which suppresses food intake (Myers et al., 2008).

Neurons from the ARC project to second order neurons in the ventromedial nucleus of the hypothalamus (VMH), dorsomedial hypothalamic nucleus (DMN), paraventricular nucleus (PVN), and lateral hypothalamic area (LHA) (Figure 2.1) (Schwartz et al., 2000). The PVN is in the anterior hypothalamus and is adjacent to the superior part of the third ventricle. It releases potent orexigenic signals while also

suppressing feeding by reducing its secretion of NPY (Kalra et al., 1999). The VMH is known as the classical satiety center. Its activation is associated with satiety perception which reduced feed intake (Kalra et al., 1999). Electrical stimulation of the DMN receives causes a voracious drive to eat while lesions of the DMN induce hypophagia, indicating that the DMN may play an orexigenic role on regulating food intake (Larsson, 1954; Bernardis, 1970). However, DMN may also contain a satiety system (Bellinger and Williams, 1983; Bellinger and Bernardis, 1984). The LHA contains two major classes of orexigenic neurons, the orexin and melanin concentrating hormone (MCH) neurons. Those neurons receive innervation from NPY-containing fibers in rodent (Broberger et al., 1998; Elias et al., 1998).

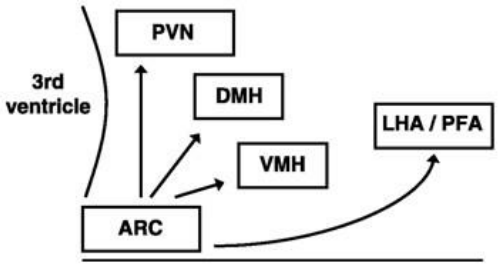

Figure 2.1. Hypothalamic nuclei defined morphologically in rats. For reference, ARC, arcuate nucleus; VMH, ventromedial nucleus; DMH, dorsomedial nucleus; PVN, paraventricular nucleus; LHA, lateral hypothalamic area; PFA, perifornical area (adapted from Stanley et al., 2005). Arrows represent projections to respective nucleus (Stanley et al., 2005).

Appetite regulation in chicks

Energy homeostasis and feeding behaviors are fundamental processes critical to the survival of animals, therefore it is reasonable to infer that the neural and endocrine

networks governing these behaviors will have similar conserved regulatory mechanisms in avian and mammalian species (Kuenzel, 1994; Kuenzel et al., 1999).

Compared with rodents, far less is known about appetite regulation in chickens and there are some different effects on food intake in response to exogenous administration of neuropeptides. For example, ghrelin and growth-hormone-releasing factor stimulate food intake in mammals while inhibiting it in chicks (Vaccarino et al., 1988; Wren et al., 2000; Furuse et al., 2001). Melanin-concentrating hormone, motilin and orexin are known orexigenic peptides in mammals, whereas central administration of these peptides did not change food intake in chicks (Presse et al., 1996; Furuse et al., 1999a; Ando et al., 2000). Somatostatin suppressed feeding behavior of fed rats while stimulated food intake in fasted rats. However ICV injection of somatostatin increased food intake in chicks regardless of feeding condition (Aponte et al., 1984; Tachibana et al., 2009a).

Research on avian appetite regulation is mainly conducted in broiler and layer chickens. Broilers and layers demonstrate different responses to the exogenous administration of some neurotransmitters, which further supports that selection for body mass alters the response to appetite regulating factors (Cline and Furuse, 2012a). For example, central administration of norepinephrine increased food intake in broilers but did not affect food intake in layers (Denbow et al., 1981; Denbow et al., 1983). Central injection of serotonin decreased food intake in fasted layers while having no effect in fasted broilers (Denbow et al., 1982). Therefore, it seems that broilers have lower sensitivity to orexigenic factors, while layers have higher sensitivity to anorexigenic factors.

Chickens genetically selected for either low body weight (LWS) or high body weight (HWS) have also shown different food intake responses to exogenous neuropeptides. It has been reported that LWS chicks are more sensitive to many of the anorexigenic neuropeptides, such as α-MSH, neuropeptide AF, insulin, insulin and amylin ,whereas HWS chicks are more sensitive to some of the orexigenic neuropeptides, including NPY and AgRP (Cline and Furuse, 2012b).

Prolactin releasing peptide

PrRP in mammals

Prolactin-releasing peptide (PrRP) was first identified as a ligand for an orphan receptor and originally named for its prolactin-releasing function (Hinuma et al., 1998). However, PrRP has many functions other than its weak prolactin-releasing effect. Plasma ACTH level was increased by ICV injection of PrRP in rats, indicating that it is associated with stress responses (Matsumoto et al., 2000; Maruyama et al., 2001). Central administration of PrRP caused a hyperthermia response in rats and the opposite response in chickens suggesting that it may be involved in energy metabolism (Ellacott et al., 2002; Tachibana et al., 2004).

PrRP decreases food intake in rats (Vergoni et al., 2002). PrRP was thought to act as a hypothalamic releasing factor at the pituitary gland for prolactin secretion. However, there were no PrRP-immunoreactive fibers found in the external region of the median eminence from where the secretion of anterior pituitary hormones is controlled by classic hypothalamic neuroendocrine hormones being released into the portal blood (Fukusumi et al., 2006). This indicates that its mechanism may be different from other hypophysiotropic hormones.

There are two main forms of PrRP with 20 or 31 amino acids (PrRP20 or PrRP31), of which the 20-amino-acid-long peptide is the truncated version of the 31-amino-acid peptide (Hinuma et al., 1998). PrRP mRNAs were distributed in the caudal portion of the VMH and DMN of the hypothalamus, the NTS and the ventral and lateral reticular nuclei (VLRN) in the medulla oblongata (Iijima et al., 1999; Minami et al., 1999). PrRP immunoreactive neuronal perikarya were identified by immunocytochemistry in the VMH and DMN in the hypothalamus, the NTS, and the VLRN in the medulla oblongata using antibody against PrRP (Chen et al., 1999; Iijima et al., 1999; Maruyama et al., 1999a), and PrRP receptors have the highest mRNA expression in DMN and PVN in hypothalamus (Roland et al., 1999). Microinjection of PrRP into the PVN (0.1 to 2 nmol) did not affect food intake at any time point. While 0.1 to 1 nmol PrRP injected into the DMN decreased food intake within the first hour, and both 0.5 and 1 nmol continued to decrease food intake after 8 h postinjection (Seal et al., 2001). This may indicate that the PVN does not play a major role in PrRP-mediated regulation of food intake though c-Fos expressions were activated in the PVN after ICV administration of PrRP (Matsumoto et al., 2000).

The anorexigenic effect of PrRP may be related to its regulation of other anorexigenic peptides. Plasma ACTH levels increased after ICV administration of PrRP following its effect of stimulating CRF in the PVN (Matsumoto et al., 2000). Central administration of PrRP also led to elevated plasma oxytocin and vasopressin levels in rats (Maruyama et al., 1999b). Double labeled immunocytochemistry using antibodies against PrRP and somatostatin synthesizing neurons demonstrated their close relationship on nerve processes or terminals. Also, somatostatin synthesizing neurons in the

periventricular nucleus of the hypothalamus expressed PrRP receptor mRNA simultaneously, which indicates that PrRP may mediate the secretion of somatostatin in the hypothalamus (Ibata et al., 2000). Oxytocin, vasopressin and somatostatin are known anorexigenic peptides in rats (Aponte et al., 1984; Langhans et al., 1991; Olson et al., 1991). CRF and oxytocin neurons in the hypothalamus are activated by the administration of PrRP and its anorexigenic effects are attenuated by pretreatment with CRF or oxytocin receptor antagonists (Bechtold and Luckman, 2006).

PrRP in chickens

PrRP stimulates food intake in chickens which is different from what is known in rats and goldfish (Vergoni et al., 2002; Tachibana et al., 2004; Tachibana et al., 2005; Kelly and Peter, 2006). Compared to what is known about PrRP in rats, less is known in chickens. Chicken PrRP20 shares 100%, 95% and 70% identity with the orthologous peptide in teleosts, *Xenopus laevis* and mammals, respectively. Additionally, chicken PrRP31 showed about 90% and 52-55% identity as from *X. laevis* and mammals, respectively (Tachibana et al., 2011). Carassius RPamide (C-RFa) is an orthologous prolactin secretagogue in fishes consisting of 20-amino acids that are identical to the chicken. However, central administration of C-RFa did not stimulate food intake or increase plasma corticosterone as mammalian PrRP with 31 amino acids (mPrRP31) did in rodents (Tachibana et al., 2005). Three receptors homologous to the mammalian PrRP receptor were cloned from the chicken brain cPrRPR1, cPrRPR2 and cC-RFaR (Wang et al., 2012). The cPrRPR1 precursor shares relatively high amino acid sequence identity to rat (58%), human (56%) and Xenopus tropicalis (80%) PrRP, and the cloned cPrRPR2

and cC-RFaR precursor share 58 and 46% of sequence identity with human PrRPR. However, the cloned cPrRPR2 and cC-RFaR share relatively higher sequence with *Xenopus* PrRPR2 (81%) and C-RFaR (69%), respectively (Wang et al., 2012).

To our knowledge c-Fos immunhistochemical analysis of the hypothalamus and brainstem following PrRP injection has not been performed in chickens. Thus, little is known which hypothalamus nucleus has been activated in response to PrRP.

Chapter III: Exogenous prolactin-releasing peptide mediates its orexigenic effect via the rostral paraventricular nucleus 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. Whilst 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's orexigenic effect 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 whole hypothalamus was scanned for c-Fos immunoreactive cells following ICV PrRP and the rostral paraventricular nucleus (PVN) was the only region found to have an increased concentration of c-Fos positive soma. 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 at the PVN. These results provide insight onto the

evolutionary aspects of appetite regulation during the course of divergent evolution of mammals and birds.

Key words: prolactin-releasing peptide, chick, hypothalamus, food intake

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; Seal et al., 2000; Maruyama et al., 2001), and affects growth (Iijima et al., 2001), pain (Kalliomaki et al., 2004) and cardiovascular functions (Samson et al., 2000). Additionally, centrally injected PrRP decreases food intake in rats and goldfish but in birds it stimulates hunger (Lawrence et al., 2000; Tachibana et al., 2005; Kelly and Peter, 2006).

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 (Lawrence et al., 2002; Bechtold and Luckman, 2006), and it was suggested that hypothalamic oxytocin and corticotrophin releasing factor (CRF) may mediate the effects of PrRP on food intake (Matsumoto et al., 2000; Ellacott et al., 2002; Bechtold and Luckman, 2006). 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 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., 2009b). 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.

Materials and methods

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 30 ± 2 °C and 50 ± 5% relative humidity with access to a mash diet (22% crude protein and 3000 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.

Intracerebroventricular (ICV) injection procedure

Chicks were ICV injected using a method adapted from Davis et al. (Davis et al., 1979) that does not appear to induce physiological stress (Furuse et al., 1999b; 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-anaesthetized 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.

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.

Experiment 2: Effect on food and water intake with lower doses

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

Experiment 3: c-Fos as an indicator of neuronal activity

Chicks were randomly assigned to receive either vehicle or 188 pmol PrRP (a dose which was expected to induce a robust expression) by ICV injection. Chicks were allowed ad libitum access to food and water until injection, at which time food was withheld to prevent c-Fos immunoreactivity associated with food consumption. One hour 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 saline 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 1 h in the same solution, after which they were blocked and placed through a series of graded sucrose incubations, consisting of 20% and 30% in 0.1 M PB, until they sank. Every other 60 μm coronal section from interaural 3.28 mm to interaural 0.16 mm corresponding to Puelles et al. (Puelles et al., 2007) was collected . Sections were collected in 0.02 M PB saline (PBS) containing 0.1% sodium azide using a cryostat at −15 °C. Procedures for c-Fos immunohistochemistry assay were based on those of Zhao and Li. (Zhao and Li, 2010). Free-floating sections were pre-blocked for 1 h with 10% normal goat serum (NGS) and 0.3% Triton X-100 in 0.02 M PBS. To inhibit endogenous peroxidase activity, sections were incubated in 1.5% hydrogen peroxide and 50% methanol in

deionized water for 30 min. Following a 3 × 10 min wash in wash buffer (0.05% NGS and 0.3% Triton X-100 in 0.02 M PBS), sections were incubated with rabbit polyclonal anti-c-Fos at a dilution of 1:20,000 (K-25, Santa Cruz, CA, USA) in PBS containing 0.3% Triton X-100, 1% NGS, and 1% blocking reagent (11096176001, Roche Diagnostics, MA, DE) for 48 h under slow oscillation at 4 °C. For assay controls, the primary antibody was substituted with normal rabbit serum. Sections were then rinsed 3 × 10 min in wash buffer and incubated with biotinylated goat anti-rabbit secondary antibody at a dilution of 1:200 (Vector Laboratories, CA, USA) in PBS containing 1% NGS for 2 h at room temperature. Following a rinse with PBS, sections were processed with avidin–biotin-horseradish peroxidase complex at a dilution of 1:200 (Vectastain Elite ABC Kit, Vector Laboratories). Reactions were visualized with the DAB Substrate Kit for Peroxidase (Vector Laboratories) for 8 s, mounted on gelatin-coated slides and cover-slipped with VectaMount (Vector Laboratories). All sections were scanned by a technician blind to treatment and any region with apparent increase in c-Fos immunoreactivity was identified. Overlays containing the respective nuclei boundaries were digitally merged with micrographs and the number of c-Fos immunoreactive cells within it was counted. Reactivity in the PVN was counted at 3.28, 3.04, 2.08, 2.56 and 2.32 mm interaural and the arcuate nucleus (ARC) at 1.60, 1.36, 1.12, 0.88, 0.64, 0.40 and 0.16 mm interaural. Data were analyzed by ANOVA using the GLM procedure of SAS.

Experiment 4: 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 min following injection, chicks were deeply anesthetized with sodium pentobarbital via cardiopuncture, decapitated, and brains 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 3.1 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.

Results

Experiment 1: Effect on food and water intake with higher doses

Chicks that received PrRP increased food intake (Figure 3.1). All dosed tested were effective at stimulating food intake up to 120 min following injection, however, from 150 to 180 min only the 188 pmol was effective. Water intake was not affected (Figure 3.2). Whole blood glucose was not affected by ICV PrRP injection at 180 min post injection. Glucose concentrations were 263.9 ± 4.4, 277.0 ± 16.3, 284.9 ± 6.8, and 301.7 ± 13.8 mg/dl for 0, 12, 47 and 188 pmol ICV PrRP injection groups, respectively.

Experiment 2: Effect on food and water intake with lower doses

Among the three doses that were tested, only the 12 pmol stimulated food intake during the 150 min after injection and the 3 pmol was only effective at increasing food intake to 60 min following injection (Figure 3.3). Water intake was not affected (Figure 3.4). Whole blood glucose was not affected by ICV PrRP injection at 180 min post injection. Glucose concentrations were 285.1 ± 12.3, 269.6 ± 9.8, 279.4 ± 12.5, and 285.8 ± 20.6 mg/dl for 0, 0.75, 3 and 12 pmol ICV PrRP injection groups, respectively.

Experiment 3: c-Fos immunoreactivity

Chicks treated with PrRP had increased c-Fos immunoreactivity (Figure 3.5) in the rostral (interaural 3.28 mm, 3.04 mm, 2.80 mm and 2.56 mm) PVN, with an approximate increase of 150%, 90%, 170% and 130% respectively, compared to the control. There was no other region of the hypothalamus that had clusters of robust c-Fos immunoreactive cells. The ARC sections were later counted based on the results of Experiment 4, but did not have increased c-Fos immunoreactivity (data not shown).

Experiment 4: Hypothalamic expression of appetite-associated factor mRNA

ICV PrRP injection significantly increased NPY mRNA expression in PrRP-treated chicks compared to vehicle-treated chicks. The abundance of agouti-related peptide (AgRP), PrRP, orexin, oxytocin, CRF, galanin and prohormone convertase 2 (PC2) was not affected by ICV PrRP injection (Figure 3.6).

Discussion

The orexigenic effect of PrRP in chicks was first reported by Tachibana et al. (Tachibana et al., 2004) using layer chicks, and the lowest dose evaluated, 47 pmol, was efficacious at increasing food intake. In the present study we used meat-type chicks and evaluated much lower doses and found that as little as 3 pmol stimulated food intake. For chicken production, broiler type chicks have been genetically selected for rapid growth and consume much more food than do layer-type chicks that have been selected for egg production (National Research Council, 1994). Broiler chicks therefore have greater basal orexigenic tone. It is for this reason that we hypothesized that the threshold in meat-type chicks would be lower than layers and designed Experiment 1. Because we did not find a non-efficacious dose in Experiment 1, we designed Experiment 2 with even lower doses. Because such low doses of PrRP were able to further stimulate the chick to eat imply that PrRP is exceptionally potent, even in an animal with high basal orexigenic tone. It would be interesting to determine if a dose lower than 3 pmols was efficacious at stimulating food intake in layer-type chicks, a thesis beyond our scope. Additionally, the efficacious doses demonstrated in the present study are much lower than the doses used in rodent studies (Lawrence et al., 2000) in which anorexia was observed. It would be informative to evaluate a lower dose in rats because sometimes high doses of exogenous neuropeptides induce opposite effects due to receptor desensitization (Changeux et al., 1992).

Because PrRP causes decreased food intake in vertebrates that are taxonomically lower (goldfish; (Kelly and Peter, 2006) and higher (rodents; (Lawrence et al., 2000) than chickens implies that the hunger stimulating effect of PrRP may be unique to birds.

Thus, it would be informative to study the effect of PrRP on food intake in an intermediate class of vertebrates, such as reptiles or amphibians.

Water intake was not affected in our study, which was in agreement with rodent reports (Lawrence et al., 2000), suggesting that PrRP is not stimulating or inhibiting food intake in a nonspecific manner. Tachibana et al. (Tachibana et al., 2013) reported that ICV injection of PrRP increased plasma glucose concentration in a dose-dependent manner, which contradicts our results where blood glucose concentration was not affected, However, in the former report food and water were removed following injection and blood was collected 30 min after injection, while in our study chicks had access to both food and water and blood was collected 180 min post injection (Tachibana et al., 2013). This difference in experimental design or the possibility that egg-type and meat-type chicks have differential glucose homeostasis regulation may explain the disparate findings (Shiraishi et al., 2011).

The hypothalamic area with the greatest c-Fos immunoreactivity was the rostral PVN from 3.28 to 2.56 mm interaural. In past work investigating the effects of neuropeptides we measured c-Fos immunoreactivity and selected only sections corresponding to interaural 2.08 and 1.12 mm as these locations provided representations of cross sections of all major hypothalamic appetite-associated nuclei. Such was the case for our initial experiments with PrRP, however after several attempts to measure c-Fos immunoreactivity at 2.08 and 1.12 mm interaural after ICV PrRP injection, we found no significant effects. This led us to the hypothesis that the effect was either 1) non c-Fos dependent or 2) that the nuclei were not behaving as a whole. To evaluate this we performed a comprehensive screening of the entire hypothalamus. This finding provided

novel insight onto our current work but also oupast reports (Cline et al., 2007; Cline et al., 2008a; Cline et al., 2009b; Newmyer et al., 2013; Webster et al., 2013; Mace et al., 2014).

Our scan of the hypothalamus included all of its nuclei, not just those associated with appetite, and that only a major homeostatic appetite regulating center was affected implies that the effect on food intake is primary. That the PVN was reactive is consistent with its role in stimulation of hunger (Leibowitz, 1978); however, this nucleus is also associated with satiety (Leibowitz and Alexander, 1998). It is for this reason that it is not surprising that in rats, where PrRP is associated with anorexia, ICV PrRP also causes increased c-Fos immunoreactivity in the PVN (Lawrence et al., 2002; Bechtold and Luckman, 2006). This led us to the hypothesis that PrRP was exerting the opposite effect in chicks and prompted the design of Experiment 4. In rats the hypothalamic mechanism has been elucidated: PrRP is associated with increased oxytocin and CRF release (Matsumoto et al., 2000; Ellacott et al., 2002; Bechtold and Luckman, 2006). Both of these factors are anorexigenic in rats (Arletti et al., 1990; Richard et al., 1996) and chicks (Jonaidi et al., 2003; Cline et al., 2009a). However, our mRNA results did not support that there was a transcriptional change for either of these factors, which implies that the mechanism is not simply the inverse of the pathways in rats. However, a reduction in the release of oxytocin or CRF may not necessarily be observed at the level of transcription. Although expression of these two genes was not affected in the present study, our analysis revealed that mRNA for NPY was up-regulated in PrRP-treated chicks. This effect is not parallel to rodent reports and may be a major component of the PrRP mechanism in chicks. This is not supported by an earlier co-injection design where dual

injection of NPY and PrRP did not cause an additive effect on food intake (Tachibana et al., 2004). However, results from such designs can have many interpretations: it is possible that the physiological ceiling had been reached by NPY alone (the NPY-only chicks had already increased food intake threefold over controls) which would not permit the detection of an additive effect.

Because NPY mRNA was increased in PrRP-treated chicks we chose to revisit our c-Fos slides and quantify the number of reactive cells within the ARC to ensure we had not overlooked a significant effect. This is because the densest concentration of NPY neurons is in the ARC, in which NPY release is dramatically increased after food deprivation in chickens (Kameda et al., 2001). That NPY expression is c-Fos dependent (Kovacs, 1998b; Wang et al., 2002a; Wu et al., 2004a) implies that the increased NPY mRNA is not of ARC origin, but rather likely of PVN origin. Thus, the PrRP that we injected likely caused release of NPY from neurons with soma in the PVN, which was associated with increased food intake. It is not clear if the PrRP that we injected bound to neurons in the PVN, as the precise localization of PrRP receptors in avian hypothalamus is unreported.

We also measured hypothalamic expression of other putative orexigenic factors including AgRP, orexin, galanin and PC2 (McCormack and Denbow, 1988; Sakurai et al., 1998; Tachibana et al., 2001; Tachibana et al., 2008; Delfino et al., 2010), but not one was affected by ICV PrRP at the time point evaluated.

Tachibana et al. (2011) identified chicken PrRP (Tachibana et al., 2011). Thereafter, Wang et al. (2012) found another type of chicken PrRP and there are two types of PrRPs in chickens while mammals have only one type of PrRP. Wang et al.

(2012) also performed synteny analysis and concluded that later PrRP is homologous to mammalian PrRP. The two types of PrRPs is not unique in chickens because it is predicted that there are at least two PrRPs in teleost, amphibian and teleost (Wang et al., 2012). Based on these facts, these two PrRPs would have been made by whole genome duplication in the ancestor of vertebrates and it is also suggested that the gene of other type of PrRP had lost in mammals through the process of evolution. Interestingly, the present study and previous studies showed that central injection of both types of PrRPs stimulates feeding behavior in chicks (Tachibana et al., 2004; Tachibana et al., 2011).

In sum, we have demonstrated that ICV PrRP is associated with increased food but not water intake. There was increased c-Fos immunoreactivity in the PVN that coincided with increased NPY mRNA expressions in the whole hypothalamus.

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Table 3.1. Primers used for real time PCR

Gene[a]	Accession ID.	Sequence 5’ to 3’ (forward/reverse)
β-Actin	NM_205518.1	GTCCACCGCAAATGCTTCTAA/TGCGCATTTATGGGTTTTGTT
NPY	M87294.1	CATGCAGGGCACCATGAG/CAGCGACAAGGCGAAAGTC
AgRP	AB029443.1	GGTTCTTCAACGCCTTCTGCTA/ TTCTTGCCACATGGGAAGGT
PrRP	NM_001082419.1	GAGCGCTCCATGGAAATCAG / ATGCCACGCCGGTGTAC
Orexin	NM_204185.2	CCAGGAGCACGCTGAGAAG/ CCCATCTCAGTAAAAGCTCTTTGC
Oxytocin	XM_001231491.3	TGGCTCTCTCCTCAGCTTGTTAT/ GGCACGGCACGCTTACC
CRF	NM_001123031.1	TCAGCACCAGAGCCATCACA/GCTCTATAAAAATAAAGAGGT GACATCAGA
Galanin	NM_001159678.1	CGAATTTCTGACTTACTTGCATCTTAA/ AAAGGTTTGTTTCCTCTGGTGAAG
PC2	XM_004940215.1	TGGGAAGGCAAGGCAATG/CCTGACTGTTTGCAATGCACTT

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

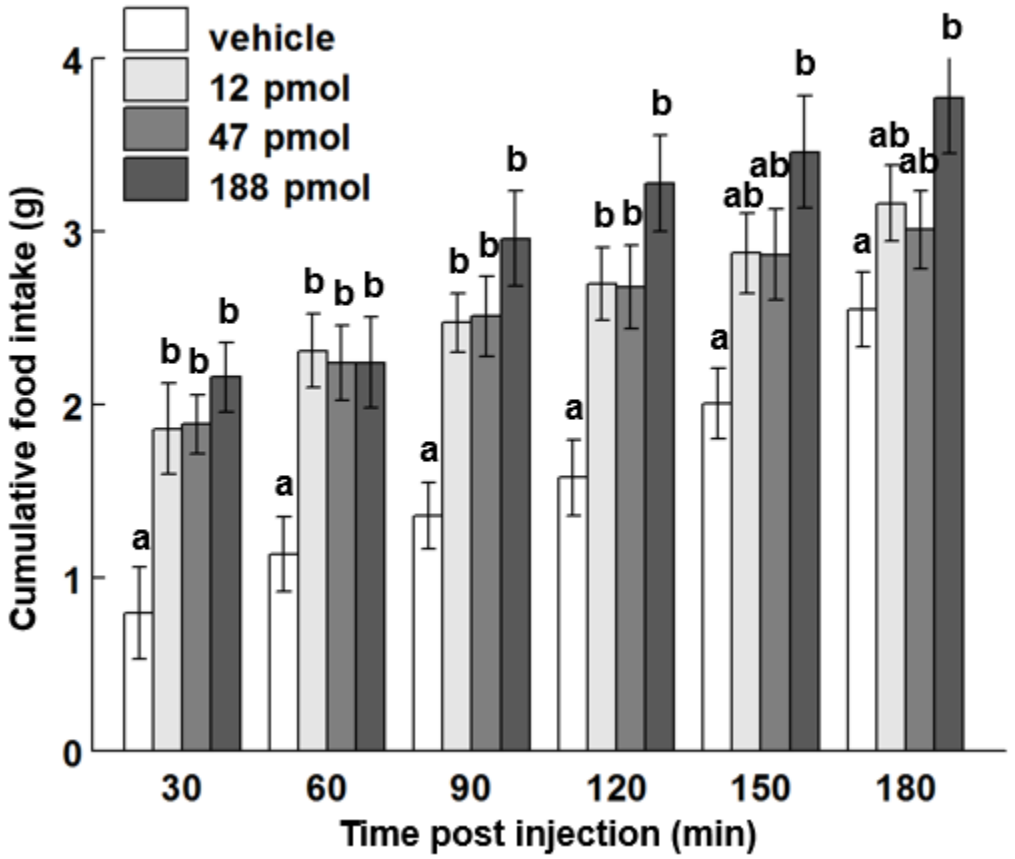

Figure 3.1. Cumulative food intake following intracerebroventricular (ICV) injection of PrRP in Hubbard x Cobb-500 broiler chicks. Values are the means ± SE; bars with different superscripts are different from each other within a time a point ($P < 0.05$). Ten, 8, 9 and 10 chicks were available for analysis at doses 0, 12, 47 and 188 pmol PrRP, respectively.

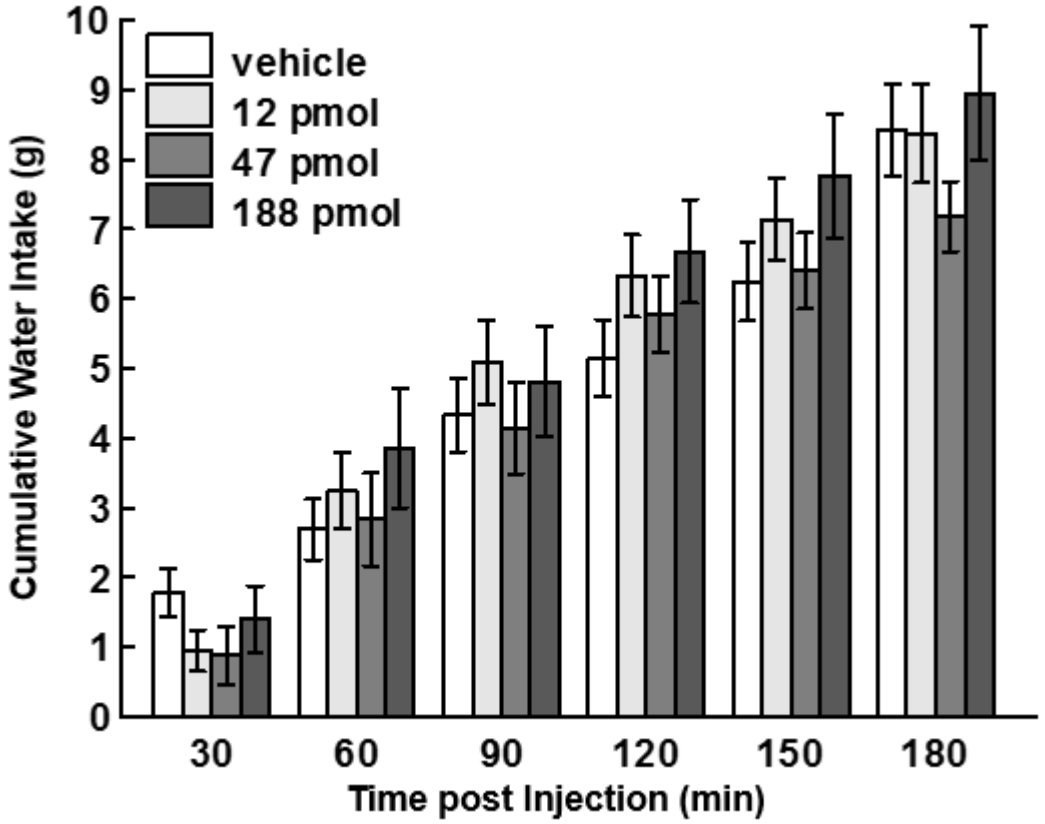

Figure 3.2. Effects of intracerebroventricular (ICV) injection of PrRP on water intake in Hubbard x Cobb-500 chicks. Data are expressed as means ± SE; no significant difference was found ($P>0.05$).

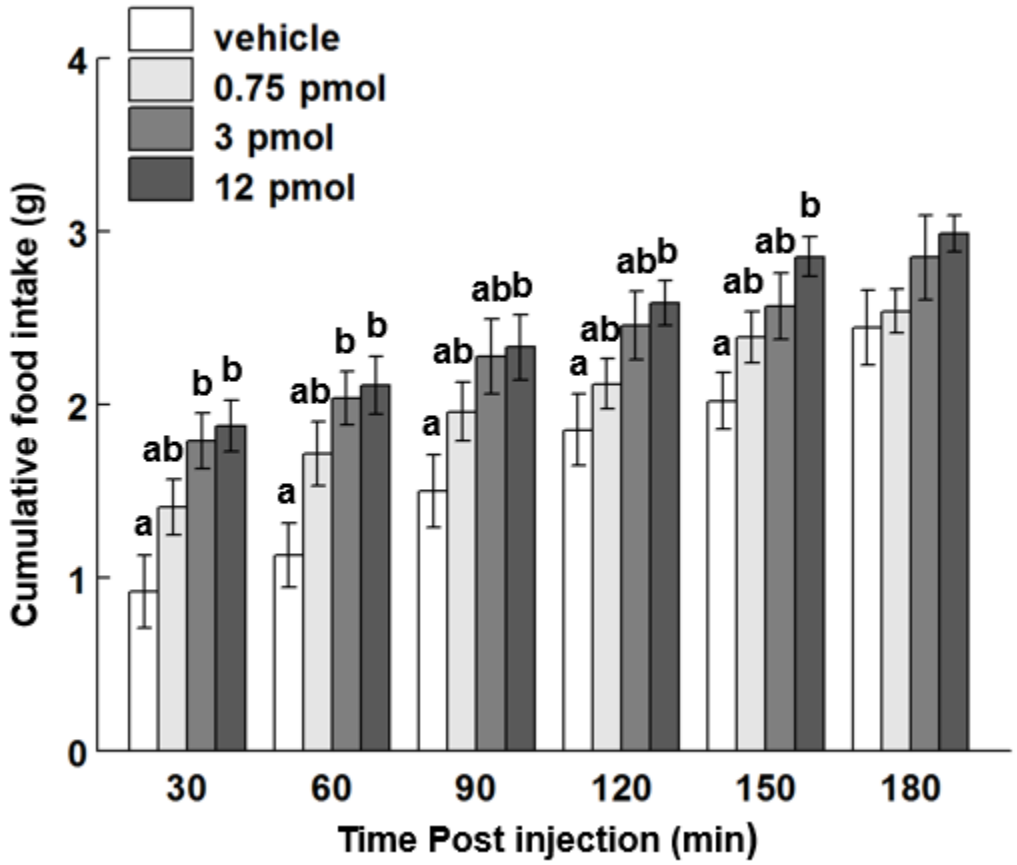

Figure 3.3. Cumulative food intake following intracerebroventricular (ICV) injection of PrRP in Hubbard x Cobb-500 broiler chicks. Values are means ± SE; bars with different superscripts are different from each other within a time point ($P < 0.05$). Nine, 10, 10 and 9 chicks were available for analysis at doses 0, 0.75, 3 and 12 pmol PrRP, respectively.

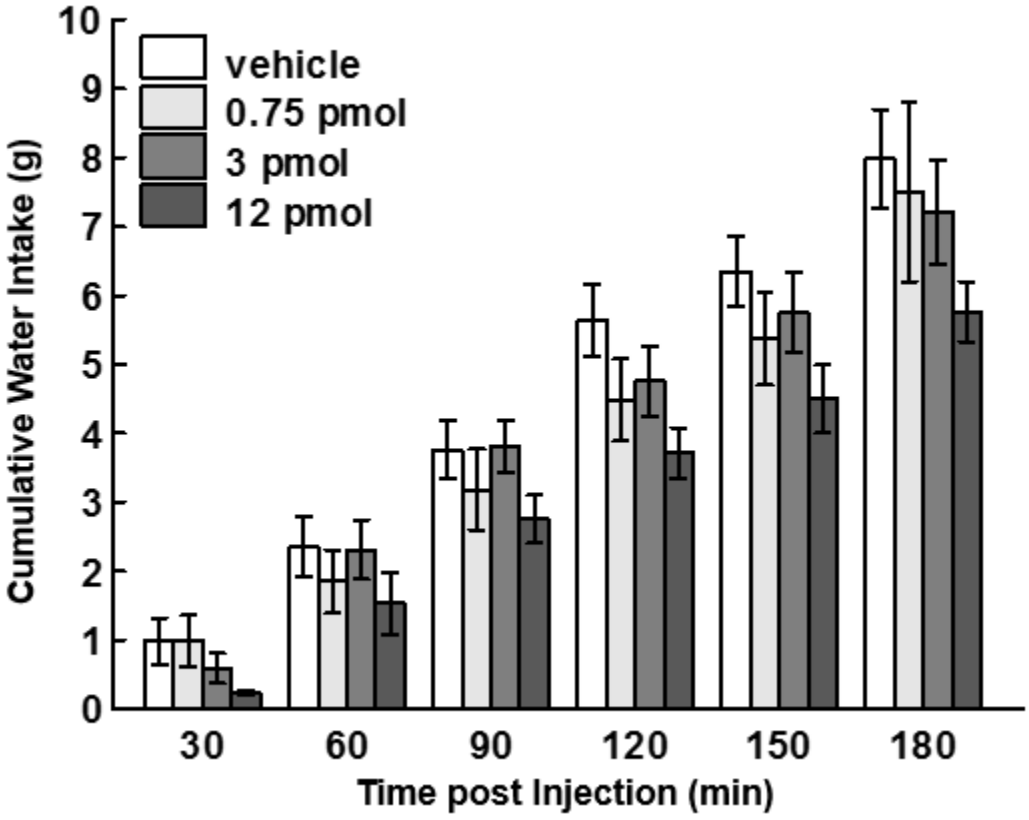

Figure 3.4. Effects of intracerebroventricular (ICV) injection of PrRP on water intake in Hubbard x Cobb-500 broiler chicks. Data are expressed as means ± SE; no significant difference was found (P>0.05).

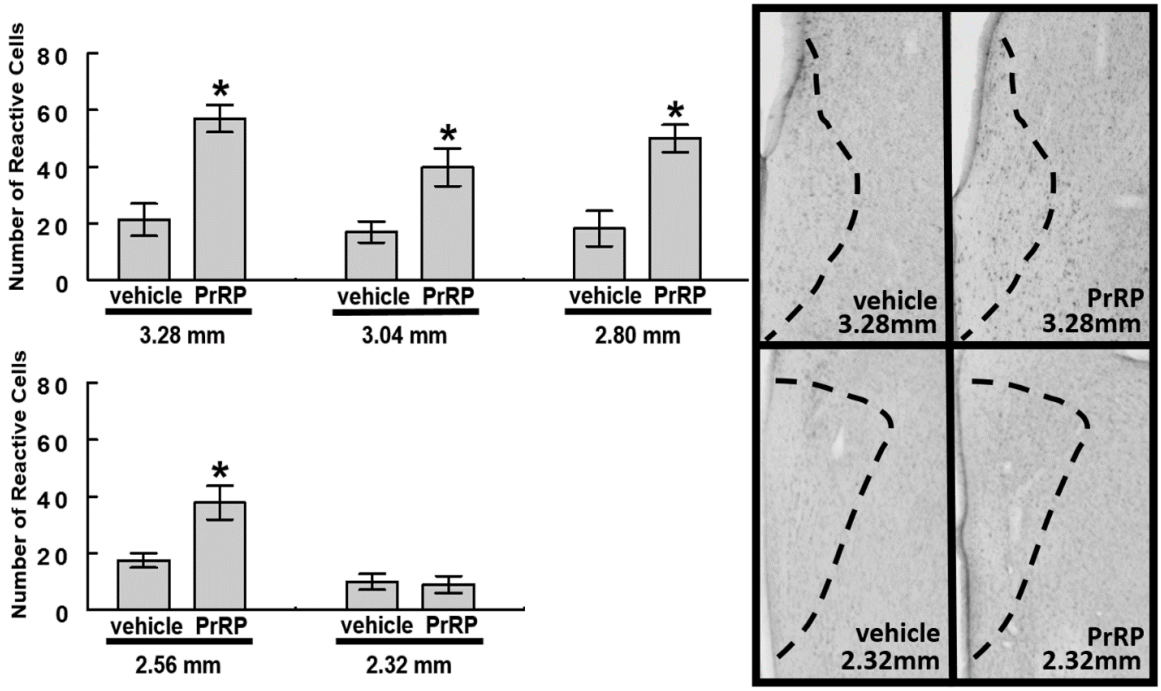

Figure 3.5. Effects of intracerebroventricular (ICV) injection of PrRP on the number of c-Fos immunoreactive cells in the paraventricular nucleus. The numbers shown in mm are Y-axis distance from interaural. Left, values are the means ± SE; asterisks denote significant difference from vehicle ($P < 0.05$). Right, representative photomicrographs captured at 3.23 and 2.32 mm for vehicle and PrRP-treated chicks. For the vehicle PrRP-treated chicks there were 6 chicks available for the analysis.

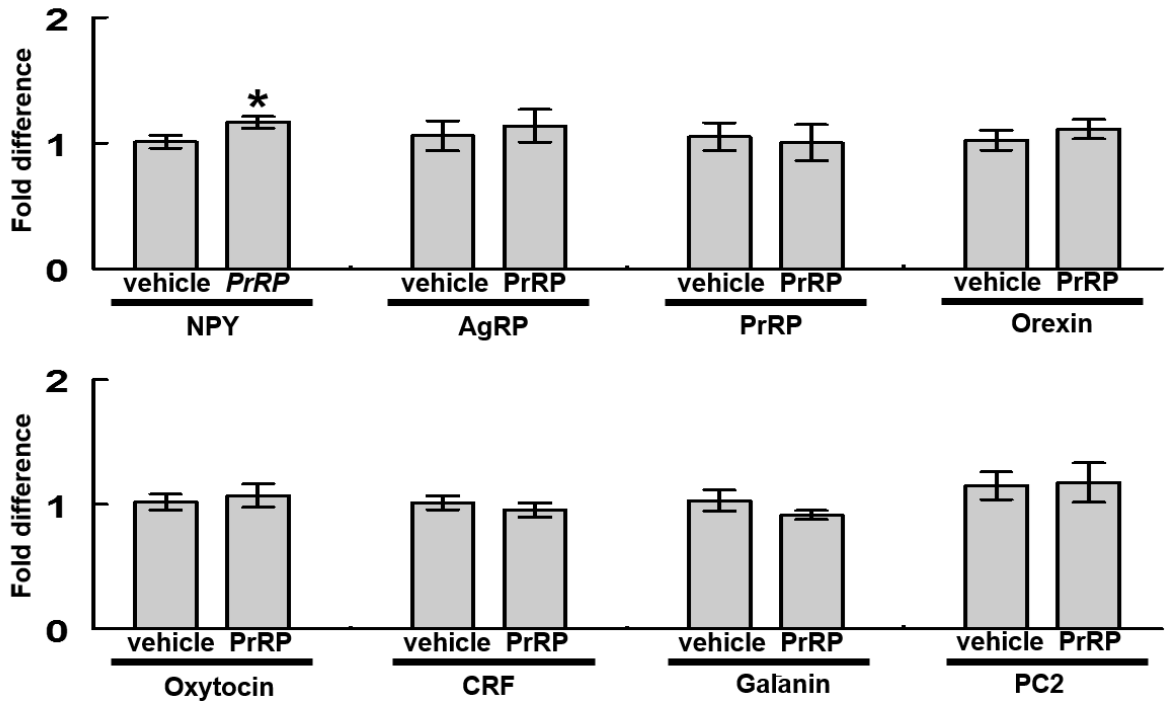

Figure 3.6. Effect of intracerebroventricular (ICV) injection of PrRP on expression of appetite-associated neuropeptide mRNA in whole hypothalamus. Asterisks denote difference from vehicle ($P < 0.05$). Values are means ± SE. For this experiment, nine vehicle and 9 PrRP-treated chicks were available for the analysis.

Chapter IV: The effect of dietary macronutrient composition on exogenous prolactin-releasing peptide's orexigenic effect in chickens

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Abstract: Prolactin-releasing peptide (PrRP) is anorexigenic in mammals but potently orexigenic in chickens. Macronutrients, including carbohydrate, fat and protein also affect food intake, but the interaction between the effects of exogenous neurotransmitters and dietary composition on appetite regulation in non-mammalian species is unclear. The objective of this study was hence to investigate the effects of exogenous PrRP and dietary macronutrient composition on food intake regulation in chicks. Three isocaloric diets were formulated: high carbohydrate (HC), high fat (HF) and high protein (HP). In Experiment 1, chicks were fed 1 of the 3 diets and received an intracerebroventricular (ICV) injection of vehicle, 3, or 188 pmol PrRP. Both 3 and 188 pmol PrRP injection increased intake of the HP diet, but only 188 pmol PrRP was efficacious at increasing HC and HF diet consumption. In Experiment 2, There was a diet effect on mRNA abundance of all appetite-associated genes measured ($P < 0.05$), with greater expression in chicks

fed the HF or HP than HC diet. While neuropeptide Y (*NPY*) mRNA abundance was similar between vehicle and PrRP-injected chicks that consumed HP or HF diets, expression was greater ($P < 0.05$) in PrRP- than vehicle-injected chicks that consumed the HC. In Experiment 3, when chicks had free access to all 3 diets, central administration of 188 pmol PrRP caused preferential intake of HP over the HC and HF diet. In conclusion, chicks fed the HP diet had a lower threshold response in food intake to ICV PrRP compared to the other diets, and chicks treated with PrRP also selected the HP over the HC and HF diet when provided free-choice access to all three diets. Results indicate that dietary macronutrient composition influences PrRP-mediated food intake while PrRP in turn affects nutrient intake and gene expression regulation. These results may have implications for eating disorders as they highlight the importance of understanding the effect of dietary background on neurotransmitter-mediated food intake regulation.

Key words: prolactin-releasing peptide, macronutrient, food intake, chicken, hypothalamus

Introduction

Dietary macronutrient composition plays an important role in regulating appetite and body weight composition and animals will select for specific nutrients in the diet. For instance, the preference of rats for protein was affected by the protein content of their previous meal, with a high-protein (HP) diet leading to selection of carbohydrates and vice versa (Li and Anderson, 1982). In general, HP diets reduce food intake (Li and Anderson, 1982; Bensaid et al., 2003), whereas diets low or deficient in protein stimulate hyperphagic behavior (Anderson, 1979; Anderson and Li, 1987; Whitedouble dagger et al., 2000). There are also reports on the effect of dietary macronutrient composition on food intake in chickens, with more research focused on the effect of different levels of dietary protein than dietary fat and carbohydrate (Swennen et al., 2007). For example, HP diets were associated with decreased food intake in 15 to 27- (Suthama et al., 1991) and 7 day-old broiler chickens (Noy and Sklan, 2002).

The effect of dietary nutrients on food intake is influenced by exogenous appetite-related neuropeptides. Central administration of galanin stimulated the ingestion of fat, and to a lesser extent carbohydrates, but not protein (Tempel et al., 1988). In rodents, central injection of neuropeptide Y (NPY) increased carbohydrate (Morley et al., 1987; Stanley et al., 1989; Welch et al., 1994; Smith et al., 1997) and fat (Stanley et al., 1989; Chavez et al., 1998) intake, leading to obesity (Chronwall et al., 1985; Giraudo et al., 1994). Macronutrient composition in turn affected NPY's effects on appetite. After four weeks of consuming diets that differed in macronutrients, free-choice rats showed increased sensitivity in their food intake response of chow and fat but not sugar to exogenous NPY (van den Heuvel et al., 2014). We recently demonstrated that NPY also

selectively influenced consumption of carbohydrate, fat and protein in chicks, with central NPY injection increasing intake of a HP and high-carbohydrate (HC) but not high-fat (HF) diet under a free-choice scenario (Nelson et al., 2015a). We also showed that diet modulated NPY's effects on food intake, with a high-fat diet enhancing NPY sensitivity (more robust increase in food intake and longer duration of response).

Prolactin-releasing peptide (PrRP) is another potent orexigenic factor (Tachibana et al., 2004). Since it was first described as a hypothalamic prolactin releasing factor in cultured mammalian pituitary cells (Hinuma et al., 1998), many other physiological functions have been ascribed to PrRP, including effects on energy metabolism, cardiovascular regulation, and sleep and pain mediation (Sun et al., 2005). Central injection of PrRP decreases food intake in rats and hypothalamic PrRP mRNA is reduced in a negative energy balance state, such as lactation and fasting in female rats (Lawrence et al., 2000). However, central administration of PrRP increases food intake in chickens, even at very low doses (Tachibana et al., 2004), although it is unknown whether its appetite-related functions are affected by dietary composition. Because we recently found that there was an interaction between exogenous NPY and dietary macronutrient composition on food intake in chicks, and because PrRP is also an extremely potent orexigenic factor in chicks that was shown to be affected by nutrition status in rodents, the objective of this study was to investigate the effects of dietary macronutrient composition on PrRP's orexigenic effects in broiler chicks.

Materials and methods

Animals

Hubbard X Cobb 500 day of hatch chicks (broiler type chicks) were obtained from a local hatchery and caged individually in a room with 30 ± 1 ˚ C and 50 ± 5% relative humidity. Chicks were handled daily to adapt to handling and to minimize stress during data collection, with ad libitum access to diet and tap water. Diets were formulated as shown in Table 5.1 and mixed at Augusta Cooperative Feed Mill (Staunton, Virginia, USA). The HC diet was formulated to meet the minimum requirements defined for the starter phase of commercial broilers (http://www.cobb-vantress.com) and serves as a broiler industry standard starter diet. The HP diet was formulated to contain 30% crude protein and the HF diet to have 60% of the metabolizable energy derived from calories in refined lard, which is designed to be similar to a common rodent obesogenic diet (Beck et al., 1994). Diets were isocaloric and isonitrogenous and formulated to meet minimum digestible amino acid requirements for commercial chicks (Table 5.1). Experimental procedures were performed according to the National Research Council publication, Guide for Care and Use of Laboratory Animals were approved by the Virginia Tech Animal Care and Use Committee.

Intracerebroventricular (ICV) injection procedure

Chicks were injected using a method adapted from Davis et al. (Davis et al., 1979) that does not appear to induce physiological stress (Furuse et al., 1999b). The head of the 4-day post hatch un-anesthetized 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.

Prolactin-releasing peptide (Rat PrRP, 3594.0 molecular weight, American Peptide, Sunnyvale, CA, USA) was dissolved in avian artificial cerebrospinal fluid and injected at a total volume of 5µL with 0.06% Evans blue dye to facilitate injection site localization. At the completion of data collection, chicks were euthanized and their brains dissected to determine accuracy of injection into the lateral ventricle. Chicks without dye present in the lateral ventricle were eliminated from the analysis. Sex was determined visually by dissection.

Experiment 1: Effect on food and water intake in chicks fed HC, HF or HP diet

In Experiment 1, chicks were randomly assigned to one of the three diets at day of hatch, with ad libitum access to food and water. On day 4 post hatch, chicks were randomly assigned 1 of 3 ICV PrRP doses: 0 (vehicle only), 3 or 188 pmol. After ICV injection, chicks were returned to their cages with continued ad lib access to food and water. Food intake was quantified up to 180 minutes following injection. Food intake data were converted to a percentage of body weight by dividing food weight consumed by the chick's body weight at injection time and multiplying by 100. All experiments were replicated and the effect of replicate was not significant, thus data were pooled. Data were analyzed using one-way ANOVA within each diet and within each time point

using the GLM procedure of SAS 9.3 (SAS Institute, Cary, NC) and the statistical model included the main effects of treatment. Sex was not significant in any experiment and was removed from the model. Tukey's method was used post hoc to separate the means. All data are presented as means ± standard error and differences considered significant at $P < 0.05$ for all experiments.

Experiment 2: Hypothalamic expression of appetite-associated factor mRNA in chicks fed HC, HF or HP diet

In Experiment 2, chicks were randomly assigned to 1 of the 3 diets at day of hatch, with ad libitum access to food and water. On day 4 post hatch, 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 min following injection, chicks were deeply anesthetized with sodium pentobarbital via cardiopuncture, decapitated, and brains 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 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-RNA Lysis reagent (5-PRIME, USA) for 2 × 2 min at 20 Hz with a Tissue Lyser II (Qiagen). After centrifugation for 10 min for 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 a 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 5. 2 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 (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. Data were analyzed by the ΔΔCt method, where β actin served as the reference gene and the average of the vehicle-treated chicks fed the HC diet served as the calibrator sample. Relative quantity values ($2^{-\Delta\Delta Ct}$) were used for statistical analysis with the GLM procedure of SAS 9.3 (SAS Institute, Cary, NC) where

the model included the effects of treatment and diet and the interaction between them. Tukey's test was used for pairwise comparisons.

Experiment 3: Effect on food and water intake in chicks fed HC, HF and HP diets

In Experiment 3, procedures were the same as in Experiment 1, except that each chick had access to all of the 3 diets prior to and after ICV PrRP injection. The position of the 3 diets was randomly assigned at day of hatch and maintained until the end of experiment. Data were analyzed using two-way ANOVA within treatment using the GLM procedure of SAS 9.3 (SAS Institute, Cary, NC) and the statistical model included time and diet and their interaction. The diet by time interaction was significant and thus secondary ANOVAs were conducted within each time point. Sex was not significant in any experiment and was removed from the model. Tukey's method was used post hoc to separate the means.

Results

Experiment 1: Effect on food and water intake in chicks fed HC, HF or HP diet

By 4 days post hatch, body weights (BW) of chicks fed HC, HF and HP diets were significantly different from each other (HC: 70.1 ± 1.9 g; HF: 52.8 ± 1.0 g; HP: 62.9 ± 1.1 g). There was no difference in the amount of the three different diets consumed among vehicle-treated chicks (Figure 4.1). For chicks fed the HC and HF diets, 188 pmol PrRP increased food intake throughout the entire observation period. ICV injection of both 3 pmol and 188 pmol PrRP increased food intake in HP-fed chicks from 30 min following injection up to 150 min and 180 min following injection, respectively. At 180

min post injection, relative to the vehicle-treated chicks, food intake after 188 pmol PrRP injection increased 47.2%, 69.1% and 29.8% in chicks fed the HC, HF and HP diets, respectively.

Experiment 2: Hypothalamic expression of appetite-associated factor mRNA in chicks fed HC, HF or HP diet

Table 5.3 Table 1 shows the hypothalamic mRNA abundance of appetite-associated factors and some of their receptors in chicks fed one of the three diets and treated with either vehicle or PrRP. There were main effects of diet ($P < 0.05$) for all genes evaluated. There was greater hypothalamic expression of agouti-related peptide (*AgRP*), *NPY* and *NPY* receptor 2 (*NPYR2*) ($P < 0.0001$) in chicks fed the HF diet, intermediate expression in the HP-fed group, and lowest expression in chicks fed the HC diet. Expression of melanocortin receptors 3 and 4 (*MC3R* and *MC4R*, respectively) was greater ($P < 0.0002$) in chicks fed the HP and HF than HC diet. Abundance of *NPYR5* mRNA was greatest ($P < 0.0001$) in the HP-fed group, intermediate in the HF-fed chicks, and lowest in the HC group. There was greater mRNA abundance of *PrRP* ($P < 0.001$), corticotropin-releasing factor (*CRF*) ($P = 0.0069$) and *NPY* receptor 1 (*NPYR1*) ($P < 0.0001$) in chicks fed the HP diet than chicks fed the HC and HF diets. The expression of oxytocin (*OXT*) ($P = 0.0004$) and orexin (*ORX*) ($P = 0.01$) was greater in chicks fed the HF diet than the HC diet.

There was a main effect of PrRP treatment on mRNA abundance of *NPYR2* ($P = 0.01$), which was decreased after PrRP administration. There was an interaction of diet and treatment ($P = 0.04$) on mRNA abundance of *NPY* (Figure 4.2), where PrRP

treatment increased *NPY* mRNA abundance in chicks that consumed the HC, but not the HF or HP diets at 1 hour post-injection.

Experiment 3: effect on food and water intake in chicks fed HC, HF and HP diets

In general, irrespective of treatment, chicks selected the HC and HP diet over the HF diet (Figure 4.3). The administration of 3 pmol PrRP did not change the preference for the HC and HP diets; however, chicks that received 188 pmol PrRP consumed more HP diet than either HC or HF diet, even though for 60 min post-injection there was no significant difference between the consumption of HC and HF diet. At 180 min post injection in vehicle-treated chicks, the percentage of HC, HF and HP diet consumption as a fraction of total diet consumed was 38%, 9% and 53%, respectively; the percentage for chicks treated with 188 pmol PrRP was 29%, 9% and 62, respectively%.

Discussion

Consistent with previous reports (Tachibana et al., 2004; Tachibana et al., 2011), ICV PrRP increased food intake in chicks. Little is known about how PrRP affects macronutrient selection (Lawrence et al., 2000), particularly in chicks where it has the opposite effect on food intake as compared to mammals. In a study that used the same diet formulations as reported for the present study, ICV NPY dose-dependently increased food intake in chicks fed the HF diet, with the highest magnitude of increase for chicks treated with 2 nmol NPY at 180 min after injection (Nelson et al., 2015b). In general, the HF diet increased the sensitivity to exogenous NPY, while NPY in turn increased selection of the HC and HP but not HF diet (Nelson et al., 2015b). In the present study, chicks that were fed the HF diet and received 188 pmol PrRP also had the highest magnitude of food intake. Unlike the NPY study, however, the dose-dependent response in food intake only occurred in chicks fed the HP diet, which implies that dietary macronutrient composition also affects the response to specific exogenous neuropeptides. That NPY and PrRP are both potently orexigenic in chicks but are affected differently by diet composition implies that their mechanism of action is different and/or that dietary macronutrient composition affects distinct neurotransmitter signaling pathways in the brain.

Unlike the previous study with NPY that focused solely on measuring food intake responses in chicks (Nelson et al., 2015b), the present study also included an experiment designed to investigate the hypothalamic molecular mechanism mediating the differential response to PrRP in chicks that consumed different diets. Overall, most of the appetite-associated genes (except for PrRP, OXT, ORX and CRF) tested were expressed less in

the hypothalamus of chicks fed the HC diet than the other two diets. There was increased hypothalamic NPY and AgRP mRNA in the hypothalamus of chicks that ate the HF diet compared to chicks fed the HC and HP diet, contradictory to a rodent study where rats fed a HC diet had increased hypothalamic NPY expression compared to rats that consumed a HF diet (Wang et al., 1999). Moreover, PrRP injection was associated with increased NPY mRNA abundance in chicks that were fed the HC diet. NPY is one of the most potent orexigenic factors in chicks and AgRP increases food intake in rats (Kuenzel et al., 1987; Hagan et al., 2001). Even though central AgRP injection did not increase food intake in broiler chickens, it is still important in the regulation of appetite as the anorexigenic effect of alpha-melanocyte stimulating hormone in chicks is attenuated by ICV AgRP (Tachibana et al., 2001).

Chicks fed the HP diet had a lower threshold response in food intake to exogenous PrRP. This increased sensitivity to PrRP could be related to the increased expression of PrRP, NPYR1, NP*YR5* and *MC4R* mRNA in chicks fed the HP diet compared to chicks fed the HF and HC diet. The NPYR2 and NPYR5 may be associated with the regulation of food intake in chickens, however, that activation of NPYR2 by NPY (13-36) only increased food intake at 30 min post-injection may indicate that the role of NPYR2 in food intake is weaker than NPYR5 (Ando et al., 2001).

The effect of ICV PrRP on NPY mRNA abundance in chicks that consumed the HC diet is consistent with a previous report (that used the same HC diet) and also coincides with the activation of the paraventricular nucleus (PVN) in the hypothalamus (Wang et al., 2015; in review). According to rodent studies, there are NPY-expressing neurons in the PVN and the expression of NPY is c-Fos dependent (Baker and

Herkenham, 1995; Kovacs, 1998a; Wang et al., 2002b; Wu et al., 2004b). Our previous study also showed that ICV PrRP activated the rostral PVN in chicks but not the arcuate, dorsal medial nucleus or ventromedial nucleus (Wang et al., 2015; in review), which are nuclei in the hypothalamus that also express NPY according to the rodent literature (Kalra et al., 1999). This may indicate that the orexigenic effect of PrRP in chicks may be associated with up-regulated NPY mRNA of PVN origin. Thus, effects of PrRP on food intake may involve transcriptional regulation of appetite-associated factors in the hypothalamus, although results should be interpreted with caution without accompanying peptide abundance data for specific hypothalamic nuclei at additional time points.

This study also revealed that when given the choice of diets, chicks selected the HC and HP diet over the HF diet and that PrRP enhanced the preference for the HP diet. When rats were offered similar diets, the HP diet was the least consumed, with HC or HF diets being the most preferred (Smith et al., 1997). To explain differences across species is beyond the scope of our study, but may involve differences in age- and species-specific physiology, source, quantity, and balance of nutrients, duration of the feeding trial and timing and type (e.g., meal vs. continuous access) of feeding, and interaction of other nutrients in affecting physiology. According to studies conducted with rodents, HP diets tend to be more satiating than HC and HF diets (Bensaid et al., 2003) but in our study the HF appeared to be the least consumed in a choice environment. In other studies, adult rodents consumed different diets before switching to the experimental diets. In the present study, chicks were fed experimental diets immediately after hatch, which is advantageous because it allows us to understand how the physiology of the animal is affected by diet without the influence of previous nutrition.

In conclusion, chicks fed the HP diet had a lower threshold response in food intake to ICV PrRP compared to the two other diets, and this may be associated with increased expression of AgRP and MC3R in the hypothalamus. Injection of PrRP increased mRNA abundance of NPY in the hypothalamus of chicks that consumed the HC, but not HP or HF diet. Chicks that were injected with PrRP also selected the HP diet over the HC and HF diet when provided free-choice access to all three diets. Results demonstrate that dietary macronutrient composition influences appetite regulation, perhaps via transcriptional regulation of appetite-associated factors in the hypothalamus, and diet also affects PrRP-mediated food intake in chicks, while PrRP in turn affects nutrient intake. In exploring strategies to affect appetite in individuals with eating disorders, these results may have implications for highlighting the importance of understanding the effect of background nutrition on neurotransmitter-mediated responses in the brain.

Table 5.1. Ingredient and chemical composition of experimental diets.

Ingredient (% as-fed)[1]	High carbohydrate	High protein	High fat
Ground corn	58.80	34.64	2.16
Soybean meal	36.12	57.48	42.48
Soybean hulls	0.00	0.00	27.71
Lard	0.00	0.00	24.00
Soybean oil	1.2	4.80	0.00
Methionine 99%	0.28	0.04	0.35
Threonine	0.10	0.00	0.07
L-Lysine 78%	0.09	0.00	0.00
Dicalcium Phosphate	1.54	1.41	1.62
Calcium carbonate	1.15	1.07	1.01
Sodium bicarbonate	0.15	0.18	0.02
SALT920831	0.37	0.36	0.37
Coban 90[2]	0.05	0.05	0.05
Phytase-RONOZYME[3]	0.05	0.05	0.05
Vitamin/ mineral premix[4]	0.10	0.10	0.10
Choline Chloride-60%	0.00	0.00	0.01
Kcal ME/kg	3,000	3,000	3,050
Crude protein	22%	30%	22%
Crude Fat	3.7%	6.7%	25%
Crude Fiber	2.5%	2.6%	12.4%

1Diets were formulated to meet or exceed minimum recommended specifications for Cobb-500 broilers during the starter phase (Cobb-Vantress).

2Coban 90 (Elanco Animal Health) contains 90 grams of Monensin sodium per pound of premix and is included in the diet as a coccidiostat.

3DSM Nutritional Products, Ltd.

[4]Guaranteed analysis (per kg of premix): Manganese, 25.6 g; selenium, 120 mg; zinc, 30 g; Vitamin A, 4,409,171.076 IU; Vitamin D_3, 1,410,934.744 ICU; 13,227.513 IU; d-biotin, 88.183 mg.

Table 5.2. Primers used for real time PCR.

Gene[1]	Accession ID	Sequence 5' to 3' (forward/reverse)
β-Actin	NM_205518.1	GTCCACCGCAAATGCTTCTAA/TGCGCATTTATGGGTTTTGTT
AgRP	AB029443.1	GGTTCTTCAACGCCTTCTGCTA/ TTCTTGCCACATGGGAAGGT
PrRP	NM_001082419.1	GAGCGCTCCATGGAAATCAG / ATGCCACGCCGGTGTAC
OXT	XM_001231491.3	TGGCTCTCTCCTCAGCTTGTTAT/ GGCACGGCACGCTTACC
NPY	M87294.1	CATGCAGGGCACCATGAG/CAGCGACAAGGCGAAAGTC
Orexin	NM_204185.2	CCAGGAGCACGCTGAGAAG/ CCCATCTCAGTAAAAGCTCTTTGC
CRF	NM_001123031.1	TCAGCACCAGAGCCATCACA/GCTCTATAAAAATAAAGAGGTG ACATCAGA
NPYR1	NM_001031535.1	TAGCCATGTCCACCATGCA / GGGCTTGCCTGCTTTAGAGA
NPYR2	NM_001031128.1	TGCCTACACCCGCATATGG / GTTCCCTGCCCCAGGACTA
NPYR5	NM_001031130.1	GGCTGGCTTTGTGGGAAA/TTGTCTTCTGCTTGCGTTTTGT
MC3R	XM_004947236.1	GCCTCCCTTTACGTTCACATGT/GCTGCGATGCGCTTCAC
MC4R	NM_001031514.1	CCTCGGGAGGCTGCTATGA/GATGCCCAGAGTCACAAACACTT

[1]AgRP: agouti-related peptide; PrRP: prolactin-releasing peptide; NPY: neuropeptide Y; CRF: corticotropin-releasing factor; NPYR1: neuropeptide Y receptor 1; NPYR2: neuropeptide Y receptor 2; NPYR5: neuropeptide Y receptor 5; MC3R: melanocortin 3 receptor; MC4R: melanocortin 4 receptor.

Table 5.3. Hypothalamic mRNA abundance in chicks fed different diets and treated with PrRP.

Diet[1]	AgRP[3]	PrRP	OXT	NPY	ORX	CRF	NPYR1	NPYR2	NPYR5	MC3R	MC4R
HC[2]	1.13 ± 0.08[a]	1.04 ± 0.07	1.01 ± 0.06	1.09 ± 0.03[a]	1.02 ± 0.05	1.12 ± 0.09	1.02 ± 0.05	1.00 ± 0.05	1.03 ± 0.05	0.98 ± 0.03[ab]	1.02 ± 0.04
HF	0.84 ± 0.08[b]	1.05 ± 0.07	0.96 ± 0.05	0.94 ± 0.03[b]	0.98 ± 0.05	1.14 ± 0.08	0.97 ± 0.05	0.96 ± 0.05	0.97 ± 0.04	0.91 ± 0.03[b]	0.96 ± 0.04
HP	1.11 ± 0.08[a]	1.10 ± 0.07	1.05 ± 0.06	1.01 ± 0.03[ab]	1.01 ± 0.05	0.99 ± 0.09	1.04 ± 0.05	0.94 ± 0.05	1.02 ± 0.04	1.03 ± 0.03[a]	1.08 ± 0.04
***P*-value**	0.03	0.80	0.57	<0.01	0.81	0.42	0.53	0.60	0.57	0.02	0.09
Dose											
0	1.02 ± 0.06	1.04 ± 0.06	0.99 ± 0.04	1.00 ± 0.02	1.02 ± 0.04	1.03 ± 0.07	1.02 ± 0.04	1.01 ± 0.04	1.01 ± 0.03	1.00 ± 0.03	1.00 ± 0.03
188	1.03 ± 0.07	1.09 ± 0.06	1.03 ± 0.05	1.03 ± 0.02	0.98 ± 0.04	1.13 ± 0.08	0.99 ± 0.04	0.92 ± 0.36	1.00 ± 0.04	0.95 ± 0.03	1.04 ± 0.03
***P*-value**	0.91	0.47	0.56	0.63	0.51	0.30	0.69	0.09	0.57	0.25	0.42
Interaction *P*-value	0.31	0.83	0.66	0.01	0.94	0.68	0.84	0.54	0.85	0.34	0.38

[1]Least squares means ± standard errors of the mean (n=7-10) for main effects of diet (Diet), prolactin-releasing peptide (PrRP) treatment (Trt), and *P*-values for the main effects and the two-way interaction of diet and treatment. Different letters within a gene and main effect indicate a significant difference, $P < 0.05$; Tukey's test. Four day-old chicks that consumed one of the three diets were injected centrally with PrRP and hypothalamus removed at 1 hour post-injection for gene expression analysis.

[2]Diets included the high carbohydrate (HC), high fat (HF) and high protein (HP).

[3]Hypothalamic mRNA abundance of AgRP, agouti-related peptide; PrRP; OXT, oxytocin; NPY, neuropeptide Y; ORX, orexin; NPYR1, NPY receptor 1; NPYR2, NPY receptor 2; NPYR5, NPY receptor 5; MC3R, melanocortin receptor 3; MC4R, MC receptor 4.

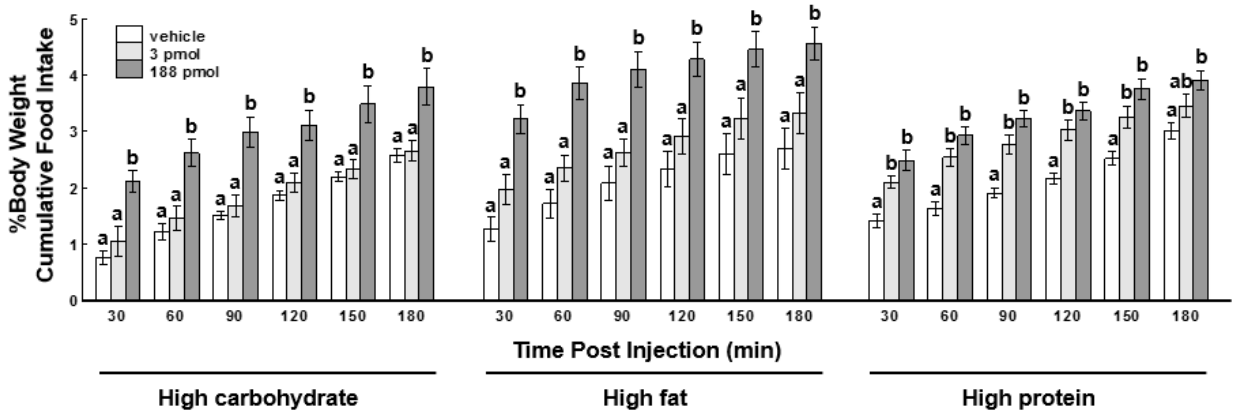

Figure 4.1. Cumulative food intake expressed as a percentage of body weight of PrRP-injected chicks fed different diets. n = 18 to 20 chicks per PrRP dose per diet; bars with different superscripts are significantly different from one another within a time and within a diet.

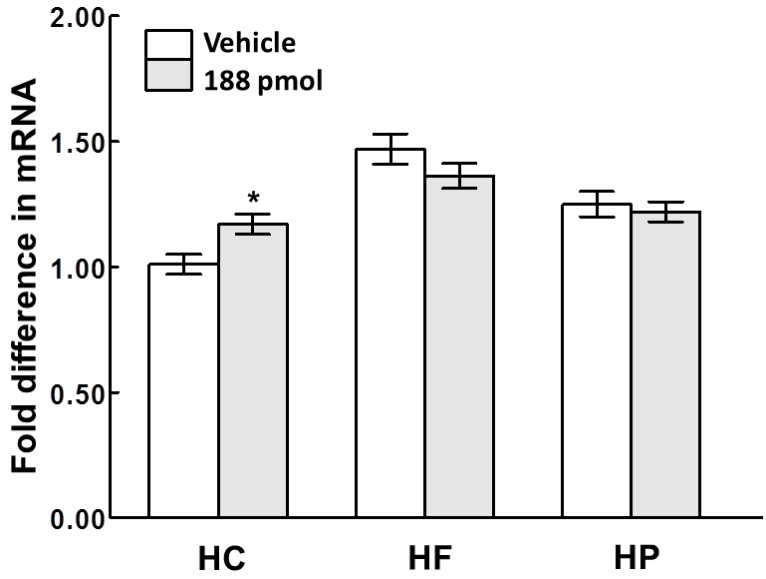

Figure 4.2. Expression of hypothalamic neuropeptide Y (NPY) mRNA in chicks fed the HC, HF or HP diet that received either vehicle or 188 pmol PrRP. There was an interaction between diet and PrRP treatment. Values represent means ± standard error (n = 7 to 10). Asterisks denote significant difference from vehicle ($P < 0.05$), within diet.

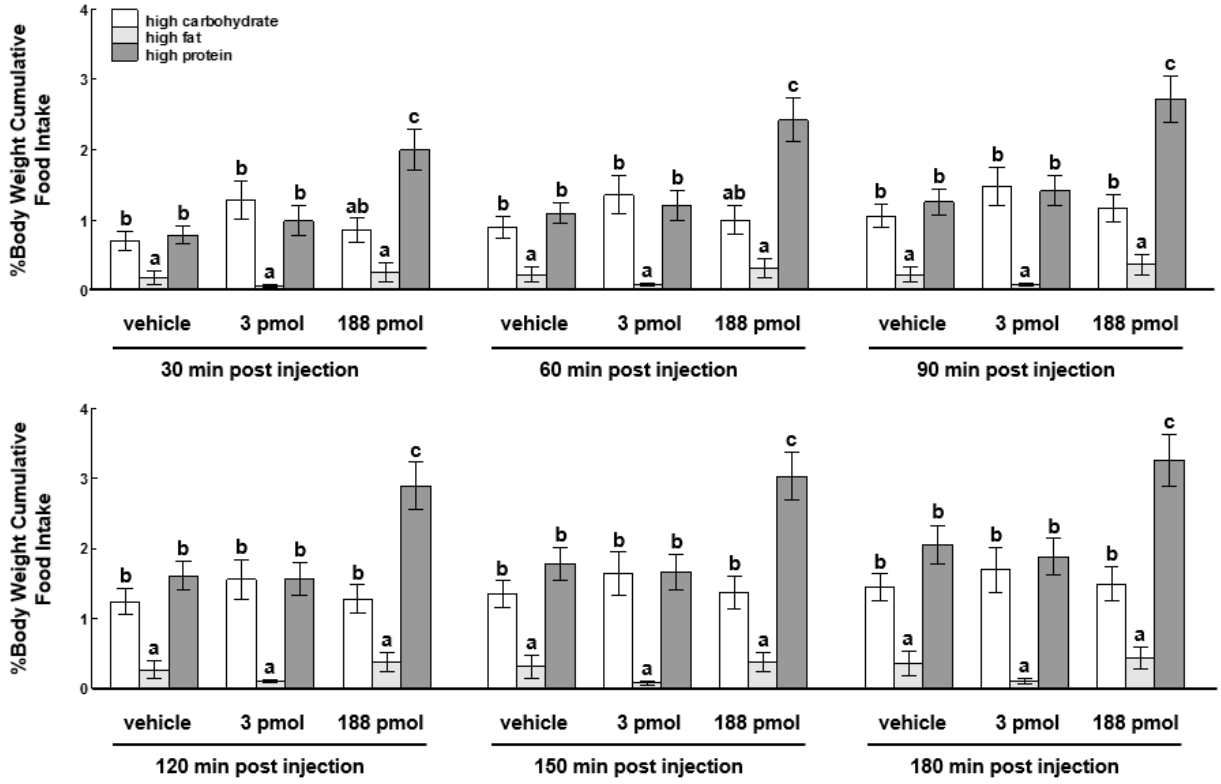

Figure 4.3. Cumulative food intake expressed as a percentage of body weight of PrRP-injected chicks provided free access to HC, HF, and HP diets simultaneously. n = 18 chicks per PrRP dose per diet; bars with different superscripts are significantly different from one another within a treatment and within a time.

Chapter V: The threshold of prolactin-releasing peptide-induced hyperphagia is lower in chicks selected for low compared to high juvenile body weight

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Abstract: Prolactin-releasing peptide (PrRP) is an orexigenic neuropeptide in chickens, whereas in rodents it decreases food intake. The effects of intracerebroventricular (ICV) injection of PrRP on food intake were evaluated in chicks selected for low (LWS) and high (HWS) bodyweight. Food and water intake were quantified after central injection of 0, 24, 94 and 375 pmol rat PrRP in LWS and HWS chicks. The LWS chicks had increased food intake when injected with each of the three doses of PrRP, however, only 375 pmol PrRP injection increased food intake in the HWS chicks. At the end of the three-hour experiment, blood glucose concentration was measured. Only the LWS that received 375 pmol PrRP blood glucose concentration increased compared to LWS chicks that received vehicle, whereas blood glucose concentration was not affected in HWS chicks after PrRP injection. These data support that the threshold of PrRP-induced hyperphagia is lower in LWS than HWS chicks.

Introduction

Prolactin-releasing peptide (PrRP) was discovered as an endogenous ligand for the G protein-coupled receptor (GPR10) and caused prolactin release from bovine

hypothalamic extracts (Hinuma et al., 1998). It is well documented that PrRP has a range of physiological responses, such as luteinizing hormone stimulation, sleep regulation and growth hormone inhibition (Seal et al., 2000; Zhang et al., 2000; Maruyama et al., 2001), which includes food intake (Lawrence et al., 2000). However, central injection of PrRP decreases food intake in rats and goldfish, it increases food intake in chicks (Lawrence et al., 2002; Tachibana et al., 2004; Kelly and Peter, 2006). PrRP and its receptors are expressed in the chicken hypothalamus, but information about individual hypothalamus nucleus expression of PrRP and its receptors in the hypothalamic is still unknown (Wang et al., 2012). The anorexigenic effect of PrRP in rodents and orexigenic effect in chicks are associated with activation of paraventricular nucleus in the hypothalamus (Bechtold and Luckman, 2006; Wang et al., 2015).

Our laboratory has been focusing on understanding how the responses of appetite-associated neurotransmitter are affected by selection for low or high body weight. The animal models we have been using are the low (LWS) and high (HWS) body weight select lines of Plymouth Rock chicks (Dunnington et al., 2013). Among the LWS line some individuals are hypophagic and others are anorexic (Zelenka et al., 1988). Unlike the LWS line, the HWS exhibits hyperphagia and after selection age (56 days), food intake must be restricted to reduce obesity-associated health complications (Siegel et al., 1984).

We demonstrated that LWS and HWS chicks have different food intake responses to some of the neuropeptides related to food intake, such as corticotrophin releasing factor (CRF), alpha-melanocyte stimulating hormone (α-MSH) and amylin (Cline et al., 2008b; Cline et al., 2009a; Cline et al., 2010). LWS chicks were more sensitive to these

anorexigenic neuropeptides compared to HWS chicks. However, there is far less research reported for effect of orexigenic neuropeptides on food intake in LWS and HWS chicks. Among those reported studies, the LWS chicks did not respond to central NPY administration and had a similar dose-dependent response to intracerebroventricular (ICV) galanin injection than HWS chicks (Hagen et al., 2013; Newmyer et al., 2013). Recently, we found that PrRP is a very potent hunger stimulating factor in broiler chicks, with an efficacious dose as low as 3 pmol (Wang et al., 2015; in review). Thus we designed the present study to investigate the effects of PrRP on food intake in the LWS and HWS chicks.

Materials and methods

Animals

The LWS and HWS lines of chickens used in this study are from a long-term divergent selection experiment for high or low body weight at 8 weeks of age. The founder population White Plymouth Rock chicks consisted of crosses of 7 inbred lines with maintained as closed populations under continuous selection. For reviews of this selection program see Marquez et al. (Marquez et al., 2010) and Dunnington et al. (Dunnington et al., 2013). Eggs obtained from age contemporary parents from S56 generation parental stocks were incubated in the same machine. After hatch, chicks were group-caged for 2 days, then individually in a room at 30 ± 2 °C and 50 ± 5% relative humidity where they had *ad libitum* access to a mash diet (22% crude protein, 3000 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 11:00 and 16:00 h using 5-day post-hatch chicks. Data in each experiment were recorded from both lines concurrently and injections were performed sequentially, LWS, HWS, LWS, HWS and so forth. 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 Animal Care and Use Committee.

Intracerebroventricular (ICV) injection procedure

Chicks were injected using a method adapted from Davis et al. (Davis et al., 1979) that does not appear to induce physiological stress (Furuse et al., 1999b). 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 for 5 s post-injection to reduce backflow. Chicks were assigned to treatments at random. Rat prolactin-releasing peptide (PrRP, 3594.0 molecular weight, American Peptide, Sunnyvale, CA, USA) was dissolved in 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. After data collection, the chicks were decapitated and their heads sectioned coronally to determine site of injection. Any chick without dye present in the lateral ventricle system was eliminated from analysis. Sex was determined visually by dissection.

Experiment 1: Effect of food and water intake in LWS and HWS chicks

Chicks from the S_{56} generation from each line were randomly assigned to receive 0 (vehicle only), 24, 94 or 375 pmol PrRP by ICV injection. After injection, chicks were returned to their individual cages and given *ad libitum* access to both food and water, with individual food and water containers weighed (0.01 g) every 30 min for 180 min post-injection. Data were analyzed using analysis of variance (ANOVA) at each time point. There was no effect of sex thus the reduced model included line, PrRP dose and the line by PrRP dose interaction. When the interaction was significant ($P < 0.05$), data were analyzed within each line for the effect of PrRP dose using Tukey's method of multiple comparisons. The LWS and HWS lines consume inherently different amounts of food due to differences in body size. Therefore, food and water intake data were normalized to body weight at each time point. This conversion was made by dividing the amount of food each chick consumed by its body weight (0.01 g) immediately prior to its ICV injection and multiplying by 100.

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) with sensitivity in the range 20 – 600 mg/dl. 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.

Results and discussion

Central PrRP significantly increased food intake in LWS chicks over the vehicle at 94 and 375 pmol doses at all observation times, while in HWS chicks only the 375 pmol dose increased food intake for the whole observation period (Figure 5.1). LWS chicks also responded to 24 pmol PrRP from 90 to 180 min post injection, whereas HWS chicks did not respond at any of the times evaluated. By 180 min post injection, there was about a 120%, 170% and 294% increase in food intake for LWS chicks that received 24, 94 and 375 pmol PrRP, respectively, and a 25% increase for 375 pmol in the HWS chicks. Water intake was not affected in LWS and HWS chicks (Figure 5.2).

Whole blood glucose concentrations were increased only in LWS chicks (Figure 5.3). LWS chicks that received 375 pmol PrRP injection had significantly higher blood glucose compared to chicks that received vehicle.

The orexigenic effect of PrRP agreed with previously published studies conducted in layer and broiler chickens (Tachibana et al., 2004)(Wang et al., 2015; in review). Many of the LWS chicks are hypophagic and some of them have anorexia (Zelenka et al., 1988), therefore, the LWS chicks were expected to have a higher threshold to orexigenic neuropeptides. However, our results indicate that the LWS chicks, which are hypophagic, have a lower threshold of response to ICV PrRP than HWS chicks which are hyperphagic. The increased food intake pattern after PrRP injection in LWS chicks was closer than HWS to that of broiler chickens (Wang et al., 2015; in review). This is the first time we have demonstrated that the LWS chicks are more sensitive to an orexigenic neuropeptide than the HWS chicks. The threshold of increasing food intake was similar to that of galanin in HWS chicks (a dose lies between 94 pmol and 375 pmol vs between

300 and 700 pmol, respectively), however, the threshold of response to PrRP was much lower than galanin in the LWS chicks (a dose between 0 and 24 pmol vs between 300 and 700 pmol, respectively) (Hagen et al., 2013). Since PrRP is a potent orexigenic neurotransmitter in chicks (Wang et al., 2015; in review), selection for juvenile body weight may not have favored a gain of sensitivity in the PrRP orexigenic system in the HWS chicks.

The anorexigenic effect of PrRP in rats may be associated with increased hypothalamic oxytocin and CRF expression, both of which are anorexigenic neuropeptides in rat (Matsumoto et al., 2000; Ellacott et al., 2002; Bechtold and Luckman, 2006). This is further supported by the fact that the PVN is activated immunochemically following ICV PrRP injection, and PVN is the major site that produces oxytocin and CRF in rat brain (Bechtold and Luckman, 2006; Bulbul et al., 2011; Dabrowska et al., 2013). According to our previous study in chickens, central PrRP injection only activated the rostral PVN in the hypothalamus and increased hypothalamic NPY mRNA expression (Wang et al., 2015; in review). Since the expression of NPY is c-Fos dependent (Kovacs, 1998b; Wang et al., 2002a; Wu et al., 2004a), and the PVN releases NPY (Kameda et al., 2001), the fact that the PVN was the only nucleus that had activated c-Fos immunochemistry from our previous study may indicate that the orexigenic effect of PrRP may be related to increased NPY expression in the hypothalamus.

However, the LWS chicks do not increase food intake after central NPY injection (Newmyer et al., 2013), therefore, the orexigenic effect of PrRP in the LWS chicks may not be associated with a change in NPY expression in the hypothalamus. Galanin is the

other orexigenic neuropeptide that has been studies in the LWS and HWS chicks, which causes a similar magnitude of food intake increase in the LWS and HWS chicks when injected centrally (Hagen et al., 2013). In our study, ICV injection of PrRP increases food intake in the LWS chicks at a much lower dose and higher magnitude than the HWS chicks. Thus we hypothesize that endogenous PrRP in the LWS chick may make up for the loss of NPY function, in order to stimulate the LWS chicks to eat.

ICV PrRP injection did not affect water intake in our study, which was consistent with rodent reports (Lawrence et al., 2000) and our previous study (Wang et al., 2015; in review), suggesting that PrRP is not affecting food intake in a nonspecific manner. At 180 min post injection, compared with chicks that received vehicle, the LWS chicks that received 375 pmol PrRP had over a 10 fold increase in food intake compared to HWS chicks that received 375 pmol PrRP (294% vs 25%). Thus, the increased blood glucose concentration in the LWS chicks may have come from the increased intake of food, which can be digested and absorbed rapidly to provide glucose.

Further studies are needed to study the molecular mechanisms that regulate the different response to exogenous PrRP administration in LWS and HWS chicks.

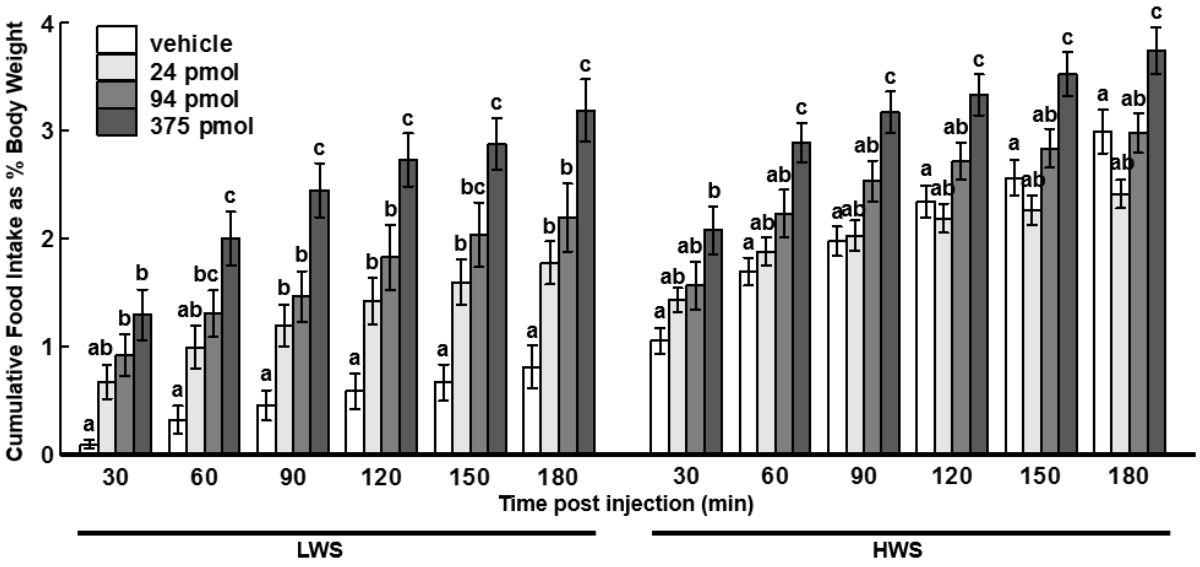

Figure 5.1. Cumulative food intake expressed as percent body weight following intracerebroventricular injection of PrRP in low (LWS) and high (HWS) body weight lines of chicks. Values are means ± SE; bars with different letters are different from each other within a time point ($P < 0.05$). n = 20–23 chicks in the line LWS and 22–24 in HWS per PrRP dose.

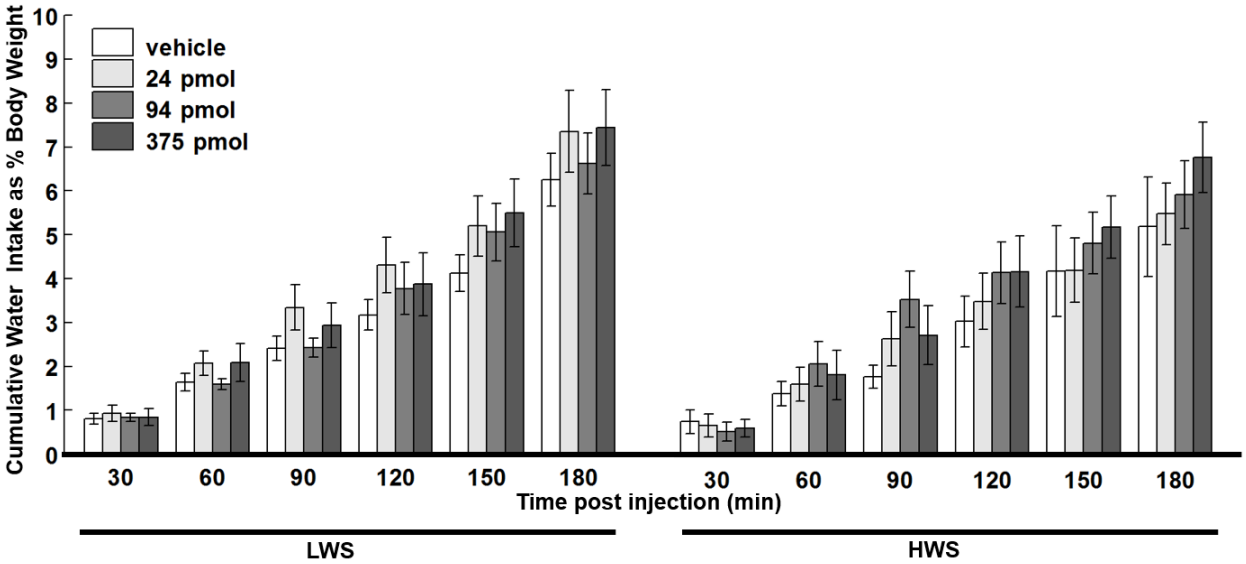

Figure 5.2. Cumulative water intake expressed as percent body weight following intracerebroventricular injection of PrRP in low (LWS) and high (HWS) body weight lines of chicks. Values are means ± SE; bars with different letters are different from each other within a time point ($P < 0.05$). n = 20–23 chicks in the line LWS and 22–24 in HWS per PrRP dose.

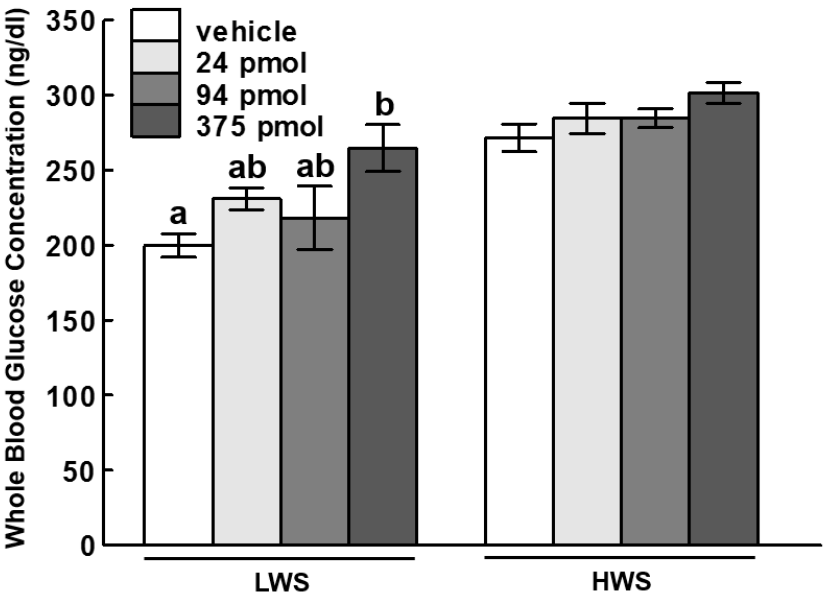

Figure 5.3. Whole blood glucose concentrations following intracerebroventricular injection of PrRP in low (LWS) and high (HWS) body weight lines of chicks. Values are means ± SE; bars with different letters are different from each other ($P < 0.05$). n = 20–23 chicks in LWS and 22–24 in HWS per PrRP dose.

Chapter VI: Synthesis

Based on our results, the orexigenic effect of PrRP may be associated with increased expression of NPY from the PVN in the hypothalamus (Figure 6.1). ICV PrRP injection increased hypothalamic NPY mRNA expression and activated c-Fos immunohistochemistry activity in the rostral PVN. Based on the rodent literature, NPY expression is c-Fos dependent. Therefore, the fact that the PVN had activated c-Fos immunohistochemistry may indicate that the increased hypothalamic NPY expression may be of PVN origin. Since NPY is a potent orexigenic neuropeptide in chicks, the increased NPY expression may explain PrRP's orexigenic effect in chicks.

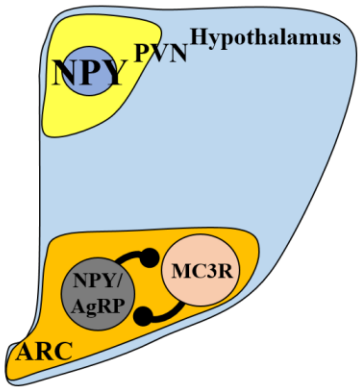

Figure 6.1. Proposed mechanisms of PrRP's orexigenic effects in chickens. Font size of neuropeptide indicates quantity of expression. For reference, NPY, neuropeptide Y; AgRP, agouti-related peptide; MC3R, melanocortin receptor 3; PVN, paraventricular nucleus; ARC, arcuate nucleus.

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