Mechanisms of hypothalamic regulation of food intake in birds

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Mechanisms of hypothalamic regulation of food intake in birds

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Abstract (Academic)

Energy homeostasis is essential for survival across all vertebrate species and involves a multitude of physiological systems that are regulated by both central and peripheral neural signaling. The hypothalamus is responsible for integrating and processing these signals and thus is regarded as the regulatory center for balancing energy homeostasis. Eating disorders, such as compulsive eating behavior associated with obesity, and anorexia, are significant public health concerns worldwide. Thus, studying appetite regulation is necessary to provide novel information for the design of solutions for health concerns that stem from altered energy intake. Such information is also relevant for improving chicken health and productivity in an agricultural setting. The objective of this dissertation research was to determine the hypothalamic mechanisms underlying appetite regulation in birds. In Experiment 1, the Virginia lines of chickens were used to elucidate the mechanisms underlying stress-induced anorexia. These chickens have been selected for low (LWS) or high (HWS) body weight at 56 days of age and have different severities of anorexia and obesity, respectively. Chicks were subjected to a combination of thermal and nutritional stress after hatch and hypothalamic nuclei, including the lateral hypothalamus (LH), paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), and arcuate nucleus (ARC), were collected 5 days later. Real-time PCR was used to measure the mRNA abundance of appetite-associated neuropeptides and receptors in each nucleus. The results showed that the two lines displayed distinct gene expression profiles in response to stress. In particular, the PVN of the LWS was significantly affected by stress, and expression of several anorexigenic factors was up-regulated including corticotropin-releasing factor (CRF), CRF receptor sub-types 1 and 2 (CRFR1 and CRFR2, respectively), melanocortin receptor 4, and

urocortin 3, suggesting that stress-induced anorexia in the LWS may result from overriding anorexigenic signaling in the PVN, primarily through CRF signaling. This CRF signaling-associated hypothesis was further supported by results showing that the original phenotypes were restored when the LWS chicks were treated with astressin (CRF receptor antagonist) before exposure to stress. In Experiments 2 and 3, we attempted to determine the mechanisms of CRF's anorexigenic effect in chickens and Japanese quail. We administered CRF by intracerebroventricular (ICV) injection and the hypothalamus was collected 1 hour later for molecular analyses. Results showed that CRF exerted a similar inhibitory effect on food intake in these two bird species, however the hypothalamic mechanisms underlying this anorexigenic effect were different. ICV injection of CRF increased c-Fos expression in the PVN, VMH, dorsomedial nucleus (DMN), and ARC in chicks while it only affected the PVN and LH in quail. Hypothalamic gene expression results suggested that CRF decreased neuropeptide Y receptor sub-type 1 (NPYR1) in chicks while it increased proopiomelanocortin (POMC), MC4R, CRF, and CRFR2 in quail. These results suggested that the anorexigenic effect of CRF may involve a dampened neuropeptide Y (NPY) system in chicks whereas it is associated with activated CRF and melanocortin systems in quail. At the nucleus level in chicks, CRF injection decreased NPY system-associated gene expression (ARC and DMN) and increased CRF (ARC and PVN) and mesotocin (MT) (VMH)-associated mRNAs, suggesting that orexigenic signaling through NPY was overridden by the heightened anorexigenic tone through CRF and MT, which led to the inhibition of food intake. In Experiments 4 and 5, we used the same experimental design as for CRF studies to determine the hypothalamic mechanisms of the anorexigenic effects of neuropeptide K (NPK) and adrenomedullin (AM) in Japanese quail. Results from Experiment 4 showed that NPK injection activated the ARC and PVN, which was associated with increased mRNAs for a group of anorexigenic factors including CRF, UCN3, cocaine and amphetamine-regulated

transcript (CART), and POMC, and decreased expression of several orexigenic factors, such as NPY and agouti-related peptide (AgRP). In Experiment 5, ICV injection of AM activated the ARC, the nucleus in which POMC and CART mRNAs were increased. In conclusion, these experiments revealed novel hypothalamic mechanisms underlying stress or exogenous neuropeptide-induced anorexia in birds and may provide insights on understanding appetite regulation from evolutionary, agricultural, and biomedical perspectives.

Abstract (Public)

Appetite regulation is important for survival across all vertebrate species and the hypothalamus is the regulatory center for control of feeding behavior. Thus, studying the functions of the hypothalamus on appetite regulation provide novel insight into the eating disorders, such as obesity and anorexia, a worldwide health issue. Also, such information is relevant for improving productivity in the modern chicken industry. The objective of this dissertation research was to determine the hypothalamic mechanisms underlying appetite regulation in birds. In Experiment 1, the Virginia lines of chickens were used to elucidate the mechanisms underlying stress-induced anorexia. These chickens have been selected for low (LWS) or high (HWS) body weight at 56 days of age and have different severities of anorexia and obesity, respectively. Chicks were subjected to a combination of thermal and nutritional stress after hatch. The results suggested the two lines displayed distinct appetite-associated gene expression profiles in response to stress in the hypothalamus. In particular, stress-induced anorexia in the LWS may result from potent feeding-inhibitory factor corticotropin-releasing factor (CRF). Thus, in Experiments 2 and 3, we attempted to determine the mechanisms of CRF's inhibitory effect on food intake in chickens and Japanese quail. We administered CRF by intracerebroventricular (ICV) injection and the hypothalamus was collected 1 hour later for molecular analyses. Results showed that CRF exerted a similar inhibitory effect on food intake in these two bird species. However, the inhibitory effect of CRF was primarily associated with a dampened neuropeptide Y (NPY) system which is a potent stimulatory factor for feeding behavior in chickens, whereas it may involve activated CRF and melanocortin systems in quail. In Experiments 4 and 5, we used the same experimental design as for CRF studies to determine the hypothalamic mechanisms of the inhibitory effects of neuropeptide K (NPK) and adrenomedullin (AM) in Japanese quail. Results from Experiment 4 showed that the feeding-inhibitory effect of NPK was associated

with a group of increased feeding-inhibitory factors such as CRF and cocaine and amphetamine-regulated transcript (CART) and decreased feeding-stimulatory factors, such as NPY and agouti-related peptide (AgRP) in the hypothalamus. In Experiment 5, AM increased gene expression of CART and proopiomelanocortin (POMC). Overall, these experiments suggested the roles of the hypothalamus in stress or exogenous neuropeptide-induced anorexia in birds and may provide insights on understanding appetite regulation from evolutionary, agricultural, and biomedical perspective

Table of contents

List of tables

List of figures

Chapter 1 Introduction and literature review

1. Appetite regulation

Appetite regulation is a complicated system through which central and peripheral signals interact to influence the response to nutrient intake (Valassi et al., 2008). Two areas in the central nervous system, the hypothalamus and brainstem, are responsible for energy homeostasis (Sam et al., 2012). These brain areas receive peripheral neural and hormonal signals that relay information about adiposity and acute nutritional status (Murphy and Bloom, 2006). These neural afferents and hormonal signals from the peripheral nervous system converge with higher brain center signals, for example, reward and mood drive, to regulate appetite and energy consumption (Schwartz et al., 2000).

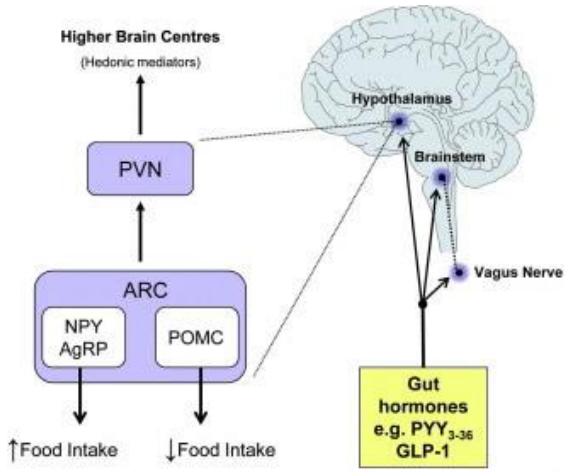

Fig. 1.1. Gut-brain axis: regulation of food intake. (Adapted from (Sam et al., 2012))

Nutrients from gastrointestinal tract digestion are proposed to activate G protein coupled receptors on the luminal side of enteroendocrine cells, such as the L-cell. This process promotes the release of gut hormones, such as peptide YY and glucagon-like peptide 1, which may affect food intake via regulation at three areas: the hypothalamus, brainstem, and vagus nerve. Two categories of neuronal populations in the arcuate (ARC) nucleus of the hypothalamus are thought to be essential conduits through which peripheral signals are integrated to influence appetite: anorexigenic, proopiomelanocortin (POMC) neurons and the

orexigenic, neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons. NPY and AgRP co-express in the ARC neurons (Broberger et al., 1998; Hahn et al., 1998), suggesting that multiple orexigenic agents can be produced by a single neuronal cell type. Subsequently, Elias et al. reported that POMC and cocaine and amphetamine-regulated transcript (CART) are co-expressed in a distinct but neighboring group of the ARC neurons, demonstrating that distinct circuits derived from these brain sites play specialized roles in energy homeostasis (Elias et al., 1998) (Fig.1.2). There may be further regulatory networks between higher brain centers and the hypothalamic nuclei that manipulate the hedonic aspects of food ingestion (Sam et al., 2012).

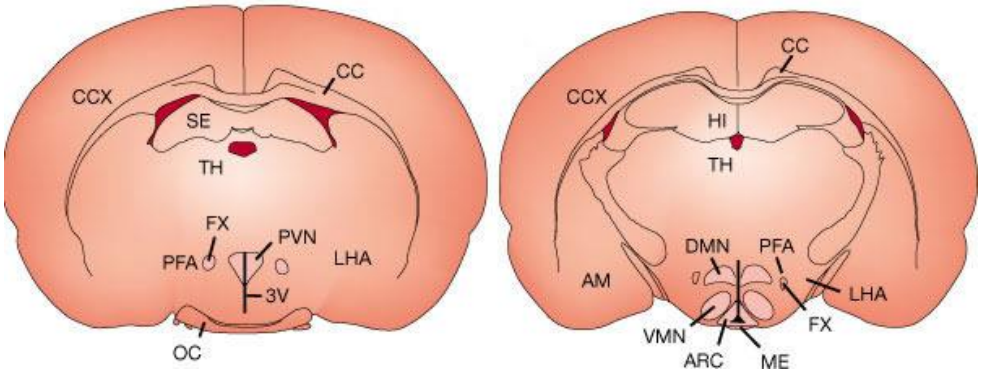

Fig.1.2. Diagrams of the rat brain, showing major hypothalamic regions implicated in adiposity signaling and regulation of food intake. The small figure at the top is a longitudinal view of a rat brain, with the olfactory bulb at the anterior end on the left and the caudal hindbrain on the right. Cross-sections of the brain at two levels (indicated by vertical lines) are shown at the left and right. First-order neurons responding to adiposity signals are located in the ARC and project anteriorly to the paraventricular nucleus (PVN) as well as the PFA adjacent to the fornix (FX) and the LH. Other regions implicated in regulating food intake include the ventromedial hypothalamus (VMH) and dorsomedial nucleus (DMN). Abbreviations of brain structures: AM, amygdala; CC, corpus callosum; CCX, cerebral

cortex; HI, hippocampus; ME, median eminence; OC, optic chiasm; SE, septum; TH, thalamus; 3V, third ventricle. (Adapted from (Benoit et al., 2000))

That a period of fasting can increase food intake (hyperphagia) is simple but compelling evidence of food intake control (Benoit et al., 2000). The subsequent restoration of lost body weight to baseline values, along with the gradual recovery to normal energy intake, is further evidence for the regulation process (Harris et al., 1986). Kennedy et al. reported that inhibitory signals derived from body fat stores function to decrease food intake via the brain (Kennedy, 1953). Therefore, weight loss caused by energy deprivation leads to decreased inhibitory signals, with food intake recovery until the energy deficit is neutralized. Also, Gibbs and Smith further demonstrated that satiety factors, such as peptides secreted from the gastrointestinal tract, can inhibit food intake and result in termination of the meal (Gibbs et al., 1973).

1.1 Roles of Hypothalamus in Appetite Regulation

The roles of the hypothalamus in appetite control were discovered via classical (but crude) experiments and some of the nuclei have been separately considered to be 'feeding' and 'satiety' centers (Arora and Anubhuti, 2006). Hypothalamic nuclei are important for the regulation of both hunger and satiety. The main hypothalamic nuclei associated with hunger and satiety are ARC, the ventromedial nucleus (VMH), dorsomedial nucleus (DMN), paraventricular nucleus (PVN) and lateral hypothalamus (LH).

1.1.1 Arcuate (ARC)

The ARC, a feeding regulation center, integrates hormonal signals associated with energy homeostasis (Funahashi et al., 2000). The ARC lies immediately above the median eminence and encloses the third ventricle. The ARC-median eminence area is one of the "circumventricular" organs where the blood-brain barrier (BBB) is specially modified to allow access of peripheral proteins and peptides, such as insulin and leptin, both of which are

considered to be signals of fat mass (Schwartz et al., 1992; Friedman and Halaas, 1998). The ARC contains two populations of neurons with opposite effects on food intake (Parkinson et al., 2008). Orexigenic neurons that produce NPY and AgRP are in the medial ARC (Hahn et al., 1998; Bewick et al., 2005). The neurons that produce alpha-melanocyte-stimulating hormone (α -MSH) (derived from POMC), and CART are laterally located (Elias et al., 1998). Thus, the ARC is in the ideal region to integrate hormonal signals due to its capability to receive peripheral signals (Arora and Anubhuti, 2006).

1.1.2 Paraventricular nucleus (PVN)

The PVN neighbors the superior part of the third ventricle in the anterior hypothalamus (Arora and Anubhuti, 2006). The PVN can integrate signals from neuronal pathways that are important to the control of energy intake, such as POMC/CART and NPY/AgRP neurons in the ARC and orexin neurons in the LH (Elmquist et al., 1998b; Elmquist et al., 1999). Food intake can be affected by infusion of diverse substances into or near the PVN, whereas lesions of the PVN cause an increase in food intake (hyperphagia) and a decrease in energy expenditure and obesity (Shor-Posner et al., 1985). The PVN can also interact with the brainstem regions that are critical for appetite regulation. Neurons in the PVN project to the nucleus tractus solitaries (NTS) (Blevins et al., 2003) and neuropharmacological manipulation of such neurons can influence hypothalamic activities in response to feeding behavior (Harrold et al., 2012).

1.1.3 Ventromedial nucleus (VMH)

The major function of the VMH is to act as a satiety center. It is an important target for leptin, which acts to inhibit feeding and energy expenditure through the hypothalamus, leading to weight loss. Lesions of either the VMH or PVN induce syndromes of hyperphagia and obesity (Satoh et al., 1997).

1.1.4 Dorsomedial nucleus (DMN)

Extensive connections exist between the DMN and other medial hypothalamic nuclei or the lateral hypothalamus (LH). The DMN plays roles in integrating and processing signals from such nuclei (Elmquist et al., 1998a).

1.1.5 Lateral hypothalamus (LH)

The LH has long been considered to be the "feeding center" since lesioning of the LH causes temporary weight loss (Brobeck, 1946; Anand and Brobeck, 1951). Two groups of neuronal populations localize in the LH: melanocyte concentrating hormone (MCH) neurons and orexin neurons.

1.1.6 Brainstem

There are extensive reciprocal connections between the hypothalamus and brainstem, specifically the NTS (Ricardo and Koh, 1978; van der Kooy et al., 1984; Ter Horst et al., 1989). There is a high density of NPY-binding sites in the NTS, including NPY receptor sub-types 1 and 5 (NPYR1 and NPYR5, respectively) (Härfstrand et al., 1986; Dumont et al., 1998; Glass et al., 2002). The fluctuation of extracellular NPY in the NTS is affected by energy status and NPY neurons in this area project to the PVN (Sawchenko et al., 1985; Yoshihara et al., 1996). Also, postprandial satiety signals are transferred to the brain stem by afferent fibers of the vagus nerve (Williams et al., 2001). The brainstem output has a role in individual meal size, increasing it with a decrease in frequency of meals (Grill and Smith, 1988).

1.2 Neuropeptide Y and food intake

NPY is a 36 amino acid peptide that was first isolated from the porcine brain (Tatemoto et al., 1982). NPY is one the most abundant neuropeptides in the brain (Allen et al., 1983). Subsequently, many studies reported that the effects of NPY are associated with the regulation of hyperphagia and obesity (Dryden et al., 1994), circadian rhythm (Sindelar et al.,

2005), cardiovascular function (Jacques and Abdel-Samad, 2007), locomotion (Heilig et al., 1989), learning and memory (Flood and Morley, 1989).

The most noticeable effect of NPY is to stimulate food intake after central administration (Wisialowski et al., 2000). Within 2 years after its discovery, NPY was found to stimulate food intake. Intracerebroventricular (ICV) administration of NPY increases food intake in a dose-dependent pattern (Levine and Morley, 1984; Clark et al., 1985). Stanley et al. identified some of the specific neuronal regions through which NPY acts, that carry out the role of increasing food intake include the LH, VMH, and PVN in the hypothalamus (Stanley et al., 1985). Additionally, long-term ICV administration of NPY induces sustained hyperphagia and promotes body weight gain bringing about obesity (Beck et al., 1992; Zarjevski et al., 1993). Kuenzel et al. first reported that NPY increases food intake in broiler chicks (Kuenzel et al., 1987), a finding that has been repeated many times since in chickens from a variety of genetic backgrounds.

The ARC is the main site of expression of NPY in the hypothalamus and NPY signals project to the DMN, PVN, LH, and other hypothalamic regions. The major projection and release of NPY onto the PVN and the synthesis of NPY in the ARC are regulated by inhibitory signals, such as insulin and leptin, and stimulatory signals, for example, glucocorticoids (Crespo et al., 2014). In the hypothalamus of rats, NPY is highly expressed in the ARC and PVN (Chronwall et al., 1985).

NPY exerts its cellular functions by binding to a family of G-protein-coupled receptors, including NPY receptor sub-types 1, 2, 4, 5, and 6 (NPYR1, NPYR2, NPYR4, NPYR5, and NPYR6, respectively). Wolak et al. found that NPY stimulates food intake in rats via both NPYR1 and NPYR5, which are widely distributed throughout the brain and are co-expressed in the ARC, LH, and PVN (Wolak et al., 2003). ICV administration of NPYR1 agonists stimulates food intake in rats (Mullins et al., 2001), while administration of NPYR1

antagonists (Kanatani et al., 1996; Wieland et al., 1998; Danielsa et al., 2001) or antisense oligonucleotides (Lopez-Valpuesta et al., 1996; Schaffhauser et al., 1998) attenuate the stimulatory effect on food intake induced by fasting or NPY stimulation in rats. There were similar effects of NPYR5 agonists in rats (Haynes et al., 1998; McCrea et al., 2000), and pigs (Lecklin et al., 2003). However, administration of NPYR5 antagonists (Kask et al., 2001; Daniels et al., 2002) or antisense oligonucleotides (Tang-Christensen et al., 1998; Flynn et al., 1999) decreases food intake in rats, suggesting that NPYR1 and NPYR5 may display different mechanisms underlying effects on mediating food intake control. Holmberg et al. first identified NPYR1 and NPYR5 in the chicken hypothalamus (Holmberg et al., 2002). Saneyasu et al. reported that the mRNA abundance of NPYR1 and NPYR5 was lower in broilers than layers at two days post-hatch and mRNA abundance of NPYR1 at four days post-hatch (Saneyasu et al., 2011).

1.3 Corticotropin-releasing factor and food intake

CRF, consisting of 41 amino acids, is best known as the potent physiological regulatory factor for pituitary adrenocorticotropic hormone (ACTH) secretion (Vale et al., 1981). This effect is carried out via projections from the PVN to the median eminence that contain a large amount of CRF (Swanson et al., 1983; Petrusz et al., 1985).

CRF inhibits food intake after its microinjection into the PVN, but not the LH or VMH, corpus striatum, or globus pallidus (Morley and Levine, 1982). ICV administration of CRF induces several stress-associated behaviors, such as increased grooming, and decreased food intake and parental care in rats (Britton et al., 1982). Also, alterations in grooming and appetite were observed in hypophysectomized rats, indicating that the response caused by CRF injection is independent of the hypothalamic–pituitary–adrenal (HPA) axis (Morley and Levine, 1982). ICV injection of CRF into different areas of the central nervous system in rats

thus demonstrates that the resulting anorexia is likely mediated through effects of CRF in the PVN. Additionally, ICV injection of CRF inhibits water intake in rats (Krahn et al., 1986). In chickens, CRF potently reduces food intake (Denbow et al., 1999; Meade and Denbow, 2003; Tachibana et al., 2006). Furuse et al. found that CRF strongly suppresses food intake in 2-day-old chicks that are fasted for 3 hours (Furuse et al., 1997). There were also potent stress-associated responses with increased concentrations of plasma corticosterone and elevated locomotion in chicks treated with CRF via ICV injection (Zhang et al., 2001; Tachibana et al., 2004).

CRF has two known receptors, CRF receptor sub-types 1 (CRFR1) (Perrin et al., 1993) and CRF receptor sub-types 1 (CRFR2) (Lovenberg et al., 1995b), which are G-protein-coupled receptors that are activated by CRF. CRFR1 is primarily associated with the release of ACTH induced by stress (Smith et al., 1998). CRFR2 is thought to a mediator in stress coping (Coste et al., 2001; Hashimoto et al., 2001). Ogino et al. reported that central CRFR2 is associated with appetite regulation, digestive tract transit, and body temperature in chicken (Ogino et al., 2014). This finding is consistent with previous findings in mammals that central CRFR2 plays roles in the suppression of appetite (Pelleymounter et al., 2004), hyperthermia (Telegdy and Adamik, 2008), and the delay of gastric emptying (Mart ńez et al., 2004). Also, CRFR2 activation was associated with the induction of jumping, wing-flapping and scratching behaviors in chicks while CRFR1 was associated with vocalizations (Ogino et al., 2014). Similar findings were reported in rats (Kehne et al., 2000; Ise et al., 2008). There is much evidence supporting the inhibitory effect of CRFR2 on food intake. For example, the antagonist of CRFR2 can completely reverse the anorexigenic role of CRF in rats (Cullen et al., 2001) while the antagonist of CRFR1 does not restore its inhibitory effect on appetite (Pelleymounter et al., 2000) in mice. In chickens, α-helical CRF, the CRF receptor antagonist that has a higher affinity for CRFR2 than CRFR1, reverses the anorexigenic effect of CRF

(Perrin et al., 1999), indicating that CRFR2 plays more of a role in appetite regulation than CRFR1.

1.4 Characteristics of appetite regulation in chickens

Integration of peripheral and central signals in the hypothalamus leads to hunger or satiety. Birds and mammals share common signaling molecules, such as AgRP/NPY, CART /POMC, cholecystokinin (CCK) and, ghrelin (Richards, 2003) to regulate food intake. It is important to note that some molecules act opposite or have different roles in birds vs. mammals and there are some proteins for which an avian ortholog has not been identified.

1.4.1 Role of anorexigenic/orexigenic peptides in mammals and avian species

The fact that birds and mammals utilize common signaling molecules does not necessarily imply that these compounds share the same function.

The neuropeptides orexin-A and orexin-B are also known as hypocretin-1 and hypocretin-2, respectively, because they are located in the hypothalamus and are associated with the incretin family of neuropeptides (Katayama et al., 2010b). Orexin-A has 33 amino acid residues while orexin-B consists of 28 amino acids. Orexin-A and orexin-B are potent orexigenic factors in mammals but do not affect food intake in chickens. ICV administration of mammalian orexins did not promote food intake in neonatal chicks (Furuse et al., 1999). On the other hand, Katayama et al. reported that monoamine metabolism increases in neonatal chicks after ICV administration of mammalian orexin-A (Katayama et al., 2010a). ICV administration of mammalian orexin-A increases the arousal of layer-type neonatal chicks (Katayama et al., 2010b). Ohkubo et al. found that orexin-A and orexin-B are highly conserved among vertebrate species (Ohkubo et al., 2002). There is no difference in orexin mRNA in the hypothalamus between 24-hour fasted and ad libitum-fed chicks (Ohkubo et al., 2002). In contrast, orexin mRNA increases in the hypothalamus of 48-hour fasted broiler

chickens, indicating that orexin plays roles in food intake regulation and/or energy homeostasis under extreme conditions (Song et al., 2012b).

ICV administration of a low dose (pmol) of growth hormone-releasing hormone (GHRH) stimulates food intake while high doses (nmol) of GHRH inhibit food intake in young male Wistar rats (Veyrat-Durebex et al., 2001). The ICV administration of Growth Hormone Releasing Peptide -2 (GHRP-2) (KP-102) which is a synthetic growth hormone secretagogue, inhibits food intake in neonatal chicks (Saito et al., 2002). Indeed, ghrelin was originally thought to be a growth hormone-releasing peptide and stimulates food intake in humans and rodents. Intriguingly, ghrelin inhibits food intake after ICV injection in chickens (Saito et al., 2002). The opposite effects of GHRH and ghrelin on food intake suggest that divergence exists in the action of the growth hormone axis on appetite regulation between mammals and birds.

1.4.2 Effect of fasting on hypothalamic neuropeptide gene expression

Alterations in hypothalamic neuropeptide abundance are the physiological adaptation to maintain energy homeostasis after being fasted. Compared to ad libitum-fed counterparts, food deprivation (dry food was provided 3 g/day for two weeks) results in increased prepro-orexin and NPY gene expression in obese mice (ob/ob and db/db) (Yamamoto et al., 2000). 48-hour fasting stimulates mRNA expression of AgRP and NPY and suppresses POMC mRNA in rats (Mizuno et al., 1999). Similarly, 24-hour fasting stimulated hypothalamic NPY mRNA expression in growing broilers (Boswell et al., 1999). POMC was decreased in 4 day-old chicks that were feed-restricted (Higgins et al., 2010). NPY, AgRP, melanocortin receptor 4 (MC4R), MCH and prepro-orexin mRNAs were upregulated by 12-hours fasting in 7- to 9-day-old chicks (Song et al., 2012a). 48-hours of fasting decreases in POMC mRNA expression is found in the hypothalamus in fasted broiler chicks (Proszkowiec-Weglarz et al., 2006). Hen et al. also reported a reduction of POMC expression is found in the hypothalamus

of 3-wk-old female meat-type and layer-type chicks treated to a 50% restriction of ad libitum food intake for one week (Arora, 2006).

2. Stress

The definition of stress is any deviation from normal physiological status and the stress response plays a critical role in maintaining homeostasis. The response to stress involves complicated interactions between the central and peripheral nervous systems and the remaining body parts (2015). Allostasis is the active response to cope with stressors through mediators such as cortisol and the autonomic, metabolic and immune systems that interact in a nonlinear way to sustain homeostasis (McEwen, 2006). Allostatic overload involves the cumulative physiological changes that can be caused by such dysregulation. Biologically, allostasis, and overload and allostatic load, are more precise definitions than 'stress' to describe maladaptation and adaptation to 'stressors', because these concepts include the physiological effects of health-damaging and health-promoting responses as well as stressful experiences (Flier et al., 1998; McEwen, 2006). There are three categories of "stress": 'toxic stress', 'tolerable stress' and 'good stress' (Shonkoff et al., 2009). When suffering stress during early life, the neural architecture might be altered and result in an increase in adverse reactions to stressors later in life (Shonkoff et al., 2009). During the critical or sensitive periods of early development, such effects of stress-associated experience may persist the entire life (Felitti et al., 1998; Halfon et al., 2014).

2.1 Mechanisms of stress in the brain

The brain plays regulatory and mediatory roles in adaptation to physical and social stressors, and manipulates the physiological and behavioral responses to stressors that may be protective or damaging. Multiple stressors and dysfunction of nonlinear interactions (for example, failure to react efficiently) result in wear and tear on the brain and body (Flier et al., 1998; McEwen and Wingfield, 2003).

The brain is one of primary targets of stressors. Glucocorticoids and excitatory amino acid neurotransmitters affect neuronal architecture by altering dendritic activity (retraction or expansion) and synapse density (increased or decreased), and can suppress dentate gyrus neurogenesis (Shaw, 1996; McEwen, 1999; Lajtha et al., 2007). Many intra- and inter-cellular processes and mediators take part in altering the brain architecture during stress or recovery from stressful experiences (McEwen, 2007, 2010).

In particular, the prefrontal cortex (PFC), hippocampus, and amygdala have important roles in behavior and cognitive function as well as in regulating the autonomic and HPA stress response.

McEwen et al. first uncovered the role of glucocorticoids and mineralocorticoid receptors (MR) in hippocampal formation (McEwen et al., 1968), suggesting that the hypothalamus is not the only target for adrenal steroids in the brain, and that other effects include mood regulation and modulation of spatial and episodic memory. In the hippocampus, glucocorticoids and stress were first found to cause loss of spines and dendritic shrinkage. The rediscovery of neurogenesis in the dentate gyrus stimulated renewed interest in the capacity for neuronal replacement in the adult brain (Shaw, 1996).

The effects of stress on the amygdala are different from those in the hippocampus. Acute traumatic stressors lead to an increase in dendritic spine density in basolateral amygdala neurons, while chronic stressors lead to the expansion of basolateral amygdala dendrites (McEwen and Morrison, 2013). On the other hand, the medial amygdala displays a chronic stress-induced reduction in spines (Bennur et al., 2007). These alterations are evidence for mechanisms involved in posttraumatic stress disorder (PTSD)-like behaviors (Rao et al., 2012; McEwen and Morrison, 2013).

In the PFC, chronic stress results in shrinkage and debranching of dendrites in medial PFC neurons that contribute to cognitive rigidity, while orbitofrontal cortical neurons display

dendritic expansion that likely contributes to increased vigilance (Radley et al., 2004; Du et al., 2009). Changes in the prefrontal cortex under stressful environments have provided significant insight to age-related loss of resilience and impaired memory as well as effects of circadian disruption and extinction of fear memory (Armanini et al., 1990).

2.2 Inverted U-shaped curve of responses

The impact of stress in the brain forms a nonlinear 'inverted-U' dose-response curve as a function of stressor severity: the transition from none-to-mild stress results in an increase in endpoint X, the transition from mild-to-moderate stress results in endpoint X to plateau, and the transition from moderate-to-severe stress decreases endpoint X (Sapolsky, 2015). The effects of mild-to-moderate stress are beneficial, while effects of severe stress are the opposite. Although major stress is harmful, mild stress can be beneficial when stress levels are optimal.

2.3 Stress in appetite regulation

The neural circuits that regulate food intake converge on the PVN, which contains CRF and urocortin neurons. Thus the HPA axis that is in charge of stress responses also manipulates appetite responses. In other words, the systems that control stress responses and appetite share anatomic structures and signaling pathways. Thus, each system can interact in initiating a response (Maniam and Morris, 2012).

In both humans and animals, stress impacts food intake regulation in a bidirectional way based on the intensity of stress and environmental factors (Bazhan and Zelena, 2013). Any stressor can affect both anorexigenic and orexigenic regulatory pathways.

2.3.1 Stress-Induced Anorexigenic Response

Emotional stress is regarded as an inhibitor to appetite in laboratory rodents (Krahn et al., 1990; Rybkin et al., 1997; De Souza et al., 2000; Harris et al., 2001; Bazhan et al., 2007) and humans (Fryer et al., 1997). The stress-induced loss in food intake has been investigated both

as a continuous decrease in food intake at 24 hours post-injection of methysergide or during repeated daily restraint stress and as an acute response after single stressor exposure (Shimizu et al., 1989; Krahn et al., 1990). Also, restrained rats lost the ability to return to normal weight after stress (Rybkin et al., 1997; Levin et al., 2000; Bazhan et al., 2007).

The primary site in the central nervous system involving in appetite regulation is the hypothalamus (especially the VMH, LH, PVN, and ARC) (Benoit et al., 2000; Palkovits, 2003), which is also the center that orchestrates the stress response. Indeed, hypothalamic melanocortin and CRF systems are known to be involved in stress along with anorexia in rodents (Ohata and Shibasaki, 2011). The CRF system is a mediator for both anorexigenic effects and stress-associated responses in fish, thus the role of CRF in regulating appetite and stress responses seems to be evolutionarily conserved (Bernier, 2006). It is also important to note that independent of the hypothalamus, the amygdala (Solomon et al., 2010; Holsen et al., 2012), dorsal raphe (Holsen et al., 2012) and dorsal vagal complex (Charrier et al., 2006) can cause stress-induced anorexia without affecting activity in the HPA axis.

2.3.2 Stress-Induced Orexigenic Response

Anorexia caused by repeated acute restraint stress is followed by increased comfort food intake (i.e., food with high fat/sugar content) (Pecoraro et al., 2004; Dallman et al., 2005; la Fleur et al., 2005; Foster et al., 2009; Dallman, 2010). Unlike normal diets, a mild pinch leads to hyperphagia and a considerable gain in body weight when sweet food is repeatedly offered to the stressed animals (Rowland and Antelman, 1976) (Table 3). Chronic life stress is likely to involve a greater preference for nutrient- and energy-dense foods (Torres and Nowson, 2007). During repeated/prolonged food intake, the sensory-specific satiety (SSS) suppresses the consumption of the same food. Stressors may lead to a disruption of this response, thereby causing an increase in consumption of the same food (Ahn and Phillips, 2012). In animals, development of obesity in response to chronic consumption of a high-fat diet (DIO

model) suggests that there is similar habituation to SSS in rodents (Auvinen et al., 2012; Ryan et al., 2012). Furthermore, rats treated with footshock stress decrease their intake of commercial chow but do not alter their consumption of comfort food (Ortolani et al., 2011). Energy stores are important for normal activity in the central stress-induced response network (Dallman, 2010). When a chronic stressor leads to elevation in glucocorticoids levels, the steroids appear in a feed-forward fashion to recruit a stress-induced response network in the brain that biases ongoing behavioral, neuroendocrine and autonomic outflow and responses to novel stressors (Dallman et al., 2006). When rats are offered sucrose or fat under conditions of repeated or acute restraint stress, the release of ACTH, glucocorticoid, and CRF in the hypothalamus and bed nucleus of stria terminalis (BNST) decrease (Pecoraro et al., 2004; la Fleur et al., 2005; Foster et al., 2009; Ortolani et al., 2011).

On the other hand, increased consumption of comfort foods during and after stress suggests a pleasurable response that alleviates the discomfort that comes from the stress. Food intake alters emotional predisposition and mood, especially decreasing arousal and irritability, meanwhile increasing calmness and positive effects (Gibson, 2006). Due to reinforcing effects of food intake, addiction disorders and severe obesity partly share some neuronal circuits (Volkow et al., 2008; Berridge, 2009; Kiefer and Grosshans, 2009). Indeed, eating high-calorie food is regarded as counteractive to some effects of stress, likely via the same regulation pathway as in alcoholics (Kiefer and Grosshans, 2009). Chronic stress has been thought to reinforce potent addictive behaviors in both experimental animals and human addicts (Stamp et al., 2008). By activating central reward pathways, high levels of corticosterone (the main glucocorticoid in rodents and birds) can relieve the averseness of stressor (Piazza and Le Moal, 1997).

Another potential mechanism related to how stress affects obesity is the bidirectional interaction between the gastrointestinal tract and HPA axis (Dinan and Cryan, 2012; Sudo,

2012). Exposure to a stressor leads to a change in several gastrointestinal parameters, such as the microbiota. Alterations in gut microbiota may be an initial factor giving rise to altered food intake behavior that lead to weight gain and metabolic syndrome (Tehrani et al., 2012). Consumption of a high-calorie diet has been associated with alterations in the gut microbiota in mice and humans. Thus stressor-induced comfort food intake causes obesity also via the effect on the microbiome (Pedersen et al., 2013).

Collectively, a simple scheme is that stress activates comfort food intake and comfort food eating relieves stress (Dallman et al., 2005). Thus, elucidating mechanisms underlying stress-induced comfort food eating is vital for understanding mechanisms of obesity development. However, such a model is mostly relevant for humans, since animals may not have the option to select "comfort food" in the wild. Although there is widespread stress-induced obesity in humans, there is no animal model where single or repeated stress exposure brings about development of obesity with a normal diet. Glucocorticoids may only stimulate the development of obesity and other metabolic syndromes in those animals, which are fed a high fat, calorie or sugar diet under stress (Rasmusson et al., 2010). Thus, stress itself does not give rise to obesity without consumption of excess calories.

2.4 Corticotropin-releasing factor

CRF, produced in the PVN as the primary regulator of the HPA axis, has widespread central and peripheral actions and distribution. CRFR1 and CRFR2 relay signals from CRF and its paralogs the Ucns (Kuperman and Chen, 2008).

The CRFR1 is distributed in the anterior pituitary corticotropes and its activation leads to the synthesis of ACTH from the POMC precursor in response to hypothalamic release of CRF (Vale et al., 1981). This release gives rise to the downstream secretion of glucocorticoids from the adrenal cortex via melanocortin receptor 2 (MC2R). The end hormones of the axis, glucocorticoids, have wide effects through ubiquitous intracellular glucocorticoid receptors

(GR), which are in most tissues and cells, and through another intracellular steroid receptor, the MR, which is widely distributed in the hippocampus and manipulates negative feedback on the HPA axis (Chrousos, 2000). Glucocorticoids also induce gluconeogenesis in the liver to increase circulating glucose levels.

Decreased food intake associated with stress reflects adaptive and defensive anorexia. Several researchers have linked stressor-induced anorexia to activation of the CRF system. Indeed, CRF affects appetite regulation and mediates physiological and behavioral responses to stress not only in laboratory rodents (Krahn and Gosnell, 1988) but also in fish (Kang et al., 2011) and steers (Yayou et al., 2011). ICV injection of CRF in rats suppresses food intake (Cullen et al., 2001). Chronic ICV administration of CRF into the hypothalamus also inhibits body weight gain and food intake in rats (Tempel and Leibowitz, 1994). Pharmacological activation of the hypothalamic CRFR2 also inhibits food intake (Fekete et al., 2007), demonstrating that CRFR2 plays an important role in mediating the anorexic effects of CRF.

2.4.1 Brain targets

The amount of CRF is increased under stress not only in the PVN but also in many other brain areas that are associated with the regulation of food intake. For example, the VMH is considered to be a satiety center due to its inhibitory effects on food intake, and CRFR2 mRNA is highly expressed in the VMH (Lovenberg et al., 1995a). The basolateral amygdala is associated with CRF-induced anorexia through CRFR1 (Jochman et al., 2005), while the lateral septum and BNST mediate CRF's effects via the CRFR2 (Smagin et al., 1998; Ohata and Shibasaki, 2011). Morphological and pharmacological studies indicate that UCN3 is the most specific and potent endogenous ligand for CRFR2 (Chen et al., 2010). In rats, ICV administration of UCN3 in the VMH decreases food intake without impacting HPA axis activity (Kuperman and Chen, 2008; Chen et al., 2010).

Knocking out CRFR1 or CRFR2 does not affect food intake or body weight (Preil et al., 2001). Thus, neither of the CRF receptors are likely to play an important role in the manipulation of food intake and the basal regulation of body weight, although functional redundancies are common in nature and there are other pathways that might compensate for a loss of either CRF receptor. Rather, CRFR1 or CRFR2 may be associated with biphasic control of UCN-mediated food intake (Bradbury et al., 2000; Carsia and Weber, 2000), as well as the interaction among the central melanocortin, leptin and CRF pathways (Uehara et al., 1998; Marsh et al., 1999). This assumption is supported by the finding that leptin infusion to the 3rd ventricle for 5 days increases expression of CRFR2 mRNA in the VMH (Huang et al., 2006).

2.4.2 Indirect effects

CRF may display orexigenic effects via elevation of plasma glucocorticoid concentrations. In line with this idea, CRF infusion into healthy, non-obese adults leads to increased cortisol levels along with an increase in food intake (George et al., 2010). Also, CRF may interact with orexigenic peptides. CRF suppresses NPY synthesis and release (White, 1993) and reduces NPY-induced food intake (Tempel and Leibowitz, 1994). Previous studies suggest that nociceptin/orphanin acts in the stria terminalis as a functional antagonist to CRF to inhibit its anorexigenic effect (Ciccocioppo et al., 2004).

2.5 Relevant stressors in chickens

Adverse environmental conditions during critical or sensitive periods of early development in any organism, including birds, may lead to the deviation of the phenotype from normal ontogenetic development, which is thought of as developmental plasticity (West-Eberhard, 2003). Exposure to either environmental or nutritional stressors during early life (critical periods) results in the alteration of hypothalamic neuronal networks and manipulates subsequent responses to stress.

2.5.1 Fasting

Previous studies suggest that both hypothalamic peptide and mRNA of NPY are increased in Japanese quail after 24 hours of fasting (Phillips-Singh et al., 2003), and in chickens subjected to 48 hours of fasting (Higgins et al., 2010). Fasting increases hypothalamic AgRP mRNA in mammalian (Ollmann et al., 1997), avian (Takeuchi et al., 2003; Song et al., 2012a) and fish (Cerdá-Reverter and Peter, 2003; Song et al., 2003). In addition to increasing hypothalamic expression of the orexigenic factors NPY and AgRP, energy restriction in rodents elevates the mRNA abundance of pre-prodynorphin, an orexigenic member of the opioid peptide family (Berman et al., 1997; Herve and Fellmann, 1997). These changes in the synthesis of orexigenic peptides can contribute to the appetite-promoting and energy-conserving effects of negative energy homeostasis.

In general, short-term energy restriction down-regulates the hypothalamic expression of anorexigenic agents, while it up-regulates the production of orexigenic peptides. There are two examples: POMC and CART in the ARC. POMC-derived α-MSH inhibits food intake by agonizing central MC4R (Vrang et al., 1999; Benoit et al., 2000; McMinn et al., 2000). CART has both anorexigenic and orexigenic effects in appetite regulation. During food deprivation, hypothalamic mRNA abundance of both POMC and CART is reduced in rodents (Brady et al., 1990; Kristensen et al., 1998; Ahima et al., 1999; Mizuno et al., 1999; Ziotopoulou et al., 2000; Savontaus et al., 2002; Swart et al., 2002).

In chickens, the amount of phosphorylated activated protein kinase α (AMPKα) significantly increased within 48 hours of fasting, while re-feeding for 24 hours restored its levels to those of counterparts that had free access to food (Song et al., 2012b). Food-deprived chickens had significantly lower plasma glucose and insulin and greater hypothalamic phospho-AMPKα to total AMPKα ratios. Re-feeding restored both plasma glucose and insulin as well as hypothalamic phospho-AMPKα to baseline levels (Song et al., 2012b).

Song et al. found that 48 hours of fasting does not alter hypothalamic POMC mRNA abundance (Song et al., 2012b). Conversely, there was a decrease in hypothalamic POMC mRNA in fasting broiler chicks (Proszkowiec-Weglarz et al., 2006; Higgins et al., 2010). Hen et al. reported a reduction in POMC mRNA in the hypothalamus of female layer-type and meat-type chicks at 3 weeks of age that were subjected to a 50% restriction of ad libitum food intake for one week (Hen et al., 2006). The effect of fasting was more potent in layer-type than meat-type chickens. Conflicting results involving the effect of food deprivation on hypothalamic POMC mRNA expression are also observed in mammals (Phillips-Singh et al., 2003). In general, one can conclude that transcriptional regulation of POMC is very dynamic and influenced by a multitude of factors, thus providing a rapid response to changes in the environment in order to regulate energy homeostasis.

Song et al. found that there was no influence of 48 hours of fasting on CART mRNA in the hypothalamus of chickens (Song et al., 2012b). Fasting elevates the hypothalamic mRNA abundance of prepro-orexins in chickens (Ohkubo et al., 2002). However, ICV administration of mammalian orexin was ineffective at altering food intake in neonatal chicks (Furuse et al., 1999). As for orexins, Ando et al. found that ICV injection of MCH does not promote food intake in chicks, whereas MCH mRNA increased after food deprivation for 48 hours (Ando et al., 2000).

In summary, fasting alters the expression levels of hypothalamic appetite-associated regulators with an up-regulation of orexigenic neuropeptides and a down-regulation of anorexigenic neuropeptides in the short-term. These changes may be important in modulating survival during food deficits by initiating energy-conserving mechanisms in a rapid and dynamic fashion to environmental perturbations.

2.5.2 Cold stress

As one of the main stressors, cold exposure impacts animal husbandry in cold regions (Li et al., 2006). The central nervous system and its central locus are major sites that exert regulatory and integratory roles in the cold stress response and carry out the regulation via the sympathetic-adrenal-medullary-axis, the hypothalamic-pituitary-adrenal-axis, and the hypothalamic-pituitary-thyroid-axis (Kim et al., 1999; Helmreich et al., 2005).

In homeotherms, environmental temperature is not only associated with energy consumption but also food intake (Brobeck, 1948; Davis, 1964; Chaffee and Roberts, 1971; Herman, 1993; Yates, 1993). Food intake serves to meet physiological nutrient requirements and to act as an important source of energy for heat generation and thermal homeostasis. When calories are insufficient, there might be a loss in body temperature (Keys et al., 1950). The heat loss to the environment depends on the environmental temperature (the lower the environmental temperature below body temperature, the more the heat loss). The correlation between environmental temperature and food intake can be visualized as a vital, inverse curve (Brobeck, 1948). This increase in food intake in response to a lowered environmental temperature is observed across a wide range of temperatures in various species (Chaffee and Roberts, 1971). Exposure to temperatures below the thermoneutral zone promotes food intake, while temperatures above the thermoneutral zone may lead to heat stress causing inhibiting of food intake while resulting in an increased metabolic response (Herman, 1993; Yates, 1993). This energetic difference between expenditure and intake above the thermoneutral zone induces acute body fat and body weight loss in animals, with available data in humans (Brobeck, 1948; Chaffee et al., 1969; Chaffee and Allen, 1973; Jakubczak, 1976; Herman, 1993; Yates, 1993).

Cold stress (10 ℃) has a delayed effect on in vitro mitogen responses to concanavalin A. (Hangalapura et al., 2004b). Hangalapura et al. reported that the antibody response is affected

by cold stress (10 ℃) (Hangalapura et al., 2003; Hangalapura et al., 2004a). Hester et al. observed that caged hens in a cold environment (0 ℃ for 72 h) had higher heterophil-to-lymphocyte ratios than their counterparts in the control environment (Hester et al., 1996). Yalcin and Siegel reported that tibia, shank, wing, and femur lengths of broiler embryos asymmetrically increased upon exposure to cold temperatures (21.0 ℃) (Yalcin and Siegel, 2003). Campo and Carnicer demonstrated that cold stress (1 ℃ for 24 h) affects tonic immobility, and the duration varied from breed to breed, the divergence between treatments (cold stress vs. control) being significant in Red-Barred Vasca (Campo and Carnicer, 1994).

3. Animal model for hypophagia and hyperphagia

Long-term divergent selection (61 consecutive generations) for low or high body weight at 56 days of age from the same founder population has produced two vastly distinct lines of chickens, the high (HWS) and low weight (LWS)-select lines, whose body weights at selection age differ by approximately 10-fold (Dunnington et al., 2013). The foundation stock consists of crosses of seven inbred lines of White Plymouth Rocks (Siegel, 1962) and a review of the selection program can be found in Dunnington and Siegel (Dunnington and Siegel, 1996) and Le Rouzic et al. (Le Rouzic et al., 2007).

The HWS chicks are compulsive eaters, their overeating resulting in obesity, whereas the LWS chicks are naturally anorexic and tend to be lean (Siegel and Dunnington, 1987; Dunnington and Siegel, 1996; Rubin et al., 2010; Ka et al., 2011). Sexual maturity of the LWS line is delayed to the age at which they reach a threshold body weight. Due to slow body weight gain, the LWS chicks are delayed in the initiation of egg laying (Liu et al., 1995) or may not reach sexual maturity (Dunnington and Siegel, 1997). The HWS chickens, at the other end of the spectrum, must be feed restricted after 56 days of age; otherwise, premature death occurs, and egg production is impaired due to metabolic disorders.

3.1 Difference in metabolism between the line chicks

3.1.1 Glucose regulation

Previous studies demonstrated that the differences in metabolism between HWS and LWS lines might be associated with divergences in glucose regulation (Parker et al., 2015). Sinsigalli et al. first documented the difference in glucose regulation and insulin sensitivity between the lines in generation S26 (Sinsigalli et al., 1987). The results showed that the LWS chicks treated with glucose after 24 hours of fasting had a greater capability to clear blood glucose than their HWS counterparts at 21, 42, 63, and 84 d of age, indicating that the HWS exhibited relative impaired glucose tolerance and hyperglycemia (Sinsigalli et al., 1987). These results were consistent with the finding that the higher concentration blood glucose was observed in the HWS chicks after 30 additional generations of selection (Sumners et al., 2014). Thus, the past 30 generations of divergent selection for juvenile body weight may have enhanced the magnitude of glucose intolerance in the HWS line.

When chicks were fasted for 72 hours after hatch and were provided ad-libitum access to food until day 15 post-hatch, the HWS line had a higher concentration of blood glucose than the LWS line in the fed state. At 72 hours post-hatch in the fasted state, the significant reduction in blood glucose and food intake seemed to then induce a compensatory response that led to an elevation of blood glucose at day 15 post-hatch in LWS, but not the HWS chicks, compared to their counterparts that were provided immediate access to food after hatching (Zhao et al., 2014). These results agree with the response of blood glucose to refeeding after fasting during an insulin sensitivity test, where compensatory changes in blood glucose were greater in LWS than LWS (Sumners et al., 2014). These findings suggest that the LWS chicks have a unique compensatory mechanism to cope with nutritional challenge and to promote survival, and such changes are observed as early as immediately after hatching and persist to older ages.

3.1.2 Functions of Insulin

Insulin plays a vital role in energy homeostasis and glucose metabolism in higher vertebrates. Unlike humans, chickens are naturally hyperinsulinemic and hyperglycemic (Zhang et al., 2013a). The lines are characterized by differences in their response to central administration of insulin. LWS chicks respond to exogenous insulin (injected ICV) with hypophagia and hypoglycemia at a lower dose as compared to HWS (Smith et al., 2011). Additionally, the lines also differ in their threshold response of blood glucose to peripheral insulin (Sumners et al., 2014).

Rice et al. reported that insulin decreased blood glucose in chicks from both lines at 4 days post-hatch (Rice et al., 2014), which is consistent with findings from studies where older chickens were used, for example at 56 days (Zhang et al., 2013b; Sumners et al., 2014). These results suggest that the HWS line individuals have a greater magnitude of response in blood glucose to insulin than the LWS line during the early post-hatch period, revealing that such responses to insulin are affected by age-associated changes, such as the larger difference in body weight and body composition (e.g., adiposity).

In order to elucidate mechanisms responsible for the differential response to insulin between the HWS and LWS lines, transcriptome profiling was performed on hypothalamic samples of 90 day-old chickens. The LWS chicks that were treated with vehicle (controls) had greater mRNA expression of genes that were associated with serotonin and dopamine biosynthesis and receptor signaling, such as tyrosine hydroxylase (TH), l-aromatic amino acid decarboxylase (DDC) and solute carrier family 18, member 2 (SLC18A2), than vehicle-injected HWS chicks (Zhang et al., 2015b). A phenomenon observed during insulin challenge studies is that the LWS chicks have greater pancreatic expression of preproglucagon mRNA than the HWS after re-feeding (Sumners et al., 2014). Although this may help explain some of the mechanisms involved in their differential responses to insulin, the correlation between

insulin, glucagon, and serotonergic and dopaminergic systems is still unclear. Some suggest that there is cross-talk between serotonin and insulin signaling pathways through POMC neurons in the ARC in rodents (Xu et al., 2010; Papazoglou et al., 2012). Consistent with this, there was 5-fold up-regulation of POMC mRNA in both lines after insulin injection, suggesting a role for POMC neurons in the physiological response to insulin (Zhang et al., 2015b). In another study, Sumners et al. reported there are differences in mRNA expression of glucose regulation factors between the two line chicks during the first hour after insulin injection (Sumners et al., 2014). For example, the LWS chicks expressed greater preproinsulin (PPI), preproglucagon (PPG), glucose transporter 2 (GLUT2) and pancreatic and duodenal homeobox 1 (PDX1) mRNAs (Sumners et al., 2014). Additionally, insulin injection is associated with a reduction in mRNA abundance of insulin receptor (IR) and glucose transporters 2 and 3 (GLUT 2 and 3, respectively) in the liver at one hour post-injection (Zhang et al., 2013b). Thus, changes in blood glucose regulation were responses to the long-term selection for low or high body weight and likely contribute to the differences in appetite regulation.

3.1.3 Lipid metabolism

At 28 days post-hatch the LWS chickens had greater lipolytic capacities in abdominal fat than the HWS line in both the fasting and fed states, which may help explain why the LWS are slow to accumulate fat after hatch (Calabotta et al., 1985; Liu et al., 2017). Administration of oral nutrient boluses was associated with minimal fat deposition in the LWS, which happened mainly through hypertrophy, while adipose tissue deposition in the HWS occurred through a combination of hyperplasia and hypertrophy (Robey et al., 1988; Robey et al., 1992). These findings indicate that the LWS and HWS differ in energy metabolism in insulin-dependent tissues, for example, skeletal muscle and white adipose tissue.

At 90 days of age, the HWS chickens are in a state of obesity with insulin insensitivity (Zhang et al., 2013b; Sumners et al., 2014; Zhang et al., 2014), which may thereby affect glucose regulation, and nutrient sensing and regulation of appetite. The LWS chicks display greater fatty acid oxidation efficiency in abdominal fat and red muscle, but no difference in found in white muscle, indicating that the red muscle which contains more mitochondria than white muscle is a site of enhanced fatty acid oxidation in the LWS chicks (Zhang et al., 2014). Collectively, these results suggest that both impaired appetite regulation and altered adipose tissue metabolism contribute to the differences in body weight between both lines (Parker et al., 2015).

3.2 Food intake in the line chicks.

Over the past 15 years, our group has screened many appetite-associated neurotransmitters in the LWS and HWS lines through exogenous administration (ICV) followed by measuring appetite-associated responses. Results showed that there are differences in appetite-associated behaviors, hypothalamic neuronal activation, and hypothalamic neuropeptide and receptor gene expression profiles between the LWS and HWS.

3.2.1 Food intake response to peptides

The hypothalamus is the regulatory center for appetite control and energy homeostasis (Zhang et al., 2015a). Burkhart et al. first reported that electrolytic lesioning of the VMH results in obesity in the LWS line, but does not affect the HWS, suggesting the importance of the hypothalamus in food intake regulation and body weight in the lines (Burkhart et al., 1983). Determining the food intake response of neuropeptides via ICV injection has revealed that the LWS chicks have a lower threshold to anorexigenic neuropeptides than the HWS line, for example, α-MSH (Cline et al., 2008b), CRF (Cline et al., 2009), insulin (Smith et al., 2011), amylin (Cline et al., 2010b), ghrelin (Xu et al., 2011), and neuropeptide AF (Newmyer et al., 2010). Conversely, the HWS line is more sensitive to neuropeptide S (Cline et al.,

2008a) and calcitonin and calcitonin gene-related peptide (Cline et al., 2010a) than the LWS. Human recombinant leptin causes a decrease in food intake in the LWS whereas it does not affect the HWS (Kuo et al., 2005). The LWS line does not respond to NPY (Newmyer et al., 2013) or AgRP (unreported data) with increased food intake. Also, both lines have similar thresholds in their food intake response to different doses of ghrelin (Xu et al., 2011) and galanin (Hagen et al., 2013). Central injection of insulin decreases food and water intake and blood glucose at a lower dose threshold in the LWS than HWS chicks (Smith et al., 2011). Similarly, the lines also display differences in their threshold response in blood glucose to peripheral insulin (Sumners et al., 2014). Collectively, various food intake and metabolic responses to neuropeptides and hormones (insulin) may contribute to differences in body weight and appetite between the lines.

3.2.2 NPY and its receptors

ICV administration of NPY induces a dose-dependent increase in food intake in rodents (Levine and Morley, 1984; Clark et al., 1985) and chickens (Kuenzel et al., 1987). During the past few decades, studies have focused on the effects of NPY on food intake in the line chicks. In prior trials, the 48th, 49th, and 50th generation line chicks were treated with a range of doses of NPY (0.3, 1, and 3 nmol) and the results showed that there was no effect on food intake in the LWS whereas all doses of NPY promoted food intake in the HWS (Newmyer et al., 2013). However, both lines displayed similar increases in c-Fos immunoreactivity in the LH and PVN, suggesting that the LWS and HWS shared similar alterations in neuronal activation in appetite-associated hypothalamic nuclei (Newmyer et al., 2013). These results suggested that NPY was activating similar pathways in LWS and HWS, but that another pathway was likely overriding the orexigenic effects of NPY in the LWS. Rice et al. reported that hypothalamic NPY, NPYR2 and NPYR5 mRNAs were greater in LWS than HWS chicks (Rice et al., 2014). Similarly, at 90 days of age, hypothalamic NPY,

NPYR1, and NPYR5 mRNAs were greater in the LWS than HWS (Zhang et al., 2013b). Collectively, these findings suggested that the lack of NPY-induced hyperphagia in the LWS was because of an effect downstream of NPY binding to its receptor rather than a deficiency or dysfunction in NPY or its receptors. Consistent with this, we were unable to find any polymorphisms or deletions in their genome that would alter the amino acid sequence and hence functionality of NPY or its receptors (Rubin et al., 2010). On the other hand, Ka et al. reported that NPY mRNA was lower in the LWS females than in the HWS at 4 days post-hatch (Ka et al., 2011). It is possible that this difference may be caused by different experimental methods, including real-time PCR (Zhang et al., 2013b; Rice et al., 2014) and oligonucleotide arrays (Ka et al., 2011), and differences in methods for brain dissection.

3.2.3 Gene expression of neuropeptides

We have described differences in the mRNA abundance of many different appetite-associated neuropeptides and receptors between LWS and HWS chicks in the whole hypothalamus and individual hypothalamic nuclei. For instance, hypothalamic POMC mRNA did not differ between the lines after overnight fasting at 90 days of age (Zhang et al., 2013b), consistent with expression differences reported at 4 days post-hatch (Ka et al., 2011). Conversely, hypothalamic POMC mRNA was greater in the HWS than LWS chicks in another study (Rice et al., 2014), although it should be noted that experimental conditions differed between studies, including nutritional status and injection status at the time of tissue sampling. Hypothalamic AgRP was greater in LWS than HWS 90 day-old chickens (Zhang et al., 2013b). Yi et al. found that 5 day-old LWS chicks expressed more hypothalamic MCH and orexin receptor 2 (ORXR2) than HWS while the HWS chicks expressed more orexin than LWS (Yi et al., 2017). The LWS chicks expressed greater amounts of hypothalamic leptin receptor (LEPR), melanocortin receptor 3 (MC3R), and oxytocin mRNAs, while the HWS

expressed more MC4R, IL1Br and POMC mRNAs than LWS (Yi et al., 2017). Such differences may be due to different experimental designs (Yi et al., 2015; Yi et al., 2017). Measuring gene expression in individual appetite-associated hypothalamic nuclei provides more detailed information because of known functional differences among these nuclei. The LWS chicks had more NPYR5 mRNA in the PVN, LH, and ARC than HWS chicks, indicating that the difference in NPYR5 expression in the whole hypothalamus may originate from the PVN, LH, and ARC (Yi et al., 2017). It is possible that greater expression of NPYR5 may serve to compensate for the refractory response to NPY in the LWS chicks. Similarly, there were differences in the mRNA abundance of NPY, ORXR2, and POMC in the whole hypothalamus that may originate from differences in the DMN, VMH, and ARC, respectively (Yi et al., 2017). However, there were also some differences in the expression profiles between the whole hypothalamus and nuclei. For example, the expression of LEPR was greater in the whole hypothalamus in LWS than HWS whereas the HWS chicks expressed more LEPR in the ARC than LWS (Yi et al., 2015; Yi et al., 2017). A similar phenomenon was observed for ORXR2 (Yi et al., 2015; Yi et al., 2017). An explanation for differences in expression between the whole hypothalamus and at the nucleus level is that there might be other regions of the hypothalamus that contribute to differences between the lines that we did not measure, and that the multiple contributions from different nuclei in the whole hypothalamus may mask some differences that are observed at a nucleus level.

3.3 Previous studies on the correlation between stress and food intake in line chicks.

3.3.1 Stress and orexigenic neuropeptides

Stress can be defined as any deviation from normal physiological status and can affect food intake in animals and humans in a bidirectional way depending on the stress intensity and environmental factors (Bazhan and Zelena, 2013). In previous studies, we reported that there is a difference in the appetite response in the line chicks after ICV administration of NPY at 5

days following exposure to a combination of nutritional and thermal stressors. NPY was expressed greater in 4 to 5 day-old LWS chicks than HWS chicks under multiple conditions: 1) after 3 hours of fasting (Yi et al., 2015), 2) 5 days after short-term (6 min) exposure to freezing temperature followed 24 hours of exposure to a cool temperature (22 ℃) and 24 hours of fasting (Yi et al., 2017) and 3) after 4 hours of fasting (Rice et al., 2014). As a potent orexigenic factor, NPY promotes food intake in mammals and chickens (Kuenzel et al., 1987; Billington et al., 1991). However, the LWS chicks at 5 days post-hatch that were stress-exposed at hatch did not respond with any magnitude of altered food intake to any dose of ICV NPY (0.3, 1, and 3 nmol) (Newmyer et al., 2013). Thus, this result showed that the LWS chicks might be more vulnerable to stress-induced anorexia than HWS chicks or that NPY has a more potent orexigenic function in the HWS. The potential causes include decreased translation or dysregulation in the processing of the bioactive peptide, finally resulting in insensitivity of NPY in stressed LWS chicks, changes in receptor activation or downstream signaling, or intensification of anorexigenic pathways that override the effect of NPY.

The LWS chicks expressed more AgRP mRNA in the ARC than HWS after 3 hours of fasting (Yi et al., 2017). However, there was no difference in whole hypothalamic AgRP mRNA between the lines after 3 hours of fasting (Yi et al., 2015). The possible reasons for these differences include different sample types and experimental designs, and again the idea that there might be other hypothalamic regions expressing AgRP that we did not sample. Additionally, hypothalamic mRNA expression of some appetite-associated neuropeptides were not affected by various stressors, such as NPYR5, oxrexinR2, NPYR2, MCH, NPYR1, visfatin and galanin (Rice et al., 2014; Yi et al., 2015; Yi et al., 2017), suggesting such neuropeptides may not play a role in coping with stress in the line chicks..

3.3.2 Stress and anorexigenic neuropeptides

CRF is a potent inhibitor of food intake in mammals and chickens (Arase et al., 1988). In mammals, the basolateral amygdala mediates CRF-induced anorexia via CRFR1 (Jochman et al., 2005), while the lateral septum and BNST does so via the CRFR2 (Smagin et al., 1998; Ohata and Shibasaki, 2011). CRFR1 and CRFR2 mRNAs were expressed greater in the LWS than HWS chicks after stress exposure (Cline et al., 2009; Yi et al., 2015). Thus, CRF may be a causative factor in the pathways leading to the insensitivity to NPY in stressed LWS chicks. POMC and CART play potent inhibitory roles in food intake as well. Our studies demonstrated that the HWS chicks expressed more POMC mRNA than LWS chicks in both the whole hypothalamus and in the ARC and stress decreased such differences, indicating that stress may stimulate food intake in the HWS line (Rice et al., 2014; Yi et al., 2015; Yi et al., 2017). Although there were no differences between the lines in whole hypothalamic CART mRNA after stress, CART mRNA expression at the nucleus level differed. For instance, CART mRNA was greater in the LWS chicks in the VMH but greater in the HWS chicks than LWS in the PVN, which may explain why we did not see differences at the level of the whole hypothalamus (Yi et al., 2015; Yi et al., 2017). Also, many anorexigenic neuropeptides were more highly expressed in the LWS chicks than HWS chicks after stress exposure, including calcitonin, neuropeptide S (NPS) (Cline et al., 2008a; Yi et al., 2015), oxytocin (Yi et al., 2015; Yi et al., 2017), LEPR (Ka et al., 2011; Yi et al., 2015), arginine vasotocin (AVT) (Yi et al., 2017), DDC (Kuo et al., 2005; Rice et al., 2014; Yi et al., 2015; Yi et al., 2017), and MC3R (Ka et al., 2009; Ka et al., 2011; Yi et al., 2015). Collectively, these results suggest that the LWS line is more vulnerable to stress-induced anorexia.

The objective of my dissertation research was thus to elucidate the hypothalamic molecular and cellular mechanisms underlying stress-induced anorexia in the LWS chicks, and to further understand the basic molecular mechanisms underlying the appetite-associated effects

of CRF and several other poorly-characterized appetite-regulatory peptides in several avian models including broiler chicks and Japanese quail. These findings provide insights on basic appetite neurobiology that have far-reaching implications for human and animal health and productivity.

Chapter 2: Stress-induced suppression of neuropeptide Y-induced hunger in anorexic chicks involves corticotropin-releasing factor signaling and the paraventricular nucleus of the hypothalamus

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Abstract

The Virginia lines of chickens have been selected for low (LWS) or high (HWS) juvenile body weight and have different severities of anorexia and obesity, respectively. The LWS that are exposed to stressors at hatch are refractory to neuropeptide Y (NPY)-induced food intake and the objective was to elucidate the underlying mechanisms. Chicks were exposed to a stressor (-20 ℃ for 6 minutes, and 22 ℃ and delayed access to food for 24 hours) after hatch and hypothalamic nuclei, including the lateral hypothalamus (LH), paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), and arcuate nucleus (ARC), were collected 5 days later. In LWS but not HWS, stress exposure up-regulated corticotropin-releasing factor (CRF), CRF receptor sub-types 1 and 2 (CRFR1 and CRFR2, respectively), melanocortin receptor 4, and urocortin 3 in the PVN, and CRFR2 mRNA in the VMH and ARC. In LWS, stress exposure was also associated with greater NPY and NPY receptor sub-type 5 mRNA in the ARC and PVN, respectively, and decreased AgRP mRNA in the ARC. In HWS, stress exposure was associated with increased CRFR1 and decreased CART in the ARC and PVN, respectively. Refractoriness of the food intake response to NPY in LWS may thus result from overriding anorexigenic tone in the PVN associated with CRF signaling. Indeed, the orexigenic effect of NPY was restored when LWS were injected with a CRF receptor antagonist, astressin, before stress exposure. These results provide insights into the molecular basis of eating disorders and suggest that CRF signaling in the PVN may exacerbate the anorexic phenotype in the presence of environmental stressors.

Key words: anorexia, chick, corticotropin-releasing factor, hypothalamus, neuropeptide Y

1. Introduction

Balancing energy intake and expenditure involves a multitude of physiological activities that are controlled by interconnected central and peripheral signaling pathways. Ultimately, these signals are integrated at the hypothalamus, which plays an essential role in maintaining energy homeostasis (Arora and Anubhuti, 2006). Within the hypothalamus, several nuclei are associated with hunger and satiety, including the arcuate nucleus (ARC), ventromedial nucleus of the hypothalamus (VMH), paraventricular nucleus (PVN) and lateral hypothalamic area (LH) (Arora and Anubhuti, 2006). As a feeding regulation center, the ARC integrates hormonal signals related to energy homeostasis (Arora and Anubhuti, 2006) and contains two functionally discrete neuronal populations that have opposite effects on appetite regulation: the anorexigenic, proopiomelanocortin (POMC)/cocaine and amphetamine-regulated transcript (CART) neurons and the orexigenic, neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons (Harrold et al., 2012). The ARC has inter-associations with other hypothalamic nuclei, such as the PVN, VMH and LH, forming an extensive system for regulating feeding behavior (Harrold et al., 2012).

Stress, defined as any deviation from normal physiological status (2015), is ubiquitous in the environment, and the consequences of it are dependent on a variety of factors including intensity, duration, and stage of the life span. The physiological effects of a stressor involve interactions between the central and peripheral nervous systems and other organs (2015), one of the most common effects being an alteration in feeding behavior modulated by the hypothalamic-pituitary-adrenal (HPA) axis, the major regulatory system for the stress response. The stress response and appetite regulation share common neuroanatomy and pathways. For example, feeding neural circuits converge on the PVN which contains corticotropin-releasing factor (CRF) and urocortin (UCN) neurons (Maniam and Morris, 2012).

The Virginia lines of chickens have been selected for low (LWS) or high (HWS) body weight (from the same original founder population) for 60 consecutive generations, and now display a more than 10-fold difference in body weight at 56 days of age, with different severities of anorexia and obesity, respectively (Jambui et al., 2017). Over the past decade, differences in their appetite and stress physiology have been documented and summarized in (Yi et al., 2015; Yi et al., 2016). For example, in the feeding response to intracerebroventricular (ICV) injection of peptides, the LWS line chicks exhibited a lower threshold to some anorexigenic neuropeptides, such as α-melanocyte stimulating hormone and CRF. In contrast, the HWS line was more sensitive than LWS to neuropeptide S and calcitonin and calcitonin gene-related peptide. The LWS chicks expressed more hypothalamic NPY mRNA than HWS chicks (Yi et al., 2015; Yi et al., 2016), and hypothalamic CRFR1 and CRFR2 mRNAs were greater in LWS than HWS chicks (Yi et al., 2015).

One of the more striking differences that we observed was with respect to the food intake-stimulating effects of NPY (Newmyer et al., 2013; Yi et al., 2016), one of the most potent orexigenic factors in mammals and birds (Furuse, 2002). While HWS chicks responded with increased food intake to a range of doses of NPY, LWS chicks did not respond to ICV-injected NPY under certain conditions (Newmyer et al., 2013; Yi et al., 2016). After much consideration about the environmental factors involved, we hypothesized that exposure to cold temperatures and transportation altered appetite regulation such that the LWS chicks were rendered refractory to the food-intake stimulating effects of NPY at 5 days of age. As it turns out, this combination of temperature exposures coupled to transportation for distances is routinely encountered in the poultry industry, where chicks may be delayed access to food for up to 48 hours post-hatch due to processing and transport from the hatchery. When exposed to this stressor combination, the LWS chicks do not respond to NPY 5 days later (Yi et al.,

2016) although hypothalamic neuronal activation is similar for both lines (Newmyer et al., 2013). The HWS however, respond robustly to NPY with increased food intake under all conditions (Yi et al., 2016). An investigation of gene expression at the whole hypothalamus level suggested that early post-hatch stressor exposure produces an intensification of anorexigenic melanocortin signaling pathways in LWS chicks that block the orexigenic effect of exogenous NPY (Yi et al., 2016). Understanding this phenomenon may provide insights on how stress and appetite converge in order to dynamically regulate energy balance in the body under different conditions.

While our previous studies have yielded information regarding the role of the hypothalamus in appetite regulation and the stress response, such experiments involved the whole hypothalamus, which does not assay the distinct functionality and diversity of the individual nuclei. The screening study as reported herein employed a punch biopsy technique coupled to real time PCR to investigate the nuclei-specific appetite- and stress-associated gene expression profiles in these two lines. Technically, the punch biopsy and real time PCR has some advantages over in *situ* hybridization and immunohistochemistry. Many genes can be screened simultaneously, real time PCR is more sensitive for detecting changes in gene expression in micro-dissected hypothalamic nuclei containing scarce mRNAs, and real time PCR is more quantitative. The present study was thus designed to determine the effects of stress and exogenous NPY on hypothalamic nuclei gene expression in LWS and HWS chicks, and to test the hypothesis that effects are being mediated via CRF signaling pathways.

2. Material and methods

2.1 Animals and experimental design

In this experiment, the lines of chickens are from a long-term divergent selection program for either low or high body weight at 56 days of age (Dunnington and Siegel, 1996). The

foundation stock consisted of crosses of seven inbred lines of White Plymouth Rocks and a review of the selection program may be found elsewhere (Dunnington and Siegel, 1996; Dunnington et al., 2013). All eggs were from age contemporary parents from the S58 generation and were incubated in the same incubator and hatcher. After hatch (day 0 post-hatch), chicks were placed inside United States Mail-approved cardboard chick shipping boxes (37 x 24 cm, n = 20 per box) and divided into four groups: LWS stress-exposed, LWS control, HWS stress-exposed and HWS control. The stress protocol in this study was identical to the one reported in (Yi et al., 2016) and was intended to mimic a series of stressors that would be encountered during post-hatch transportation during cold weather in the commercial chicken industry (Hunter et al., 1999). The chicks in the stress-exposed group were subjected to -20 ℃ for 6 minutes and then transferred to 22 ℃ for 24 hours during which time neither feed nor water was provided. For the control groups, chicks were housed in a room at 32 ± 1 ℃ and 50 ± 5% relative humidity with free access to a mash diet (21.5% crude protein and 3,000 kcal ME/kg) and water. At 1 day post-hatch, all chicks were transferred to individual cages in which rearing conditions and feed were the same as for the control group. Chicks had visual and auditory contact with each other in the individual cages and were handled twice daily to adapt to handling. 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 Experiment 1: Hypothalamic nuclei mRNA abundance

At 5 days post-hatch, each chick was deeply anesthetized with sodium pentobarbital via cardiopuncture and then perfused via the carotid artery with 2.5 mL of RNA stabilizing buffer (16.7 mM sodium citrate, 13.3 mM EDTA, and 3.5 M ammonium sulfate; pH=5.2). Within 30 minutes of perfusion, brains were sectioned in a cryostat at -10 ℃ into 500 μm

thick coronal sections in the direction from rostral to caudal: LH, PVN, and VMH were collected at 8.0, 7.4, and 6.8 interaural respectively, and the ARC was collected at 5.4 interaural based on the Kuenzel and Masson chicken stereotaxic atlas (Kuenzel and Masson, 1988). Punches were collected using sterile disposable biopsy punch instruments (1 mm, Braintree Scientific Inc., Braintree, MA) and were immediately transferred to sterile microcentrifuge tubes containing RNA lysis buffer with 1% beta-mercaptoethanol (Norgen Biotek, Thorold, ON, Canada), vortexed, snap-frozen in liquid nitrogen, and stored at -80 ℃. In order to ensure anatomical accuracy, the remaining brain section was photographed and verified via the overlays containing the respective nuclei boundaries according to Kuenzel and Masson chicken stereotaxic atlas (Kuenzel and Masson, 1988). There were 10 to 15 samples per nuclei in each group that were verified for total RNA isolation.

The punches were thawed, vortexed vigorously for 30 seconds, and incubated at room temperature for 5 minutes before adding 70% molecular biology-grade ethanol, and total RNA was isolated following the manufacturer's instructions for the Total RNA Purification Micro Kit (Norgen Biotek). The concentration and purity of total RNA was assessed by spectrophotometry at 260/280/230 nm with a NanoDrop 2000 (Thermo Fisher Scientific Inc., West Palm Beach, FL, USA). Total RNA integrity was verified using an Experion (Bio-Rad, Hercules, CA) and RNA StdSens analysis kit (Bio-Rad), according to the manufacturer's instructions.

First-strand cDNA was synthesized in 20 µl reactions from 100 ng of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's instructions. Reactions were performed under the following conditions: 25 ℃ for 10 minutes, 37 ℃ for 120 minutes and 85 ℃ for 5 minutes. Primers for real time PCR were designed with Primer Express 3.0 software (Applied Biosystems) (Table 2.1) and validated for amplification efficiency before use (95-105%). Real-time PCR

reactions were performed in 10 µl reactions that contained 5 µl Fast SYBR Green Master Mix (Applied Biosystems), 0.5 µl primers (0.25 µl of 5 uM forward primer and 0.25 µl of 5 µM reverse primer), 1.5 µl nuclease-free water, and 3 µl 5-fold diluted cDNA using a 7500 Fast Real-Time PCR System (Applied Biosystems). Real-time PCR was performed under the following conditions: 95 ℃ for 20 seconds and 40 cycles of 90 ℃ for 3 seconds plus 60 ℃ for 30 seconds. A dissociation step consisting of 95 ℃ for 15 seconds, 60 ℃ for 1 minute, 95 ℃ for 15 seconds and 60 ℃ for 15 seconds was performed at the end of each PCR reaction to ensure amplicon specificity.

Real-time PCR data were analyzed using the ΔΔCT method (Livak and Schmittgen, 2001) with β-actin as the reference gene. After verification of anatomical accuracy following punch biopsy, there were 10 to 15 samples per group that were available for analysis. Because not every factor was measured in all four nuclei, the analysis of each gene employed a different calibrator sample. The average of the control LWS chicks in the VMH (leptin receptor and orexin receptor 2), and LH (orexin) was used for the genes indicated in parentheses. The average of the control LWS chicks in the PVN was used as the calibrator sample for all other genes. Relative quantities calculated as $2^{-\Delta\Delta CT}$ were used for statistical analysis. The statistical model included the main effect of genetic line (HWS and LWS), hypothalamic nuclei (LH, PVN, VMH and ARC; not all genes were measured in all nuclei), treatment (control and stress-exposed) and their interactions. Data were analyzed by analysis of variance (ANOVA) using JMP Pro 11 (SAS institute, Cary, NC, USA). Means were separated using Tukey's test when the interaction or effect of nuclei was significant. For all experiments, differences were considered to be significant at $P < 0.05$.

2.3 Experiment 2: Food intake following NPY injection in astressin-treated chicks

After hatch (day 0 post-hatch), chicks were placed inside shipping boxes and randomly assigned to eight groups (n=20 per group): LWS astressin + NPY, LWS astressin + vehicle,

LWS control + NPY, LWS control + vehicle, HWS astressin + NPY, HWS astressin + vehicle, HWS control + NPY, and HWS control + vehicle. Chicks were assigned to receive 0 (control) or 6 nmol astressin by ICV injection. After injection, all chicks were subjected to the stress protocol described in experiment 1. On day 1 post-hatch, chicks were transferred to individual cages in a room at 32 ±1 ℃ and 50 ±5% relative humidity. All chicks had free access to food and water. On day 5 post-hatch, chicks were assigned to receive 0 (vehicle) or 0.2 nmol NPY by ICV injection. After injection, chicks were returned to their individual home cages and given ad libitum access to both food and water. Food intake was monitored (0.01 g) for 30 minutes post-injection.

Chicks were injected using an adapted method (Davis et al., 1979) that does not appear to induce physiological stress (Furuse et al., 1999). The head of the chick was briefly inserted into a restraining device that leaves the cranium exposed and allows 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 the injection depth in the un-anesthetized chick for 10 seconds post-injection to reduce backflow. Astressin (CPC Scientific, Inc., Sunnyvale, USA) or chicken NPY (YPSKPDSPGEDAPAEDMARYYSALRHYINLITRQRY, AnaSpec, San Jose, CA, USA) was dissolved in artificial avian cerebrospinal fluid (Anderson and Heisley, 1972) as a vehicle for a total injection volume of 5 µl with 0.1% Evans Blue dye to facilitate injection site localization. After food intake data collection, the chick was decapitated and its head sectioned along the frontal plane to determine the site of injection. Any chick without dye present in the lateral ventricle system was eliminated from analysis. Sex of chicks was determined visually by dissection.

Food intake data were analyzed with the GLM procedure of SAS 9.3 (SAS Institute, Cary, NC, USA), with analyses conducted within each line. The statistical model included sex (male or female), astressin (0 or 6 nmol), NPY dose (0 or 0.2 nmol), and their interactions. Neither sex nor interactions associated with it were significant, hence sex was removed from the statistical model. When interactions were significant means were separated using Tukey's test.

3. Results

3.1 Anorexigenic neuropeptide and related receptor mRNA in hypothalamic nuclei

Results for the mRNA abundance of factors classified as being anorexigenic are summarized in Table 2.2. Results will be described in rank from highest-order interactions to main effects.

Three-way interactions of line, stress, and nucleus. There was an interaction of line, stress treatment, and nucleus distribution on mRNA abundance of CART ($P < 0.0001$), CRF ($P = 0.0075$), CRF receptor sub-type 1 (CRFR1; $P < 0.0001$), CRFR2 ($P = 0.0496$), UCN3 ($P < 0.0001$), and melanocortin receptor sub-type 4 (MC4R; $P = 0.0015$). For CART, expression was greater in stressed than non-stressed LWS in the PVN but greater in control than stressed HWS chicks in the PVN, with non-stressed HWS having greater expression than LWS in either state, and expression being greater in the PVN than in other nuclei in HWS (Fig. 2.1A). Stress increased expression of CRF in the PVN of LWS but not HWS chicks although in both states expression was greater in HWS than LWS in the PVN and PVN expression was greater as compared to other nuclei (Fig. 2.1B). The mRNA abundance of CRFR1 was increased by stress in the PVN of LWS but in the ARC of HWS with expression greater in LWS than HWS in the ARC in the non-stressed state (Fig. 2.1C). Expression of CRFR2 on the other hand was increased by stress in the ARC, PVN, and VMH of LWS but was not affected by

stress in HWS in any of the nuclei in which it was measured, and was more highly expressed in LWS than HWS in the ARC, PVN, and VMH in the stressed state (Fig. 2.1D). Urocortin 3 mRNA was greater in the PVN than in other nuclei and in the PVN was greater in LWS than HWS and was increased in the LWS by stress (Fig. 2.2A). Melanocortin receptor 4 mRNA was increased by stress in the PVN of LWS but not HWS and was greater in HWS than LWS in the PVN in the non-stressed state (Fig. 2.2B).

Interactions of genetic line and nucleus. There were interactions of genetic line and nucleus for growth hormone secretagogue receptor (GHSR; $P = 0.04$), leptin receptor (LEPR; $P = 0.0093$), and POMC ($P < 0.0001$). Expression of GHSR was greater in the PVN than VMH of LWS but not HWS chicks (Fig. 2.3A). Leptin receptor mRNA was similarly expressed among LWS and HWS in the ARC but was greater in the HWS than LWS in the VMH and greater in the VMH than ARC in HWS but not LWS chicks (Fig. 2.3B). Expression of POMC mRNA was greater in the LWS than HWS in the ARC, but did not differ among lines in the other nuclei (Fig. 2.3C). The mRNA abundance of POMC was at least 30-fold greater in the ARC than other nuclei in both lines.

Main effects of stress, genetic line, and nucleus. Stress-exposure increased expression of GHSR ($P = 0.0055$) and mesotocin (MT; $P = 0.0011$), and reduced expression of POMC ($P = 0.019$). Expression of ghrelin ($P = 0.0009$) and MT ($P = 0.001$) was greater in LWS than HWS while POMC ($P = 0.0003$) was greater in HWS than LWS (Table 2.2). MC3R mRNA was greater in the ARC than LH, PVN, or VMH ($P < 0.0001$).

3.2 Orexigenic neuropeptide and related receptor mRNA in hypothalamic nuclei

Three-way interactions of line, stress, and nucleus. Results for factors associated with orexigenic effects are summarized in Table 2.3. There was an interaction of genetic line, stress treatment, and nucleus on expression of NPY ($P = 0.048$), NPYR5 ($P = 0.0126$), and AgRP ($P = 0.0006$). Neuropeptide Y mRNA was increased by stress in the ARC of LWS but

not HWS and was greater in LWS than HWS in the ARC regardless of stress state (Fig. 2.2C). The expression of NPYR5 was up-regulated by stress in the PVN of LWS but not HWS and was greater in LWS than HWS in the stressed state (Fig. 2.2D). Quantities of AgRP mRNA in the ARC were decreased by stressor-exposure in the LWS but not HWS, and were not affected by treatment in any other nucleus (Fig. 2.2E). Expression of AgRP was at least 100-fold greater in the ARC than other nuclei in both lines.

Interactions of genetic line and nucleus. There was an interaction of genetic line and nucleus on expression of ORXR2 ($P = 0.0448$; Fig. 2.3C). In the LH, there was greater expression of ORXR2 in LWS than HWS, while in the VMH expression was similar between the lines.

Main effects of genetic line, stress, and nucleus. Expression of NPYR1 was greater ($P = 0.0083$) in HWS than LWS (Table 2.3). Stress was associated with increased expression of orexin receptor 2 (ORXR2; $P = 0.0242$) and orexin ($P = 0.0286$). NPYR1 mRNA was differentially expressed among nuclei with the order of magnitude as follows: ARC > PVN > VMH > LH ($P < 0.0001$).

3.3 Food intake and body weight

There were interactions of astressin and NPY treatment in LWS ($P < 0.0001$) and HWS ($P < 0.0001$) chicks. In stressed LWS chicks, at 30 minutes post-injection, food intake was increased by NPY relative to the vehicle-injected chicks treated with astressin whereas food intake was not affected by NPY injection in chicks that did not receive astressin (Fig. 2.4). In the HWS line, NPY increased food intake in both astressin and non-astressin-injected chicks exposed to stress although the magnitude of increase was greater in the absence of astressin treatment (Fig. 2.4). At the time of NPY injection, there was an interaction of astressin and genetic line on body weight ($P = 0.0017$), where LWS body weight was not affected by astressin treatment (20.0 vs. 18.3 g $\pm$ 0.91 pooled SEM for astressin and vehicle treated,

respectively) whereas HWS body weights were reduced (37.9 vs. 44.3 g ± 1.3 pooled SEM for astressin and vehicle treated, respectively).

4. Discussion

The interplay between appetite regulation and stress coping mechanisms is complex and these systems share overlapping neural circuitry (Maniam and Morris, 2012). In our study of correlated responses to long-term divergent selection for body weight in the LWS and HWS lines, we observed differences in their food intake, one of the most profound being the stress-dependent difference in their appetite response to exogenous NPY (Newmyer et al., 2013). Although the LWS chicks did not respond to NPY, hypothalamic nucleus activation was similar, suggesting that NPY was activating similar pathways but that its effect was being overridden by other systems in the LWS chicks (Newmyer et al., 2013). Thus, we repeated the study, with the hypothesis that refractoriness to NPY in the LWS was dependent upon exposure to nutritional and thermal stressors at hatch, as our previous studies had been conducted under conditions where the chicks were transported to a more distant facility at hatch during cold weather (Yi et al., 2016). Indeed, LWS but not HWS chicks that were exposed to a stress protocol at hatch did not increase their food intake in response to centrally-injected NPY at 5 days post-hatch, whereas non-stressed LWS responded to NPY with increased food intake (Yi et al., 2016).

It is important to note the growth and overall health of the LWS in response to the stress protocol. If the chick was in a state of morbidity there would be a confound of health with the appetite response to NPY at 5 days of age. The body weight of LWS at day 5 post-hatch was not affected by the stress protocol ($P = 0.6669$) whereas HWS body weight was decreased in response to stress when compared to their control HWS counterparts ($P < .0001$) (data not shown). With respect to the food intake, the stress treatment did not affect the food intake

LWS (P = 1.0000) or HWS (P = 0.7863). Hence, the results collectively demonstrate that the stressed LWS chicks were not in a state of depressed growth that exerted confounding effects on appetite.

To identify the molecular mechanisms underlying stress-dependent anorexia in the LWS, we measured hypothalamic mRNA abundance of various appetite-associated factors and concluded that increased expression of MC3R may amplify anorexigenic melanocortin signaling pathways in LWS chicks that block the orexigenic effect of exogenous NPY (Yi et al., 2016). As there are distinct hypothalamic nuclei that mediate various aspects of appetite regulation and stress response mechanisms, measuring gene expression at the whole hypothalamus level likely masks differences that are nucleus-specific. For example, the PVN integrates signals from first-order appetite neurons in the ARC, and produces CRF, which plays a role in mediating the stress response and regulating appetite. Thus, to dissect the hypothalamic pathways that integrate stress coping mechanisms and appetite regulation, molecular studies focusing on the individual hypothalamic nuclei are more meaningful, hence the design of the present study. As summarized in Figure 2.5, more factors were affected by stress at the transcriptional level in LWS than HWS, with most of the effects occurring in the PVN and no changes observed in the LH. The following discussion will focus on uniting the results into a proposed mechanism that may explain the stress-induced anorexia in LWS chicks.

Neuropeptide Y and NPYR5 mRNAs increased in the ARC and PVN, respectively, in LWS but not HWS in response to stress exposure. NPY-ergic neurons project from the ARC to the PVN, which plays an important role in mediating the effects of NPY in feeding and stress coping regulation (Reichmann and Holzer, 2016). NPYR5 is highly expressed in the PVN and is thought to be the major receptor that mediates the orexigenic effects of NPY (Zhang et al., 2011). In addition to modulatory effects on feeding behavior, NPYR5 is

implicated in stress coping responses. For instance, a selective NPYR5 antagonist suppressed the anxiolytic effect of NPYR5 in anxious rats (Walker et al., 2009), and anxiolytic effects mediated via NPYR5 were enhanced by its selective agonist (Morales-Medina et al., 2012). Thus, in view of its multi-functional properties, the NPYR5 may be a major participant in mediating NPY signals in both feeding behavior and stress adaption in the LWS chicks.

In fact, a number of studies have demonstrated that NPY plays an important role in stress-induced adaptations in feeding behavior (Reichmann and Holzer, 2016) and its abundance is an indicator that reflects a dynamic physiological state. For example, fasting initiates a gradual, time-related elevation in NPY expression that peaks on the 4th day of food deprivation, thereafter falling sharply to the level of control rats following one day of refeeding (Sahu et al., 1988a). There are multiple reports that stressful events are associated with an increase in hypothalamic NPY signaling as reviewed (Reichmann and Holzer, 2016), suggesting that ARC-derived NPY plays important roles in integrating feeding behavior and stress coping. Collectively, results suggest that differences in the abundance and regulation of NPY and associated factors reflects a difference in physiological status and that the lines may require different time scales to adapt to a stressful event. Elevated ARC NPY and PVN NPYR5 mRNAs in the LWS in response to stress might thus serve as an adaptive mechanism to attempt to compensate for the overriding anorexigenic tone.

Such overriding anorexigenic tone may originate from CRF and UCN3 in the PVN. In the present study, CRF, UCN3, and both receptor sub-types were up-regulated in the LWS but not HWS in the PVN in response to stress. The CRF family, including CRF and UCNs 1-3, is highly conserved among mammalian, non-mammalian, and invertebrate species (Stengel and Taché, 2014). Their biological functions are mediated by two G-protein-coupled receptors, CRFR1 and CRFR2 (Stengel and Taché, 2014). CRF and UCN3 are multi-functional neuropeptides that have anorexigenic effects and also participate in the stress response

(Stengel and Taché, 2014). Typically, the adaptive response to stress is classified into three phases including initiation, maintenance, and recovery (Carsia and Weber, 2000). It is generally accepted that the CRF-CRFR1 system is essential for the initiation phase of the stress response while the UCN-CRFR2 system is critical for the termination of the stress response or recovery from allostasis (Neufeld-Cohen et al., 2010). The CRF and UCN3 have a high affinity for CRFR1 and CRFR2, respectively, indicating that CRF-CRFR1 and UCN3-CRFR2 are the primary ligand-receptor forms that carry out their biological functions (Smith and Vale, 2006). Thus, the results of the present study suggest that on day 5 post-hatch, LWS chicks are still in the initiation/maintenance phase of the response to the stressor. This thesis is supported by the observation that stressed LWS had higher plasma corticosterone (CORT) concentrations than HWS (Yi et al., 2016). In general, the HPA axis can be activated by stress and there is a tight correlation between CRF production and CORT in the circulation (Smith and Vale, 2006).

CRFR2 mRNA was also up-regulated in response to stress in the LWS but not HWS chicks in the VMH and ARC. Previous studies showed that the VMH, the major site of CRFR2 expression, mediates the effects of UCN3 on energy homeostasis and that the CRFR2-UCN3 system is an essential signaling pathway in metabolic regulation (Chen et al., 2012). Moreover, CRFR2-UCN3 has been identified as a major neural pathway in the response to physiological and/or psychological perturbations (Kuperman et al., 2010). Thus, CRFR2-UCN3 signaling in the VMH may mediate effects on energy intake regulation and stress adaption in the VMH of the LWS, which expressed more CRFR2 in the VMH in the stressed state. There is also high expression of CRFR2 in the ARC, directly associated with the regulation of energy balance and feeding responses (Kuperman and Chen, 2008). Because CRFR2 signaling has been implicated in modulation of the anxiety and stress response (Bale et al., 2002), increased expression of CRFR2 in the ARC, VMH and PVN of stressed LWS

may be responsible for mediating stress-induced changes in physiology that render them refractory to the orexigenic effects of NPY. CRFR1 mRNA was increased in HWS in the ARC and LWS in the PVN in response to stress. About half of the CRFR1 neurons in the ARC co-express AgRP and play critical roles in the adaptation to stress, including facilitating transition from an anabolic to catabolic status (Kuperman et al., 2016). Thus, results collectively suggest that the lines have different regulatory systems to cope with environmental stressors, and that the anorexigenic effects of stress in LWS chicks may stem from increased CRF-ergic tone originating from the PVN that overrides NPY signaling.

The NPY and CRF systems exert opposite roles in food intake and stress adaptation: orexigenic NPY mediates anxiolytic functions whereas anorexgenic CRF induces anxious and stressful behavior (Ehlers et al., 1997). To date, extensive studies have confirmed anatomical and functional crosstalk between NPY and CRF systems with respect to feeding regulation and stress adaptation, and the competitive balance between these two systems is the determinant for feeding behavior and physiological state outcomes (Li et al., 2000; Reichmann and Holzer, 2016). Thus, we hypothesize that the refractoriness to the orexigenic effect of NPY in stressor-exposed LWS results from asymmetric competition between NPY and CRF systems; specifically that orexigenic NPY is overridden by CRF.

To test this hypothesis, we injected astressin, a non-selective CRF receptor antagonist, at hatch before application of the stress protocol. As a potent antagonist of both CRFR1 and CRFR2, astressin reversed anxiety-like behavior induced by social stress (Spina et al., 2000) or CRF administration, and blocked ACTH to suppress stress (Pelleymounter et al., 2002). In chicks, central injection of astressin blunted ghrelin- (Ocłoń and Pietras, 2011) vasoactive intestinal peptide-(Khan et al., 2013) CRF- (Cline et al., 2009) and α-MSH (Tachibana et al., 2007)- induced anorexia, and reduced ghrelin- (Ocłoń and Pietras, 2011) and α-MSH-induced corticosterone release (Tachibana et al., 2007). Injection of astressin alone did not affect food

intake or affect sulfated cholecystokinin (26–33)- (Tachibana et al., 2012) or Substance P- (Tachibana et al., 2010) induced food intake in chicks. Thus, astressin has been used in many food intake studies with chicks to evaluate the contribution of CRF receptor signaling to food intake regulation. In a previous study, astressin treatment alone increased food intake in 5 day-old LWS but not HWS, and dampened CRF-induced food intake in both lines (Cline et al., 2009). Such a line-dependent effect of astressin was also observed in the present study. Namely, NPY increased food intake in astressin-treated LWS chicks after stress exposure, demonstrating CRF (and/or UCN) signaling as a major pathway that blocks NPY-induced food intake in the stressed LWS, further underscoring the physiological importance of the competitive interaction between NPY and CRF in regulating feeding behavior and the stress response. In stressed HWS, however, astressin treatment alone did not affect food intake, and astressin injection reduced the NPY-induced orexigenic response, which was unanticipated. In addition to feeding behavior, astressin also exerted a line-dependent effect on body weight, that is, a decrease in HWS but not LWS. Although the mechanisms behind this line-dependent difference is unclear, data suggest that CRF-ergic tone differs between LWS and HWS and is affected by early-life exposure to stress.

In addition to the NPY and CRF systems, the central melanocortin system may also be involved in appetite regulation and the stress response in the stressed LWS. In the present study, MC4R mRNA was increased in response to stress in LWS but not HWS in the PVN and stress decreased AgRP in the ARC of LWS but not HWS. The effect of stress on increasing MC3R was not statistically significant, and there were no effects of stress involving POMC. Notably, these findings in the present study are different from the ones in our previous experiment. For example, the whole hypothalamic gene expression of AgRP displayed a three-way interaction of genetic line, stress, and NPY treatment, where ICV NPY administration decreased AgRP mRNA in stressed LWS and increased its expression in non-

stressed LWS and stressed HWS (Yi et al., 2016). There was no main effect of stress or two-way interaction of line and stress, demonstrating that the effect on AgRP mRNA was NPY-dependent. Also, stress was associated with an increase in hypothalamic MC3R in both lines and a decrease in POMC mRNA in HWS but not LWS (Yi et al., 2016). Regarding the differences between the present and previous experiment, two factors should be considered. Firstly, the presence of exogenous NPY, because chicks in the previous experiment were ICV-injected before hypothalamus collection but none were injected in the present experiment and secondly, different samples, since we sampled several appetite-associated nuclei vs. the whole hypothalamus that was assayed in the former experiment. Therefore, differences in experimental design and type of tissue collected likely influenced the stress effects that were observed in both studies.

As a well-studied system, the central melanocortin system plays important roles in both the regulation of appetite and the stress response and shows neuroanatomical and functional conservation between birds and mammals (Boswell and Dunn, 2015). The melanocortin system contains two separate neuronal populations that generate ligands for MC3R and MC4R: one population synthesizes AgRP which functions as an antagonist to melanocortin receptors and the second population releases POMC, a precursor of α, β, and γ-melanocyte-stimulating hormones (MSH) that are agonists for MC3R and MC4R (Cone, 2005). Although NPY and AgRP are highly co-localized in the ARC and function similarly in the regulation of food intake, their gene expression regulation can be dissociated following exposure to stress, indicating they may have separate functions in stress adaptation. In previous studies, AgRP mRNA was down-regulated in the ARC of rats following foot-shock (Kas et al., 2005) and both acute and repeated restraint stress treatment (Chagra et al., 2011), which is consistent with results in the present study. Since AgRP is known to be an antagonist for MC4R which is one of the primary receptors mediating the anorexigenic effect of the melanocortin system

(Adan et al., 2006) and the stress response and HPA axis activation(Yamano et al., 2004), stress-induced reduction of AgRP in the ARC and increased MC4R in the PVN may thus contribute to the inhibition of appetite, which is likely an adaptive response of the central melanocortin system. There is also evidence to support that MC4R has anatomical and functional interactions with the CRF system and that CRF is a downstream mediator of the melanocortin system (Lu et al., 2003; Yamano et al., 2004). Collectively, results suggest that MC4R may act synergistically with CRF to override NPY signaling in stress-treated LWS.

Hypothalamic CRF-ergic tone can be magnified by CART, which was increased and decreased in the PVN of the LWS and HWS, respectively, in response to stress. As an anorexigenic factor, CART is involved in appetite regulation and HPA actions in response to a stress-inducing challenge (Koylu et al., 1997). The behavior of CRF is augmented by CART, indicating that there is a positive correlation in the activity of these two neuropeptides (Smith et al., 2004). Astressin B treatment attenuated the activation of CART and the release of ACTH and CORT induced by CRF, suggesting that the regulatory effects of CART in the HPA axis is mediated by central CRF signaling (Smith et al., 2004). Thus, the distinct differences in the hypothalamic expression pattern of CART between the lines may contribute to differences in hypothalamic CRF signaling. Due to the CRF-dependent effect of CART, we may hypothesize that increased expression of CART may contribute to greater CRF-ergic tone in the PVN of stressed LWS.

In conclusion, exposure to a combination of thermal and nutritional stress at hatch renders chicks from an anorexic but not obese line refractory to the food intake-stimulating effects of NPY at a later age. Gene expression analysis revealed distinct changes in response to stress that were different between the lines within hypothalamic nuclei. In general, most stress-induced changes occurred in the LWS chicks in the PVN, where there was increased expression of mRNAs encoding several anorexigenic factors and associated receptors,

including CRF, CRFR1, CRFR2, MC4R, and UCN3. These findings suggest that refractoriness of the food intake response to NPY in stressor-exposed LWS may result from overriding anorexigenic tone in the PVN associated with CRF signaling. This was supported by results showing that the orexigenic effect of NPY was restored when stressed LWS chicks were injected with a CRF receptor antagonist, astressin, before stress exposure, demonstrating that CRF receptor-mediated signaling pathways are the major contributors to stress-induced anorexia in LWS chicks. These results provide insights into the molecular basis of eating disorders and suggest that CRF signaling in the PVN may exacerbate the anorexic phenotype in the presence of environmental stressors.

Table 2.1 Primers for real-time PCR[1]

Gene	Accession No.	Sequences (forward/reverse)
β-actin	NM_205518.1	GTCCACCGCAAATGCTTCTAA / TGCGCATTTATGGGTTTTGTT
AgRP	XM_004950992.1	GGTTCTTCAACGCCTTCTGCTA / TTCTTGCCACATGGGAAGGT
CART	XM_003643097.2	GCTGGAGAAGCTGAAGAGCAA / GGCACCTGCCCGAACTT
CRF	NM_001123031.1	TCAGCACCAGAGCCATCACA / GCTCTATAAAAATAAAGAGGTGACATCAGA
CRFR1	NM_204321.1	CTGCTGTCCTTGCTGGGAAT / ATCCTCCCCCGGATTGAC
CRFR2	NM_204454.1	GGATCAAATACAACACCACAAAAAAT / GGCCCATGTCCCATTGC
Ghrelin	NM_001001131.1	GAAGCACTGCCTAACGAAGACA / GGATGCTGAGAAGGAGAATTCCT
GHSR	AB095994.1	TCTGCGAGCGAAGGTGATC / AGACGGCCCAGAGGATGAG
LEPR	NM_204323.1	GCAAGACCCTCTCCCTTATCTCT / TCTGTGAAAGCATCATCCTGATCT
MC3R	XM_004947236.1	GCCTCCCTTTACGTTCACATGT / GCTGCGATGCGCTTCAC
MC4R	NM_001031514.1	CCTCGGGAGGCTGCTATGA / GATGCCCAGAGTCACAAACACTT
NPY	NM_205473.1	CATGCAGGGCACCATGAG / CAGCGACAAGGCGAAAGTC
NPYR1	NM_001031535.1	TAGCCATGTCCACCATGCA / GGGCTTGCCTGCTTTAGAGA
NPYR5	NM_001031130.1	GGCTGGCTTTGTGGGAAA / TTGTCTTCTGCTTGCGTTTTGT
Orexin	NM_204185.2	CCAGGAGCACGCTGAGAAG / CCCATCTCAGTAAAAGCTCTTTGC
ORXR2	NM_001024584.1	TGCGCTACCTCTGGAAGGA / GCGATCAGCGCCCATTC
Mesotocin	XM_001231491.3	TGGCTCTCTCCTCAGCTTGTTAT / GGCACGGCACGCTTACC
POMC	NM_001031098.1	GCCAGACCCCGCTGATG / CTTGTAGGCGCTTTTGACGAT
UCN3	XM_001231710.2	GGGCCTTCCGTCTCTACAATG / GGTGAGGGCCGTGTTGAG

[1]Primers were designed with Primer Express 3.0 (Applied Biosystems). Abbreviations: agouti-related peptide (AgRP), cocaine and amphetamine-regulated transcript (CART), corticotropin-releasing factor (CRF), CRF receptor sub-types 1 and 2 (CRFR1 and CRFR2, respectively), growth hormone secretagogue receptor (GHSR), leptin receptor (LEPR), melanocortin receptors 3 and 4 (MC3R and MC4R, respectively), neuropeptide Y (NPY), NPY receptor sub-types 1 and 5 (NPYR1 and NPYR5, respectively), orexin receptor 2 (ORXR2), pro-opiomelanocortin (POMC), urocortin 3 (UCN3).

Table 2.2 Anorexigenic neuropeptide and receptor mRNA in hypothalamic nuclei[1]

Effect	CART	GHSR	LEPR	CRF	CRFR1	CRFR2	UCN3	Ghrelin	MC4R	Mesotocin	MC3R	POMC
Line												
HWS	0.86±0.07	0.77±0.05	1.60±0.08	2.16±0.13	1.02±0.04	0.98±0.08	0.12±0.03	0.72±0.07	1.07±0.08	1.08±0.14	1.76±0.20	18.54±1.78
LWS	0.70±0.07	0.88±0.05	1.07±0.08	0.79±0.13	1.09±0.04	1.28±0.07	0.33±0.03	1.07±0.07	0.92±0.07	1.89±0.15	2.22±0.18	9.66±1.61
***P*-value**	<.0001	0.1303	<.0001	<.0001	0.0178	0.0012	<.0001	0.0009	0.1274	<.0010	0.0933	0.0063
Treatment												
Control	0.83±0.07	0.72±0.05	1.27±0.08	1.38±0.13	0.98±0.04	0.90±0.08	0.17±0.03	0.88±0.07	0.96±0.08	1.06±0.14	1.75±0.19	12.42±1.68
Stress	0.73±0.06	0.92±0.05	1.39±0.07	1.54±0.12	1.12±0.04	1.33±0.07	0.27±0.03	0.90±0.06	1.02±0.07	1.83±0.14	2.28±0.20	15.08±1.70
***P*-value**	0.0040	0.0055	0.2412	0.0123	0.0838	0.0007	0.0008	0.8965	0.5036	0.0011	0.6015	0.7131
Nucleus												
ARC	0.47±0.10[b]	NM	1.17±0.08	0.25±0.16[b]	2.27±0.06[a]	1.63±0.10[a]	NM	NM	0.38±0.07	NM	5.02±0.24[a]	46.73±2.22[a]
LH	0.36±0.10[bc]	NM	NM	0.22±0.17[b]	0.42±0.05[c]	0.22±0.11[c]	0.03±0.03[b]	NM	NM	NM	0.22±0.30[b]	0.23±2.62[b]
PVN	2.74±0.11[a]	0.91±0.05	NM	6.83±0.21[a]	1.28±0.05[b]	1.41±0.09[ab]	0.82±0.04[a]	0.90±0.05	1.67±0.07	1.45±0.10	1.15±0.26[b]	1.04±2.26[b]
VMH	0.16±0.08[c]	0.75±0.05	1.50±0.08	0.13±0.18[b]	0.42±0.06[c]	1.21±0.10[b]	0.01±0.03[b]	NM	NM	NM	0.42±0.29[b]	0.59±2.45[b]
***P*-value**	<.0001	0.0191	0.0038	<.0001	<.0001	<.0001	<.0001	.	<.0001	.	<.0001	<.0001
Interactions (*P*-values)												
Line * treatment	<.0001	0.4735	0.1527	0.0256	0.9172	<.0001	0.0006	0.8367	0.0004	0.1298	0.3099	0.8064
Line * nucleus	<.0001	0.0400	0.0093	<.0001	0.2453	0.6802	<.0001	.	0.8410	.	0.4418	<.0001
Treatment * nucleus	<.0001	0.5054	0.4695	0.0012	0.0164	0.1329	0.0001	.	0.5156	.	0.9129	0.9139
Line * treatment * nucleus	<.0001	0.2281	0.9733	0.0075	<.0001	0.0496	<.0001	.	0.0015	.	0.1383	0.9845

[1]Relative quantity values were analyzed by ANOVA and the model included the main effects of genetic line (low weight-selected; LWS, and high weight-selected; HWS), treatment, hypothalamic nucleus (arcuate nucleus; ARC, lateral hypothalamic area; LH, paraventricular nucleus; PVN, and ventromedial hypothalamus; VMH), and the interactions between them. Data are expressed as means ± standard error with corresponding *P*-values for main effects and the interactions (n =10 to 15 per group). Unique letters denote a difference within the gene and effect, $P < 0.05$ (Tukey's test). Abbreviations: cocaine and amphetamine-regulated transcript (CART), growth hormone secretagogue receptor (GHSR), leptin receptor (LEPR), corticotropin-releasing factor (CRF), CRF receptor sub-types 1 and 2 (CRFR1 and CRFR2, respectively), urocortin 3 (UCN 3), melanocortin receptors 3 and 4 (MC3R and MC4R, respectively), and pro-opiomelanocortin (POMC). NM indicates that the respective mRNA were not measured in those nuclei.

Table 2.3 Orexigenic neuropeptide and receptor mRNA in hypothalamic nuclei

Effect	NPY	NPYR1	ORXR2	NPYR5	Orexin	AGRP
Line						
HWS	2.20 ±0.52	1.19 ±0.05	0.96 ±0.10	0.59 ±0.03	1.46 ±0.46	70.38 ±11.63
LWS	6.66 ±0.51	1.01 ±0.04	1.14 ±0.10	0.77 ±0.03	3.22 ±0.58	38.14 ±10.86
***P*-value**	<.0001	0.0083	0.2027	<.0001	0.0529	0.4652
Treatment						
Control	3.88 ±0.54	1.06 ±0.05	0.87 ±0.10	0.62 ±0.03	1.11 ±0.58	56.27 ±11.28
Stress	4.90 ±0.49	1.12 ±0.04	1.21 ±0.07	0.73 ±0.02	2.99 ±0.46	49.69 ±11.22
***P*-value**	0.0808	0.2385	0.0242	0.0061	0.0286	0.3271
Nucleus						
ARC	15.46 ±0.73[a]	1.68 ±0.06[a]	NM	0.95 ±0.04[a]	NM	274.48 ±17.66[a]
LH	NM	0.65 ±0.06[d]	0.99 ±0.10	0.44 ±0.03[b]	0.37 ±2.17	0.20 ±16.68[b]
PVN	1.16 ±0.58[b]	1.17 ±0.07[b]	NM	0.95 ±0.04[a]	NM	1.22 ±14.07[b]
VMH	0.86 ±0.58[b]	0.88 ±0.06[c]	1.11 ±0.10	0.38 ±0.04[b]	NM	0.37 ±14.99[b]
***P*-value**	<.0001	<.0001	0.3368	<.0001	.	<.0001
Interactions (*P*-values)						
Line * treatment	0.0388	0.6641	0.2843	0.0246	0.8200	0.0089
Line * nucleus	<.0001	0.1370	0.0448	0.0027	.	0.6858
Treatment * nucleus	0.1229	0.1093	0.8359	0.4575	.	0.4719
Line * treatment * nucleus	0.0480	0.1669	0.3394	0.0126	.	0.0006

[1]Relative quantity values were analyzed by ANOVA and the model included the main effects of genetic line (low weight-selected; LWS, and high weight-selected; HWS), treatment, hypothalamic nucleus (arcuate nucleus; ARC, lateral hypothalamic area; LH, paraventricular nucleus; PVN, and ventromedial hypothalamus; VMH), and the interactions between them. Data are expressed as means ± standard error with corresponding *P*-values for main effects and the interactions (n =10 to 15 per group). Unique letters denote a difference within the gene and effect, $P < 0.05$ (Tukey's test). Abbreviations: neuropeptide Y (NPY), NPY receptor sub-types 1 and 5 (NPYR1 and NPYR5, respectively), orexin receptor 2 (ORXR2), and agouti-related peptide (AgRP). NM indicates that the respective mRNA were not measured in those nuclei.

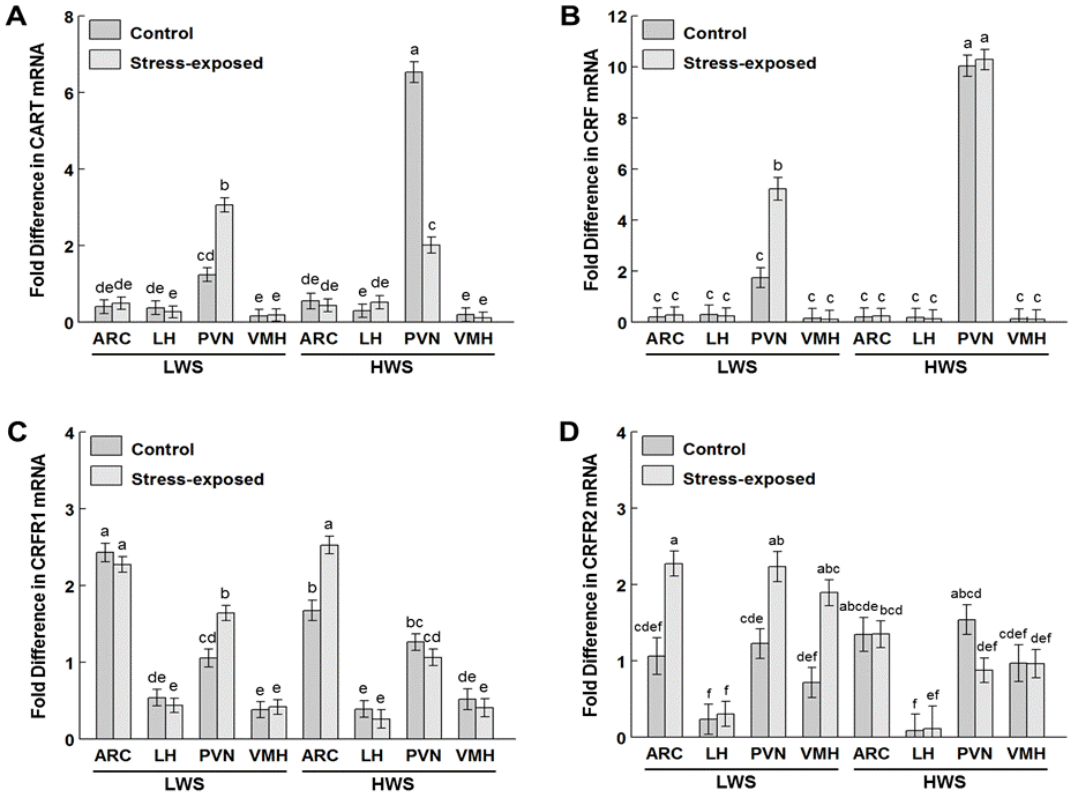

Fig. 2.1 Significant interactions among genetic line, treatment, and hypothalamic nuclei for the expression of genes. Interactions of genetic line (low weight-selected; LWS, and high weight-selected; HWS), treatment, and hypothalamic nucleus (arcuate nucleus; ARC, lateral hypothalamic area; LH, paraventricular nucleus; PVN, and ventromedial hypothalamus; VMH) for A) cocaine and amphetamine-regulated transcript (CART), B) corticotropin-releasing factor (CRF), C) CRF receptor sub-type 1 (CRFR1), and D) CRFR2 mRNAs. Values represent means ± SEM (n = 10 to 15). The interactions were separated using Tukey's test; bars with unique letters indicate a difference at $P < 0.05$.

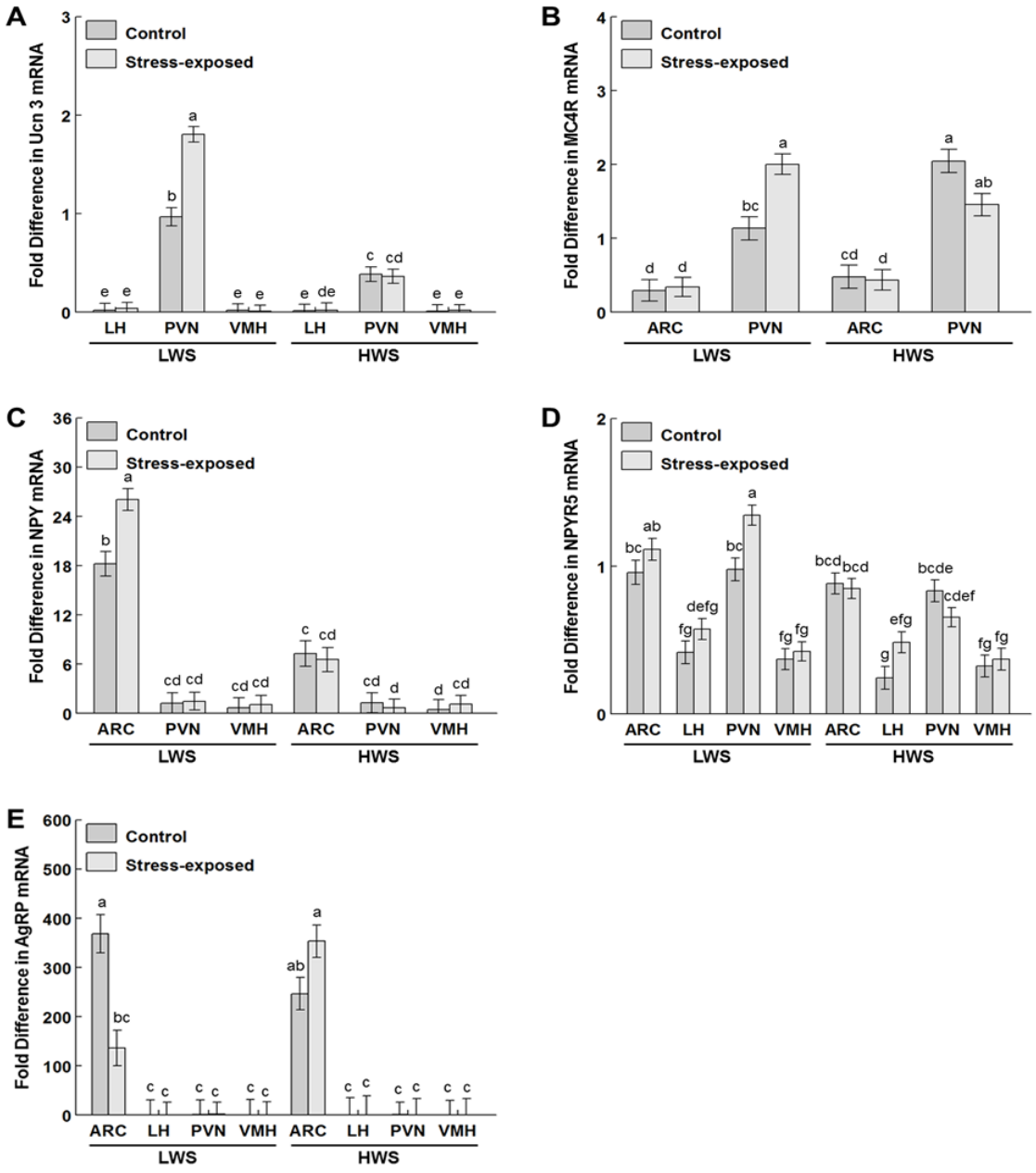

Fig. 2.2 Significant interactions among genetic line, treatment, and hypothalamic nuclei for the expression of genes. Interactions of genetic line (low weight-selected; LWS, and high weight-selected; HWS), treatment, and hypothalamic nucleus (arcuate nucleus; ARC, lateral hypothalamic area; LH, paraventricular nucleus; PVN, and ventromedial hypothalamus; VMH) for A) urocortin 3 (UCN3), B) melanocortin receptor 4 (MC4R), C) neuropeptide Y (NPY), D) NPY receptor sub-type 5 and E) agouti-related peptide (AgRP) mRNAs. Values represent means ± SEM (n = 10 to 15).

The interactions were separated using Tukey's test; bars with unique letters indicate a difference at $P < 0.05$.

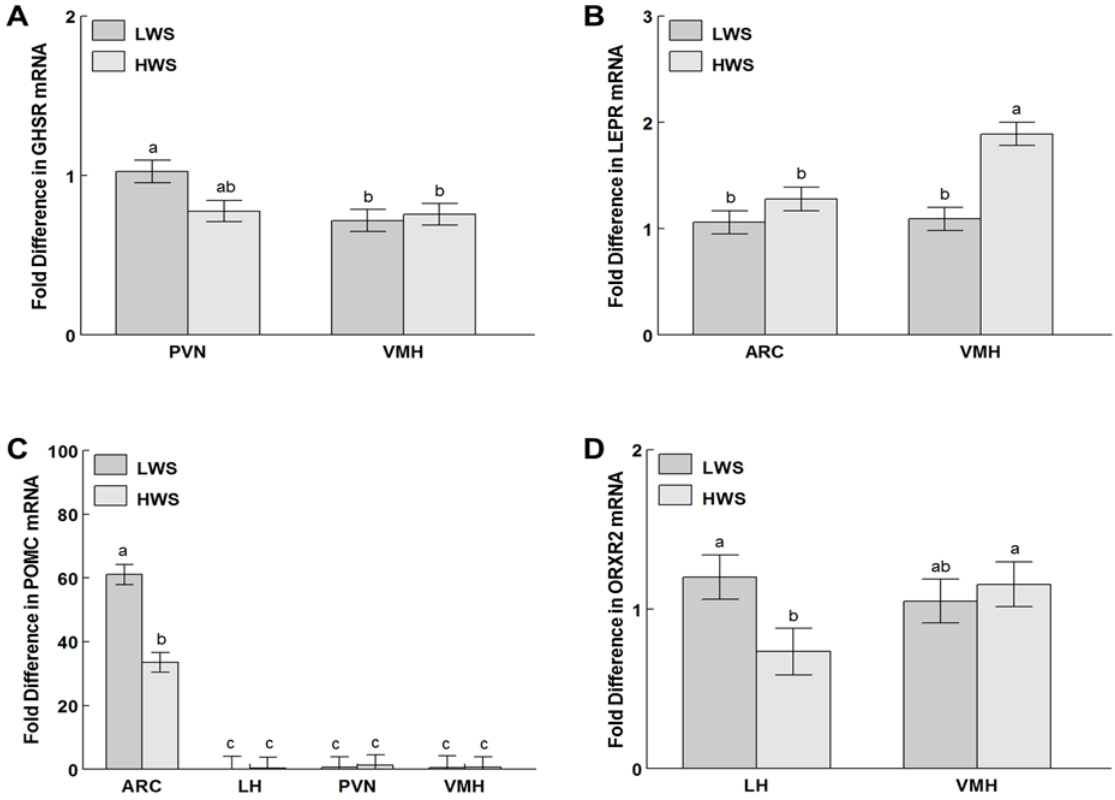

Fig. 2.3 Significant interactions between genetic line and hypothalamic nuclei for the expression of genes. Interactions of genetic line (low weight-selected; LWS, and high weight-selected; HWS) and hypothalamic nucleus (arcuate nucleus; ARC, lateral hypothalamic area; LH, paraventricular nucleus; PVN, and ventromedial hypothalamus; VMH) for A) growth hormone secretagogue receptor (GHSR), B) leptin receptor (LEPR), C) pro-opiomelanocortin (POMC) and D) orexin receptor 2 (ORXR2) mRNAs. Values represent means ± SEM (n = 10 to 15). The interactions were separated using Tukey's test; bars with unique letters indicate a difference at $P < 0.05$.

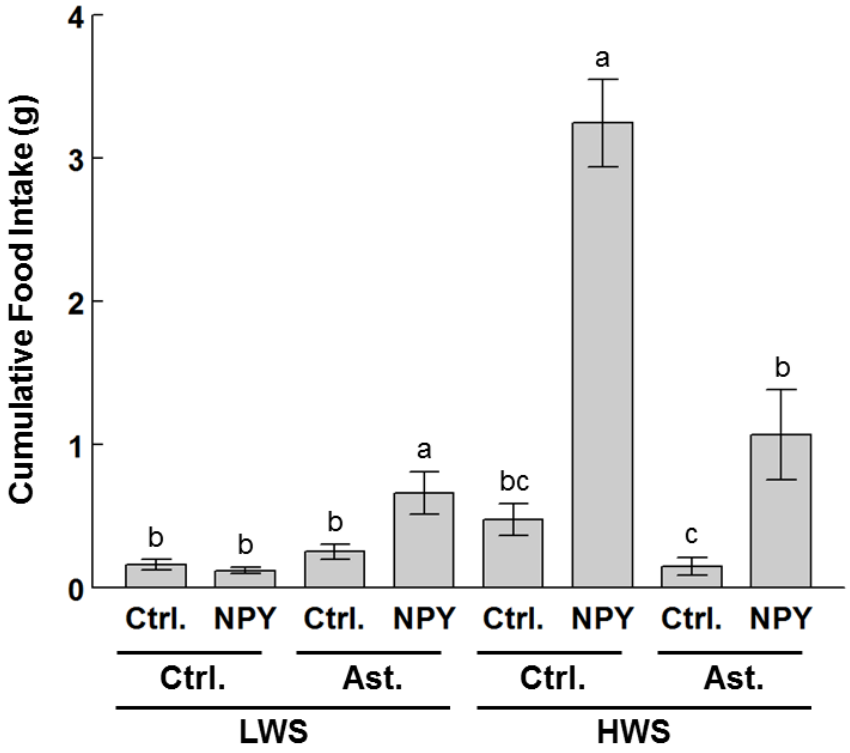

Fig. 2.4 Interaction of astressin and neuropeptide Y treatments on cumulative food intake at 30 minutes post-NPY injection in the LWS and HWS. Ctrl. = vehicle-injected group. Different letters within genetic line, $P < 0.05$, Tukey's Test. Values are expressed as means ± SEM (n =18 to 20 per group).

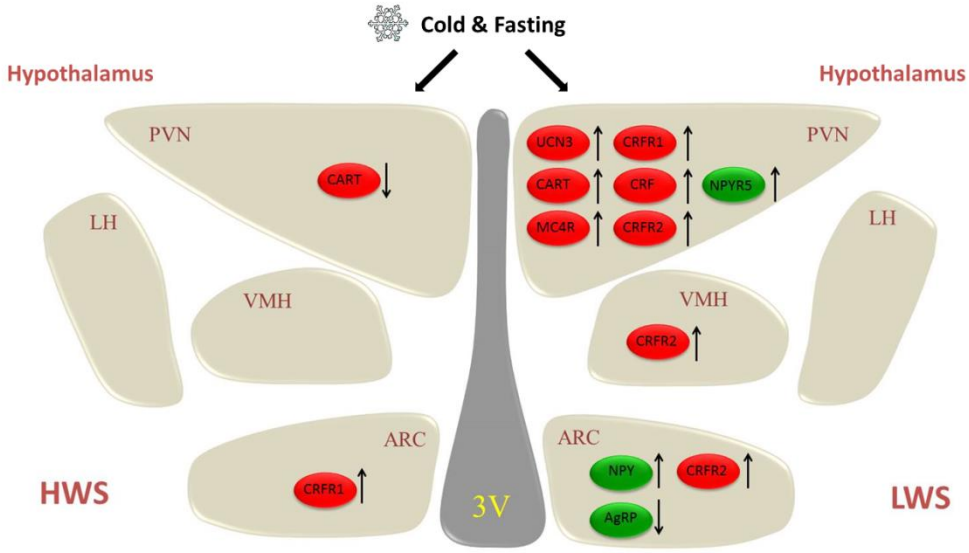

Fig. 2.5 Graphical summary of three-way interactions of on mRNA abundance ($P <$ 0.05). "↑" and "↓" indicate mRNA increase or decrease, respectively, within line and nucleus relative to the non-stressed state. Red and green indicate anorexigenic and orexigenic factors, respectively. Gene abbreviations: agouti-related peptide (AgRP), cocaine and amphetamine regulated transcript (CART), corticotropin-releasing factor (CRF), CRF receptor sub-types 1 and 2 (CRFR1 and CRFR2, respectively), melanocortin receptors 4 (MC4R), neuropeptide Y (NPY), NPY receptor sub-types 1 and 5 (NPYR1 and NPYR5, respectively) and urocortin 3 (UCN3).

Chapter 3: Hypothalamic mechanisms associated with corticotropin-releasing factor-induced anorexia in chicks

Abstract

Central administration of corticotropin-releasing factor (CRF), a 41-amino acid peptide, is associated with potent anorexigenic effects in rodents and chickens. However, the mechanism underlying this effect remains unclear. Hence, the objective of the current study was to begin to elucidate the hypothalamic mechanisms that mediate CRF-induced anorexia in chicks. Four day-old chicks that were intracerebroventricularly injected with 0.02 nmol of CRF ate less than vehicle-injected chicks but did not differ in water intake at 30 minutes post-injection. At 1 hour post-injection, the CRF-injected chicks had more c-Fos immunoreactive cells in the arcuate nucleus (ARC), dorsomedial nucleus (DMN), ventromedial hypothalamus (VMH), and paraventricular nucleus (PVN) of the hypothalamus than vehicle-treated chicks. CRF injection was associated with decreased whole hypothalamic mRNA abundance of neuropeptide Y receptor sub-type 1 (NPYR1) at 1 hour post-injection. In the ARC, CRF-injected chicks expressed more CRF and CRF receptor sub-type 2 (CRFR2) mRNA but fewer quantities of agouti-related peptide (AgRP), NPY, and NPYR1 mRNA than vehicle-injected chicks. CRF-treated chicks expressed greater amounts of CRFR2 and mesotocin mRNA than vehicle chicks in the PVN and VMH, respectively. In the DMN, CRF injection was associated with reduced NPYR1 mRNA. In conclusion, the results provide insights into understanding CRF-induced hypothalamic actions and suggest that the anorexigenic effect of CRF involves increased CRFR2-mediated signaling in the ARC and PVN that overrides the effects of NPY and other orexigenic factors.

Keywords: anorexia, appetite, chick, hypothalamus, obesity

1. Introduction

Corticotropin-releasing factor (CRF), a 41-amino acid peptide, is an essential component of the hypothalamo-pituitary-adrenal (HPA) axis, stimulating the secretion of pituitary adrenocorticotropin (ACTH) and in turn the release of corticosterone or cortisol from the adrenal glands (Vale et al., 1981; Gillies and Grossman, 1985). CRF is associated with multiple, diverse physiological functions, such as immunity (Blalock, 1989; Trout and Mashaly, 1994), reproduction (Sirinathsinghji et al., 1983; Astheimer et al., 1992), stress responses (Luo et al., 1994) and arousal and locomotor activity (Morley and Levine, 1982; Mönnikes et al., 1992).

Over the past few decades, many studies have demonstrated that CRF regulates feeding behavior. Administration of CRF into the central nervous system (CNS) suppresses food intake and increases energy expenditure in mammals (Dunn and Berridge, 1990), fish (Bernier and Peter, 2001; Volkoff et al., 2005), amphibians (Crespi and Denver, 2004) and birds (Furuse et al., 1997; Denbow et al., 1999; Ohgushi et al., 2001; Zhang et al., 2001; Cline et al., 2009). The paraventricular nucleus (PVN) is the major site of synthesis and release of CRF in the hypothalamus (Mönnikes et al., 1992; Heinrichs et al., 1993; Wang et al., 2011a). The CRF antagonist, α-helical CRH, attenuated the anorectic effects of CRF injection into the PVN but not other hypothalamic nuclei such as the ventromedial hypothalamus (VMH) (Kalra et al., 1999b).

Cellular effects of CRF are mediated via CRF receptor sub-types 1 (CRFR1) and 2 (CRFR2) (Perrin et al., 1993; Lovenberg et al., 1995b). The CRFR2 is regarded as the

primary receptor through which the anorexigenic effects of CRF are mediated, as demonstrated with pharmacological inhibitors (Pelleymounter et al., 2000; Cullen et al., 2001) and gene deletion experiments (Bradbury et al., 2000). Besides the direct activation of anorexigenic networks in the CNS, there are several proposed mechanisms for CRF-induced anorexia. For instance, intracerebroventricular (ICV) injection of CRF leads to an inhibition of gastric emptying (Stengel and Taché, 2010) and hyperglycemia (Brown et al., 1982), both of which lead to a reduction in food intake. Specifically, the slowing of gastric emptying causes the accrual of food in the stomach and the transmission of satiety signals to the brain (Phillips and Powley, 1996). Meanwhile, increased glucose can be sensed by hypothalamic neurons and the pancreas, leading to an induction of satiety (Levin, 2006; Cha et al., 2008).

In chickens, the avian species for which the most information exists, CRF inhibits food intake (Furuse et al., 1997; Denbow et al., 1999; Ohgushi et al., 2001; Zhang et al., 2001; Cline et al., 2009) and increases plasma corticosterone (Ogino et al., 2014). CRF may also mediate the anorexigenic effect of other neuropeptides, such as ghrelin (Saito et al., 2005), α- and β-melanocyte-stimulating hormone (α- and β-MSH) (Tachibana et al., 2007; Kamisoyama et al., 2009), leptin (Gardner et al., 1998), and cholecystokinin (CCK) (Tachibana et al., 2012).

The hypothalamus is thought to be the ultimate regulator of energy intake and expenditure, as it integrates a diverse array of peripheral and central signals (Maniam and Morris, 2012; Parker and Bloom, 2012; Sinha and Jastreboff, 2013). Within the hypothalamus, several important nuclei and many neuropeptides constitute a complex

network to regulate feeding behavior. The greatest amounts of chicken CRF immunoreactivity were observed in several CNS areas that are involved in food intake control, such as the PVN of the hypothalamus, the dorsal vagal complex, and the parabrachial area (Richard et al., 2004). To date, although the effect of CRF on feeding behavior in chickens and other species has been replicated many times, the CRF-induced hypothalamic mechanisms are still unclear. Therefore, the objective of this study was to identify the hypothalamic nuclei and associated signaling factors that mediate the anorexigenic effects of CRF.

2. Materials and methods

2.1 Animals

Day-of-hatch Cobb-500 chicks (broiler-type) were obtained from a local hatchery. Chicks were caged individually with 30 ± 2 ℃ and 50 ± 5 % relative humidity and 24 hours of light. Chicks were handled daily to adapt to handling and minimize stress, with ad libitum access to food (21.5 % crude protein and 3,000 kcal ME/kg) and water. Chicks had visual and auditory contact with each other in the individual cages. All experiments were conducted at 4 days post-hatch and there were 12 chicks in each treatment group. 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.

2.2 Intracerebroventricular injection procedure

On the day of the experiment, chicks were injected intracerebroventricularly (ICV; lateral ventricle) using a method that does not appear to induce physiological stress (Davis et al., 1979). The head of the chick was briefly inserted into a restraining device that left the cranium exposed to allow for free-hand injection. Injection coordinates were 2 mm anterior to the coronal suture, .75 mm lateral from the sagittal suture, and 1.5 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 5 seconds post-injection to reduce backflow. Chicks were assigned to treatments at random. Ovine CRF (Sigma, St. Louis, MO, USA) was dissolved in artificial cerebrospinal fluid (Anderson and Heisley, 1972) as a vehicle for a total injection volume of 5 µl with 0.1 % Evans Blue dye to facilitate injection site localization. After data collection, the chick was decapitated and its head sectioned along the frontal plane to determine the site of injection. Any chick without dye present in the lateral ventricle system was eliminated from analysis. Sex of chicks was determined visually by dissection at the time of decapitation.

2.3. Experiment 1: food and water intake

Chicks, fasted for 180 minutes, were randomly assigned to receive either vehicle or 0.02 nmol CRF, dose based on (Zhang et al., 2001) and (Cline et al., 2009), by ICV injection (n=10 in each group). After injection, chicks were returned to their individual home cages and given ad libitum access to both food and water. Water weight (g) was converted to volume (ml; 1 g = 1 ml). Food and water intake were

recorded (0.01 g) at 30 minutes post-injection. Data were analyzed using analysis of variance (ANOVA), with the statistical model including the main effect of treatment. Statistical significance was set at $P < 0.05$ for all experiments.

2.4 Experiment 2: c-Fos immunohistochemistry

Chicks were randomly assigned to receive either 0 or 0.02 nmol CRF via ICV injection (n=12 for each group). Chicks were provided ad libitum access to food and water until 180 minutes prior to injection, after which food was withheld to prevent c-Fos immunoreactivity associated with food consumption. Sixty minutes post-injection as this is the time expected for the most robust c-Fos expression (Müller et al., 1984), chicks were deeply anesthetized with sodium pentobarbital via cardiopuncture, then perfused via the carotid artery with ice-cold 0.9 % NaCl followed by 4 % paraformaldehyde in 0.1 M phosphate buffer (PB) containing 0.2 % picric acid at pH 7.4. Brains were removed from skulls and post-fixed for 60 minutes in the same solution, after which they were blocked and placed through a series of graded sucrose incubations, consisting of 20, 30 and 40 % in 0.1 M PB, until they sank. Several 60 µm coronal sections that contained appetite-related nuclei based on anatomies described by Kuenzel (Kuenzel and Masson, 1988) were collected in 0.02 M phosphate-buffered saline (PBS) containing 0.1% sodium azide using a cryostat at –15 ℃. Sections were processed immediately after collection. Procedures for c-Fos immunohistochemistry were performed as described (Hagen et al., 2013) using rabbit polyclonal anti-c-Fos at a dilution of 1:20,000 (K-25, Santa Cruz, CA, USA). Anatomy was confirmed and a digital micrograph captured for each section. Overlays

containing the respective nuclei boundaries were digitally merged with micrographs and the number of c-Fos immunoreactive cells within each respective nucleus counted by a technician blind to treatment. Data were analyzed by ANOVA and the model included the main effect of CRF treatment within each nucleus.

2.5 Experiment 3: Whole hypothalamic mRNA abundance of appetite-associated factors

Chicks were randomly assigned to receive either 0 or 0.02 nmol CRF via ICV injection (n=12 for each group). Chicks were provided ad libitum access to food and water until 180 minutes prior to injection, at which time food was withheld to prevent molecular changes associated with differences in food consumption. At sixty minutes post-injection, chicks were deeply anesthetized with sodium pentobarbital via cardiopuncture, decapitated, and brains removed. The whole upside-down brain was snap frozen in liquid nitrogen for 9 seconds. This duration freezes the outermost portion of the brain, providing firmness while leaving the center unfrozen to permit dissection without shattering. Cuts were made visually as per the following anatomy: perpendicular to the midline suture a cut was made at the septopallio-mesencephalic tract and at the third cranial nerves. 1.8 mm parallel to the midline two cuts were made and finally the dorsal cut was made from the anterior commissure to 0.8 mm ventral to the posterior commissure (McConn et al., 2014). This block (comprised primarily of the hypothalamus) was immediately stored in RNAlater (Qiagen). Hypothalamus was homogenized using 5 mm stainless steel beads (Qiagen, Valencia, CA, USA), Isol Lysis reagent (5-Prime, Gaithersburg, MD, USA) and a Tissue Lyser

II (Qiagen) and total RNA was extracted following the manufacturer's instructions (5-Prime). The RNeasy Mini Kit (Qiagen) and RNase-free DNase I (Qiagen) were then used for total RNA purification. The concentration and purity of total RNA was assessed by spectrophotometry at 260/280/230 nm with a Thermo NanoDrop 2000 (Thermo Fisher Scientific Inc., West Palm Beach, FL, USA). RNA integrity was verified using Biorad's automated electrophoresis system Experion (RNA StdSens analysis kit), according to the manufacturer's instructions.

First-strand cDNA was synthesized in 20 µl reactions from 200 ng of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's instructions. Reactions were performed under the following conditions: 25 ℃ for 10 minutes, 37 ℃ for 120 minutes and 85 ℃ for 5 minutes. Primers for real-time PCR were designed with Primer Express 3.0 software (Applied Biosystems) (Table 3.1) and validated for amplification efficiency before use (95–105%). Real-time PCR reactions were performed in 10 µl reactions that contained 5 µl Fast SYBR Green Master Mix (Applied Biosystems), 0.5 µl primers (0.25 µl of 5 µM forward primer and 0.25 µl of 5 µM reverse primer), 1.5 µl nuclease-free water, and 3 µl 10-fold diluted cDNA using a 7500 Fast Real-Time PCR System (Applied Biosystems). The real-time PCR was performed under the following conditions: 95 ℃ for 20 seconds and 40 cycles of 90 ℃ for 3 seconds plus 60 ℃ for 30 seconds. A dissociation step consisting of 95 ℃ for 15 seconds, 60 ℃ for 1 minute, 95 ℃ for 15 seconds and 60 ℃ for 15 seconds was performed at the end of each PCR reaction to ensure amplicon specificity.

Real-time PCR data were analyzed using the ΔΔCT method with β-actin as the reference gene and the average of the chicks in the vehicle group as the calibrator sample (Livak and Schmittgen, 2001). Relative quantities calculated as $2^{-\Delta\Delta CT}$ were used for statistical analysis. For ANOVA, the statistical model included the main effect of treatment, sex, and their interactions. The mRNA abundance was not affected by sex for any gene measured, thus we removed sex from the model.

2.6 Experiment 4: mRNA abundance of appetite-associated factors in hypothalamic nuclei

The design was the same as for Experiment 3 except for that at sixty minutes post-injection, chicks were deeply anesthetized with sodium pentobarbital via cardiopuncture and then perfused via the carotid artery with 2.5 mL of RNA stabilizing buffer (16.7 mM sodium citrate, 13.3 mM EDTA, and 3.5 M ammonium sulfate; pH = 5.2). Within 30 minutes of perfusion, brains were sectioned in a cryostat at -10 ℃ into 500 μm thick coronal sections in the direction from rostral to caudal: lateral hypothalamic area (LHA), PVN and VMH were collected at plate 8.0, 7.4, and 6.8 respectively, and the arcuate nucleus (ARC) and dorsomedial hypothalamic nucleus (DMN) were collected at plate 5.4 based on described anatomy (Kuenzel and Masson, 1988). Punches were collected using sterile disposable biopsy punch instruments (1 mm, Braintree Scientific Inc., Braintree, MA). The punches were immediately placed in RNA lysis buffer with 1% beta-mercaptoethanol (Norgen Biotek, Thorold, ON, Canada), vortexed, snap-frozen in liquid nitrogen, and stored at

-80 ℃. The remaining brain section was photographed and punch accuracy verified via the overlays containing the respective nuclei boundaries.

The punches were vortexed vigorously for 30 seconds and incubated at room temperature for 5 minutes before adding 70% molecular biology-grade ethanol, and total RNA was isolated following the manufacturer's instructions for the Total RNA Purification Micro Kit (Norgen Biotek). The concentration and purity of total RNA was assessed by spectrophotometry at 260/280/230 nm with a Thermo NanoDrop 2000 (Thermo Fisher Scientific Inc., West Palm Beach, FL, USA). RNA integrity was verified using Biorad's automated electrophoresis system Experion (RNA StdSens analysis kit), according to the manufacturer's instructions.

Subsequent reactions were performed under the same conditions as those described in Experiment 3 except that 100 ng of total RNA was used for the reverse transcription and 5-fold diluted cDNA was used for the real-time PCR. Data were analyzed the same as for Experiment 3 except that the statistical model included the main effect of CRF treatment within nucleus.

3. Results

3.1 Food and water intake

At 30 minutes post-injection, CRF-injected chicks ate about 75% less than vehicle chicks ($P = 0.0019$) while water intake was not affected by CRF injection ($P = 0.1852$) (Fig. 3.1).

3.2 c-Fos immunohistochemistry

The number of c-Fos immunoreactive cells in chicks treated with CRF increased by about 740% (P = 0.0044) and 390% (P = 0.0057) over the vehicle-injected chicks in the ARC and DMN, respectively (Fig. 3.2). In the VMH and PVN, immunoreactive cell numbers increased 2,500% (P = 0.0004) and 1,200% (P <.0001), respectively, in CRF-treated compared to vehicle-injected chicks. The c-Fos expression in the LHA was not affected by CRF injection.

3.3 Whole hypothalamic mRNA abundance of appetite-associated factors

Not one of the anorexigenic-associated factors that was measured was differentially expressed in this study (Fig. 3.3A). Among all measured orexigenic factors, only the mRNA abundance of neuropeptide Y receptor sub-type 1 (NPYR1) was affected by CRF injection, with less whole hypothalamic expression in CRF-injected than vehicle chicks at 1 hour post-injection (P = 0.0492) (Fig. 3.3B).

3.4 mRNA abundance of appetite-associated factors in hypothalamic nuclei

In the ARC, CRF-injected chicks expressed greater CRF (P = 0.0352) and CRF receptor sub-type 2 (CRFR2) (P = 0.0463) mRNA (more than four-fold up-regulation) but less agouti-related peptide (AgRP) (P = 0.0014), neuropeptide Y (NPY) (P = 0.0079), and NPYR1 (P = 0.0146) mRNA than vehicle chicks (Fig. 3.4A). In the PVN, CRF-injected chicks expressed more CRFR2 mRNA than vehicle-treated chicks (P = 0.0022) and in the DMN, NPYR1 mRNA was down-regulated in CRF-injected chicks (P = 0.0105) (Fig. 3.4B and C, respectively). In the VMH, CRF-injected chicks had almost four times greater quantities of mesotocin (MT) mRNA than chicks from the vehicle group (P = 0.0094) (Fig. 3.4D).

4. Discussion

CRF is a potent anorexigenic factor in chickens (Furuse et al., 1997; Denbow et al., 1999; Tachibana et al., 2006; Cline et al., 2009). Central administration of 2-200 pmol of CRF suppressed food intake dose-dependently in 2 day-old broiler chicks that were fasted for 3 hours before injection (Furuse et al., 1997). A dose range of 1-4 nmol was effective at dose-dependently reducing food intake in leghorn and broiler cockerels, at 7 and 4 weeks of age, respectively (Denbow et al., 1999). Chicks from lines that have been selected for low or high juvenile body weight displayed differences in their dose threshold responses to the food intake-suppressing effects of centrally injected CRF (Cline et al., 2009). In the present study, a 0.02 nmol dose of CRF, which was used in an earlier experiment (Zhang et al., 2001) potently reduced food intake in the Cobb-500 chicks during the first 30 minutes post-injection. We did not observe changes in water intake, consistent with previous research (Denbow et al., 1999). Collectively, results not only confirm the potent anorexigenic effect of CRF in chickens, but demonstrate that the dose threshold of the feeding response is influenced by several factors, such as age, body weight, and genetic background.

To understand the role of the hypothalamus in mediating the effects of CRF on feeding behavior, we first aimed to identify whether appetite-related nuclei showed indications of being activated in response to CRF injection. The c-Fos protein is an early intermediate transcription factor that is typically expressed when a neuron is activated, as a means to replenish the neurotransmitter (Kovács, 1998). Thus, the

presence of c-Fos implies that a region of the brain was recently activated. The c-Fos immunoreactivity was increased in response to central injection of CRF in the ARC, DMN, PVN, and VMH, indicating these hypothalamic sites may be involved in mediating the anorexgenic effect of CRF. All of these nuclei are important in the regulation of feeding behavior. As the 'first order neurons'(Hillebrand et al., 2002), the ARC is a feeding control center that integrates hormonal signals associated with energy homeostasis from peripheral and central nervous systems (Funahashi et al., 2000). Moreover, the ARC has extensive connections with 'second order neurons' including the PVN, VMH, DMN, and LHA (Benoit et al., 2000; Hillebrand et al., 2002), forming a complex hypothalamic network to regulate feeding behavior. The c-Fos immunoreactivity data thus imply that the ARC, DMN, PVN, and VMH are involved in the CRF-induced physiological response.

We then measured the mRNA abundance of a variety of appetite-associated factors (genes encoding neuropeptides and receptors) in the whole hypothalamus and in the nuclei that were activated in response to CRF injection. It is worthwhile to note that studying the whole hypothalamus is a safeguard such that if a hypothalamic region is not c-Fos reactive (and thereby not selected for gene expression analysis) but is still responsive to CRF, those mRNA changes should be captured in the analysis of the whole hypothalamus. Whole hypothalamic NPYR1 mRNA decreased following CRF injection while other genes were not affected. As one of the primary receptors mediating the potent orexigenic effect of NPY, NPYR1 is widely distributed throughout the hypothalamus, with relatively high expression in the ARC, LHA, and

PVN (Jacques et al., 1996; Parker and Herzog, 1999; Wolak et al., 2003). The NPYR1 agonist (Mullins et al., 2001) and antagonist (Kanatani et al., 1996; Danielsa et al., 2001) enhanced and attenuated, respectively, the orexigenic effect of NPY in rodent studies. Overall, decreased NPYR1 gene expression supports that hunger signaling may be dampened by overriding anorexigenic tone via CRF signaling. Reduced NPYR1 mRNA was also observed in the ARC and DMN, suggesting that the decrease in NPYR1 at the whole hypothalamus level may have originated from the ARC and DMN. Because it is reported that the major receptors for NPY involved in feeding regulation, NPYR1 and NPYR5, are highly expressed in the DMN (Kishi et al., 2005; Chance et al., 2007), the DMN likely plays an important role in mediating the orexigenic effect of NPY. Thus, reduced NPYR1 mRNA in the DMN and ARC may have contributed to reduced orexigenic tone in the hypothalamus of CRF-treated chicks.

The mRNAs for neuropeptide Y and AgRP were also down-regulated in the ARC of CRF-treated chicks. The ARC is comprised of a subset of neurons that produce orexigenic neuropeptides such as NPY and AgRP (Hillebrand et al., 2002), and NPY and AgRP are known to be co-localized in appetite-related nuclei in the hypothalamus (Hahn et al., 1998). Thus, CRF signaling may attenuate orexigenic signaling by down-regulating ARC production and release of NPY and AgRP, although further studies are needed at the peptide level to determine whether CRF affects the abundance and release of these neuropeptides.

On the other hand, expression of CRF and CRFR2 mRNAs were increased in the ARC of CRF-treated chicks, suggesting that CRF treatment enhanced anorexigenic tone in the ARC. We also observed that CRFR2 increased in the PVN in response to CRF. The CRF-CRFR2 system is the primary signaling pathway that mediates the anorexigenic effect of CRF (Stengel and Taché, 2014). Anatomically, the axons of NPY-producing neurons contact cell bodies and dendrites of CRF-containing neurons in the PVN in rats and the dendrites of CRF neurons also connect to NPY-producing terminals (Liposits et al., 1988). Functionally, CRF and NPY exert opposite effects on food intake and serve to counteract each other (Goebel et al., 2009; Reichmann and Holzer, 2016). Thus, the exogenous administration of CRF in chickens may enhance anorexigenic signaling via CRF/CRFR2, while dampening NPY/NPYR1 signaling in the ARC, resulting in overriding anorexigenic tone.

Mesotocin, the non-mammalian equivalent of oxytocin in birds, increased in the VMH in response to CRF treatment. As an anorectic neuropeptide, ICV injection of mesotocin results in decreased food intake in 5 day-old layer chicks (Masunari et al., 2013). The anorexigenic effect of CRF is mediated by oxytocin; the antagonist of the oxytocin receptor is able to completely eliminate the suppression of food intake induced by ICV injection of CRF (Olson et al., 1991). On the other hand, the VMH is considered to be a satiety center because the lesioning of the VMH induces syndromes of hyperphagia and obesity (Satoh et al., 1997). The VMH receives signals from the ARC and projects onto other nuclei, such as the ARC, PVN, LHA, DMN, and the nucleus of the solitary tract (NTS) (Roh and Kim, 2016). Collectively, we can

speculate that the anorexigenic tone produced in the ARC was transmitted to the VMH, leading to an increase in the production of mesotocin, although without accompanying protein/peptide data at different time points, mRNA results should be interpreted with caution.

In conclusion, data suggest that exogenous CRF activated CRF (ARC and PVN) and MT signaling (VMH) pathways that overrode the NPY system (ARC and DMN) to eventually suppress food intake. This study provides insights into understanding CRF-induced actions in the hypothalamus at the nuclei and transcriptional level.

Table 3.1 Primers for real-time PCR[1].

Gene	Accession No.	Sequences (forward/reverse)
β-actin	NM_205518.1	GTCCACCGCAAATGCTTCTAA / TGCGCATTTATGGGTTTTGTT
AgRP	NM_001031457.1	GGTTCTTCAACGCCTTCTGCTA / TTCTTGCCACATGGGAAGGT
CART	XM_003643097.3	GCTGGAGAAGCTGAAGAGCAA / GGCACCTGCCCGAACTT
CRF	NM_001123031.1	TCAGCACCAGAGCCATCACA / GCTCTATAAAAATAAAGAGGTGACATCAGA
CRFR1	NM_204321.1	CTGCTGTCCTTGCTGGGAAT / ATCCTCCCCCGGATTGAC
CRFR2	NM_204454.1	GGATCAAATACAACACCACAAAAAAT / GGCCCATGTCCCATTGC
Ghrelin	NM_001001131.1	GAAGCACTGCCTAACGAAGACA / GGATGCTGAGAAGGAGAATTCCT
LEPR	NM_204323.1	GCAAGACCCTCTCCCTTATCTCT / TCTGTGAAAGCATCATCCTGATCT
MC3R	XM_004947236.2	GCCTCCCTTTACGTTCACATGT / GCTGCGATGCGCTTCAC
MC4R	NM_001031514.1	CCTCGGGAGGCTGCTATGA / GATGCCCAGAGTCACAAACACTT
MCH	NM_001195795.1	GTGGGCAGAAAGCAACTACCTT / TCAGTGTCAGCTGGAAAAGCA
MT	XM_004936280.2	TGGCTCTCTCCTCAGCTTGTTAT / GGCACGGCACGCTTACC
NPS	XM_015289049.1	GTGGGCAGGAGCGAAGAG / CCACACCGTTGCGAAAGG
NPY	NM_205473.1	CATGCAGGGCACCATGAG / CAGCGACAAGGCGAAAGTC
NPYR1	XM_015285306.1	TAGCCATGTCCACCATGCA / GGGCTTGCCTGCTTTAGAGA
NPYR2	NM_001031128.1	TGCCTACACCCGCATATGG / GTTCCCTGCCCCAGGACTA
NPYR5	NM_001031130.1	GGCTGGCTTTGTGGGAAA / TTGTCTTCTGCTTGCGTTTTGT
POMC	NM_001031098.1	GCCAGACCCCGCTGATG / CTTGTAGGCGCTTTTGACGAT
UCN3	XM_001231710.3	GGGCCTTCCGTCTCTACAATG / GGTGAGGGCCGTGTTGAG

[1]Abbreviations: agouti-related peptide (AgRP), cocaine and amphetamine-regulated transcript (CART), corticotropin-releasing factor (CRF), corticotropin-releasing factor receptor sub-types 1 and 2 (CRFR1 and CRFR2, respectively), leptin receptor (LEPR), melanocortin receptors 3 and 4 (MC3R and MC4R, respectively), melanin-concentrating hormone (MCH), Mesotocin (MT), neuropeptide S (NPS), neuropeptide Y (NPY), neuropeptide Y receptor sub-types 1, 2 and 5 (NPYR1, NPYR2 and NPYR5, respectively), Pro-opiomelanocortin (POMC), and Urocortin 3 (UCN3).

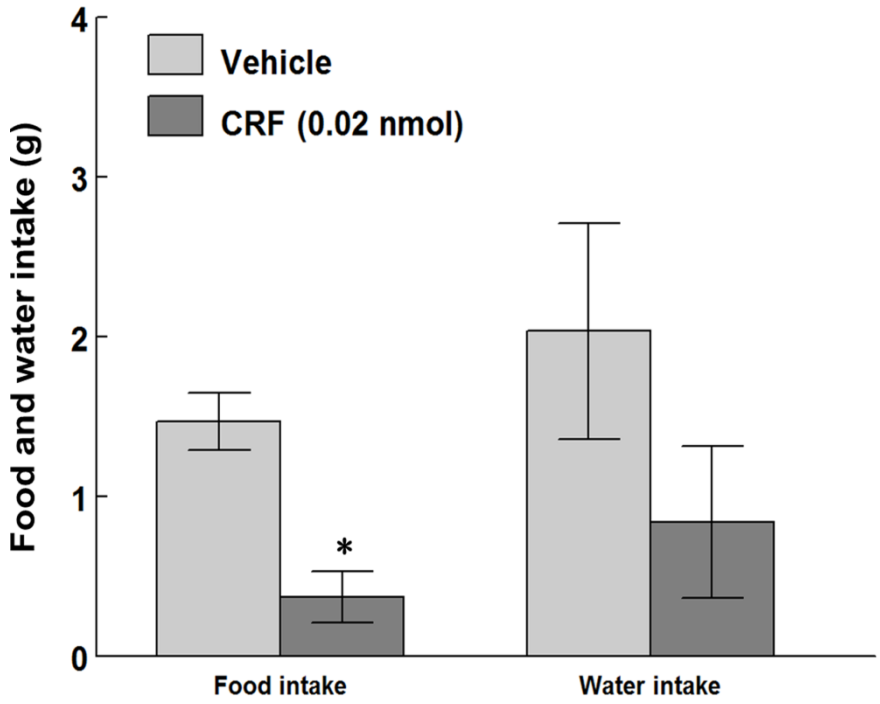

Fig. 3.1 Effect of intracerebroventricular injection of corticotropin-releasing factor (CRF; 0.02 nmol) in 4 day-old Cobb-500 chicks on food and water intake at 30 minutes post-injection. (*) denotes difference from vehicle-injected group ($P < 0.05$). Values are means ± SEM. For this experiment, 10 vehicle- and 10 CRF-treated chicks were available for the analysis.

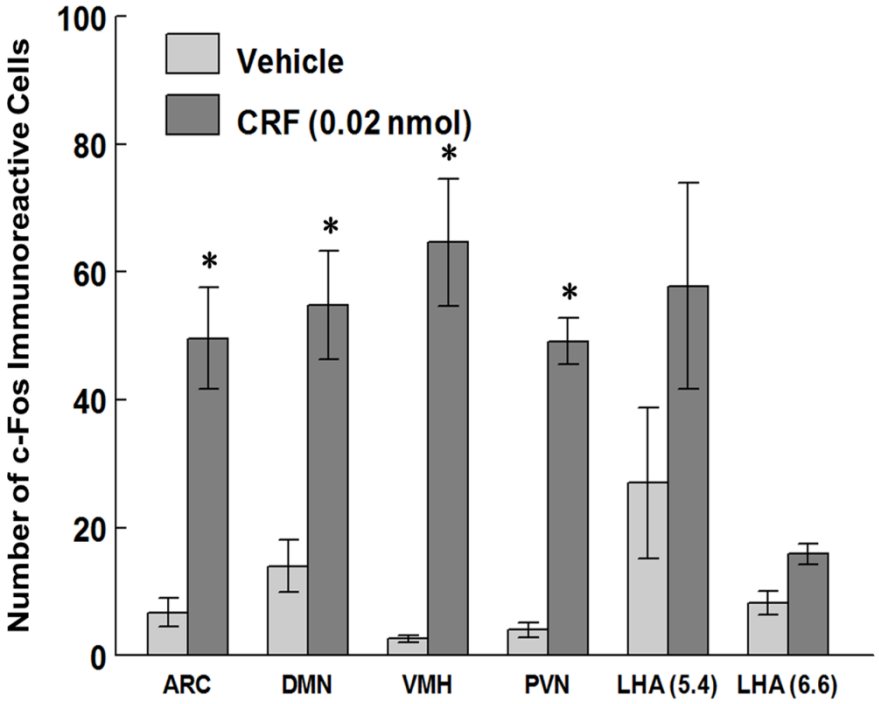

Fig. 3.2 Effect of intracerebroventricular injection of corticotropin-releasing factor (CRF; 0.02 nmol) in 4 day-old Cobb-500 chicks on the number of c-Fos immunoreactive cells in the arcuate nucleus (ARC), dorsomedial hypothalamic nucleus (DMN), ventromedial hypothalamus (VMH), paraventricular nucleus (PVN), and lateral hypothalamic area (LHA). LHA (5.4) and LHA (6.6) represent the 5.4 and 6.6 interaural, respectively, based on the Kuenzel and Masson chicken stereotaxic atlas (Kuenzel and Masson, 1988). (*) denotes difference from vehicle-injected group ($P < 0.05$). Values are means ± SEM. For this experiment, 6 vehicle- and 8 CRF-treated chicks were available for the analysis.

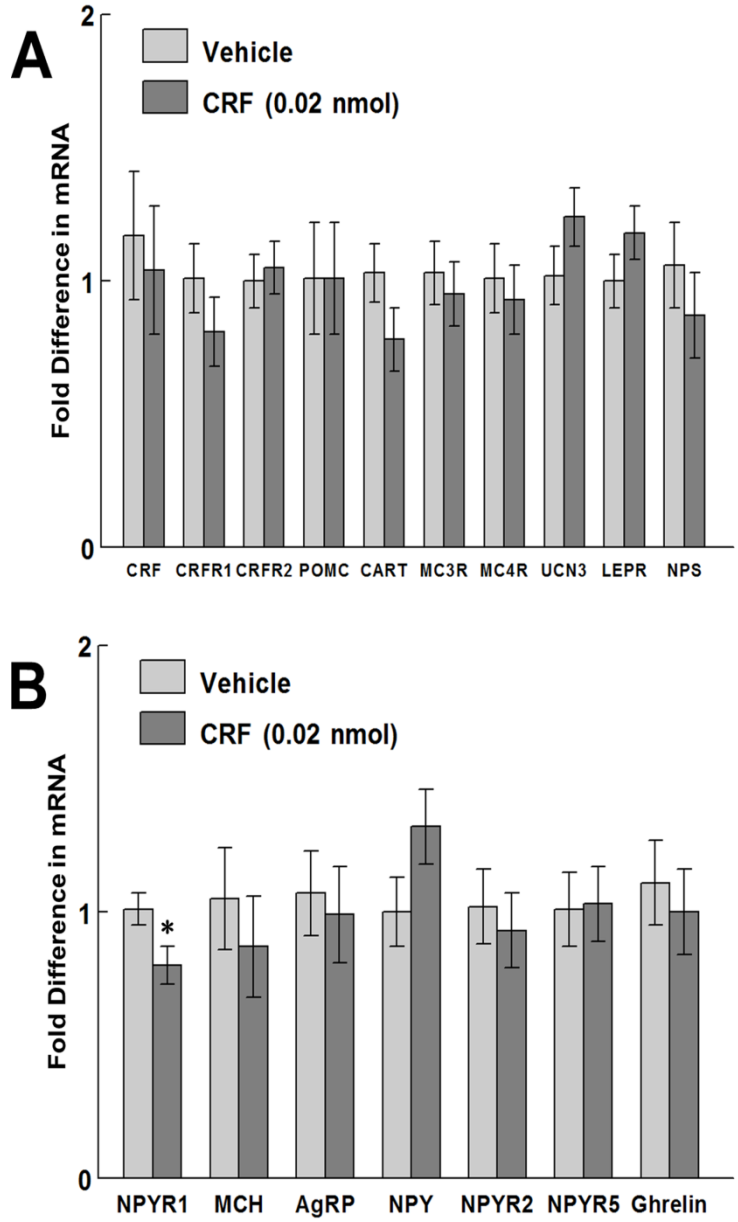

Fig. 3.3 Effect of intracerebroventricular administration of corticotropin-releasing factor (CRF; 0.02 nmol) in 4 day-old Cobb-500 chicks on expression of anorexigenic (A) and orexigenic (B) –associated factors in whole hypothalamus at 1 hour post-injection. (*) denotes difference from vehicle-injected group ($P < 0.05$). Values are means ± SEM. For this experiment, 8 vehicle and 8 CRF-treated chicks were available for the analysis. Abbreviations: agouti-related peptide (AgRP), cocaine and amphetamine-regulated transcript (CART), CRF receptor sub-types 1 and 2 (CRFR1 and CRFR2, respectively), leptin receptor (LEPR), melanocortin receptors 3 and 4 (MC3R and MC4R, respectively), melanin-

concentrating hormone (MCH), Mesotocin (MT), neuropeptide S (NPS), neuropeptide Y (NPY), NPY receptor sub-types 1, 2 and 5 (NPYR1, 2 and 5, respectively), Pro-opiomelanocortin (POMC), and Urocortin 3 (UCN3).

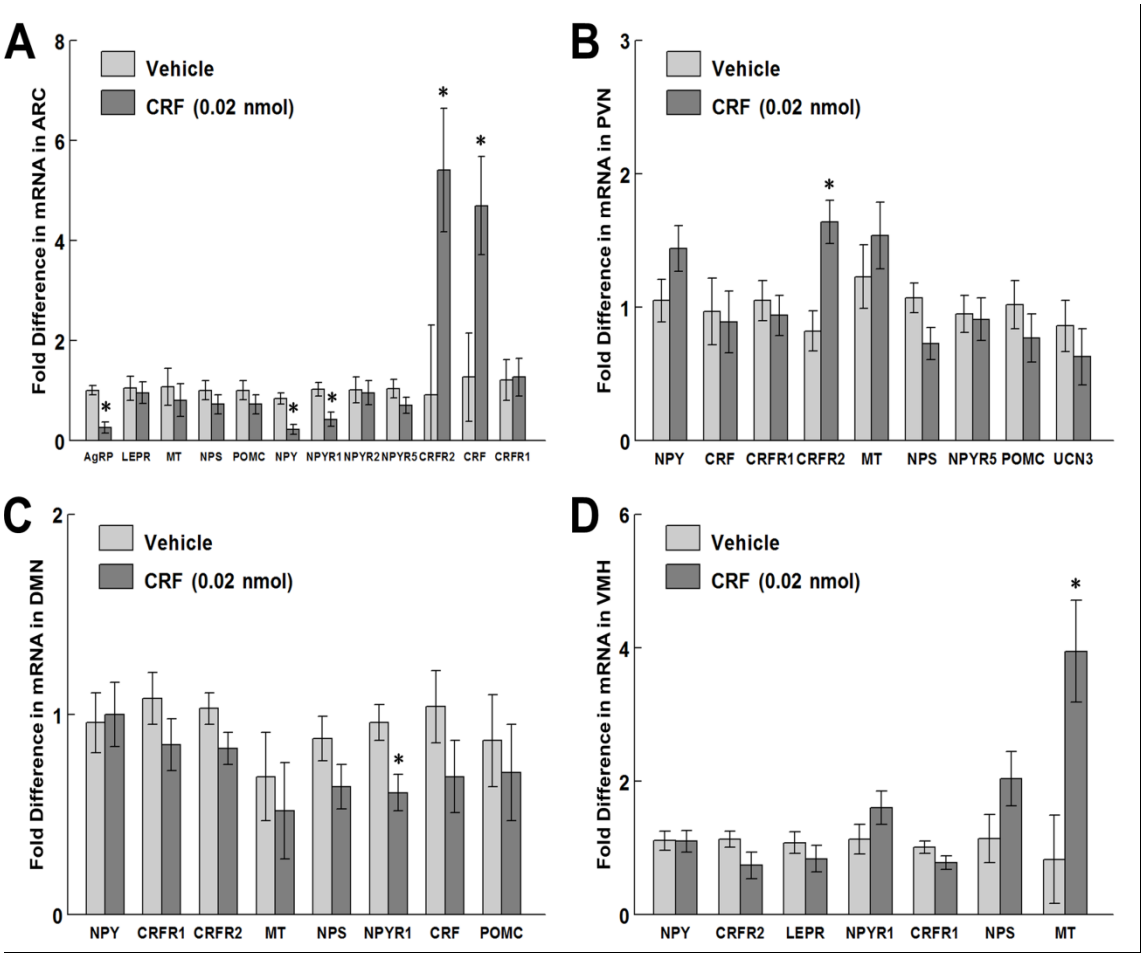

Fig. 3.4 Effect of intracerebroventricular administration of corticotropin-releasing factor (CRF; 0.02 nmol) in 4 day-old Cobb-500 chicks on mRNA abundance in the arcuate nucleus (ARC) (A), paraventricular nucleus (PVN) (B), dorsomedial hypothalamic nucleus (DMN) (C), and ventromedial hypothalamus (VMH) (D) at 1 hour post-injection. (*) denotes difference from vehicle-injected group ($P < 0.05$). Values are means ± SEM. For this experiment, 12 vehicle and 12 CRF-treated chicks were available for the analysis. Abbreviations: agouti-related peptide (AgRP), cocaine and amphetamine-regulated transcript (CART), CRF receptor sub-types 1 and 2 (CRFR1 and CRFR2, respectively), leptin receptor (LEPR), melanocortin receptors 3 and 4 (MC3R and MC4R, respectively), melanin-concentrating hormone (MCH), Mesotocin (MT), neuropeptide S (NPS), neuropeptide Y

(NPY), NPY receptor sub-types 1, 2 and 5 (NPYR1, NPYR2 and NPYR5, respectively), Pro-opiomelanocortin (POMC), and Urocortin 3 (UCN3).

Chapter 4: Hypothalamic mechanism of corticotropin-releasing factor's anorexigenic effect in Japanese quail (Coturnix japonica)

Abstract

Central administration of corticotropin-releasing factor (CRF), a 41-amino acid peptide, is associated with anorexigenic effects across various species, with particularly potent reductions in food intake in rodents and chickens (*Gallus gallus domesticus*), a species for which the most is known. The purpose of the current study was to determine the hypothalamic mechanism of CRF-induced anorexigenic effects in 7 day-old Japanese quail (*Coturnix japonica*), a less-intensely-selected gallinaceous relative to the chicken that can provide more evolutionary perspective. After intracerebroventricular (ICV) injection of 2, 22, or 222 pmol of CRF, a dose-dependent decrease in food intake was observed that lasted for 3 and 24 hours for the 22 and 222 pmol doses, respectively. The 2 pmol dose had no effect on food or water intake. The numbers of c-Fos immunoreactive cells were increased in the paraventricular nucleus (PVN) and lateral hypothalamic area (LHA) at 1 h post-injection in quail injected with 22 pmol of CRF. The hypothalamic mRNA abundance of proopiomelanocortin, melanocortin receptor subtype 4, CRF, and CRF receptor sub-type 2 was increased at 1 h in quail treated with 22 pmol of CRF. Behavior analyses demonstrated that CRF injection reduced feeding pecks and jumps and increased the time spent standing. In conclusion, results demonstrate that the anorexigenic effects of CRF in Japanese quail are likely influenced by the interaction

between CRF and melanocortin systems and that injection of CRF results in species-specific behavioral changes.

Key words: quail; corticotropin-releasing factor; hypothalamus; paraventricular nucleus; lateral hypothalamus

1. Introduction

Corticotropin-releasing factor (CRF), a 41 amino acid peptide, is perhaps best known for its role as a regulator of the hypothalamic–pituitary–adrenal (HPA) axis (Vale et al., 1981). The amino acid sequence of CRF is highly conserved, underscoring its evolutionary importance as a physiological regulator (Ehlers et al., 1992). The biological functions of CRF are primarily mediated through two G-protein-coupled receptors, CRF receptor sub-type 1 (CRFR1) (Perrin et al., 1993) and CRF receptor sub-type 2 (CRFR2) (Lovenberg et al., 1995b). In the central nervous system, CRF is widely distributed in the paraventricular nucleus (PVN), hippocampus, amygdala and the Barrington's nucleus (Stengel and Taché, 2014) and has similar distribution pattern in various species (Richard et al., 2004).

Treatment with CRF suppresses food intake in various species (Zorrilla et al., 2003; Wang et al., 2011b), including chickens (Furuse et al., 1997; Denbow et al., 1999; Meade and Denbow, 2003; Tachibana et al., 2006; Cline et al., 2009), and promotes energy expenditure (Richard et al., 2002). Appetite regulation is a complex system in which central and peripheral signals interact to modify the body's response to nutrient intake and energy storage and expenditure (Valassi et al., 2008). As a primary

regulatory center, the hypothalamus plays important roles in energy homeostasis (Sam et al., 2012). Several hypothalamic nuclei are essential for the regulation of feeding behavior, including the PVN, arcuate nucleus (ARC), ventromedial nucleus of the hypothalamus (VMH), dorsomedial hypothalamic nucleus (DMN), and lateral hypothalamic area (LHA) (Arora and Anubhuti, 2006). The ARC is a "first-order" feeding regulation center containing two functionally discrete neuronal populations. The anorexigenic population is composed of proopiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART)-releasing neurons. The orexigenic population consists of neuropeptide Y (NPY) and agouti-related peptide (AgRP)-expressing neurons (Hillebrand et al., 2002; Parkinson et al., 2008). In general, signaling from the ARC is projected to 'second order" regulatory centers including the PVN, VMH, DMN, and LHA (Schwartz et al., 2000). These neural signals and pathways form a complex regulatory mechanism for modulating food intake that is evolutionarily conserved across species (Allen et al., 2005).

To date, the majority of research on the anorexigenic effect of CRF in avian species has used the chicken as the animal model. However, the chicken has undergone extensive artificial selection for various growth-related traits, which may have resulted in alterations to the hypothalamic circuitry involved in regulating feeding behavior. The Japanese quail (*Coturnix japonica*) is less-selected and is also well-adapted to the experimental environment and may thus provide more perspective on the role of CRF in appetite regulation in birds. Hence, the purpose of the present study was to evaluate the effect of central CRF injection on food intake, behavior, and

hypothalamic changes in neuronal activity and gene expression in 7 day-old Japanese quail.

2. Materials and Methods

2.1. Animals

Japanese quail were bred and hatched in our vivarium. The breeder flock was established with eggs donated by Mike Lacy at the University of Georgia. Upon removal from the hatcher, quail were group caged in a brooder for 4 days, then individually caged in galvanized wire cages (8 cm wide, 7 cm deep, and 8 cm high) in a room maintained at a constant temperature of 35 ± 1 °C and a relative humidity of 50 ± 5% with a 14-hour light/10-hour dark period (lights on at 05:00 hours). At all times, unless otherwise noted, quail had ad libitum access to a mash diet (energy: 2,900 kcal ME/kg and 24% crude protein) and tap water. The individual cages allowed visual and auditory contact with other quail. After the quail were individually caged, they were handled twice daily to reduce the effects of stress on the day of data collection. The handling procedure was conducted as we described (Lear et al., 2017). All quail in this experiment were 7 day-old and there were 12 chicks in each treatment group. All procedures were performed according to the National Research Council publication, Guide for Care and Use of Laboratory Animals and were approved by the Virginia Tech Animal Care and Use Committee.

2.2. ICV injection procedure

On the day of the experiment, quail were injected using an adapted method that does not appear to induce physiological stress (Lear et al., 2017; Yuan et al., 2017). The

head of the quail was briefly inserted into the restraining device. Injection coordinates were 2 mm anterior to the coronal suture, .75 mm lateral from the sagittal suture, and 1.5 mm deep, targeting the left lateral ventricle. Anatomical landmarks were determined both visually and by palpation. Injection depth was controlled by placing a plastic tubing sheath over the base of the needle. The needle remained at injection depth in the un-anesthetized quail for 5 seconds post-injection to reduce backflow. Quail were assigned to treatments at random. Ovine CRF (Sigma, St. Louis, MO, USA) was dissolved in artificial cerebrospinal fluid (Anderson and Heisley, 1972) as a vehicle for a total injection volume of 5 µl with 0.1% Evans Blue dye to facilitate injection site localization. After data collection, the quail was decapitated and its head sectioned along the frontal plane to determine the site of injection. Any quail without dye present in the lateral ventricle system was eliminated from analysis. The sex of the quail was determined visually by dissection at the time of decapitation.

2.3. Experiment 1: Food and water intake

The quail were fasted for 6 hours prior to ICV injection of CRF. Quail were randomly assigned to receive either 0 (vehicle only), 2, 22, or 222 pmol of CRF by ICV injection (n=12 for each group). After injection, quail were returned to their individual home cages and given ad libitum access to both food and water. Food and water intake were monitored (0.01 g) every 30 minutes for 180 minutes and then 6, 16, 21 and 24 hours post-injection. The data were analyzed using analysis of variance (ANOVA) within each time point, and the statistical model included the main effect of dose. When dose effects were significant, Tukey's method of multiple comparisons

was used to separate the means within each time point. Statistical significance was set at $P < 0.05$ for all experiments. Food and water intake results are shown on a cumulative and non-cumulative basis.

2.4. Experiment 2: Hypothalamic mRNA abundance of appetite-associated factors

The quail were fasted for 6 hours prior to ICV injection of CRF. Quail were randomly assigned to receive either vehicle or 22 pmol of CRF (based on results of Experiments 1) via ICV injection (n=12 for each group). After injection, food was withheld. Each quail was deeply anesthetized at 60 min post-injection with sodium pentobarbital via cardiopuncture, decapitated, and its brain removed. The whole upside-down brain was then snap frozen in liquid nitrogen for 9 seconds. This duration freezes the outermost portion of the brain, providing firmness while leaving the center unfrozen to permit dissection without shattering. Cuts were made while using visual cues to follow the anatomy: a cut was made perpendicular to the midline suture at the septopallio-mesencephalic tract and at the third cranial nerves. Two cuts were made 1.8 mm parallel to the midline and finally the dorsal cut was made from the anterior commissure to 0.8 mm ventral to the posterior commissure (McConn et al., 2014). This block (comprised primarily of the hypothalamus) was immediately stored in RNAlater (Qiagen).

The hypothalamus was homogenized using 5 mm stainless steel beads (Qiagen, Valencia, CA, USA), Tri reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and a Tissue Lyser II (Qiagen). The total RNA was extracted following the manufacturer's instructions (5-Prime). The RNeasy Mini Kit (Qiagen) and RNase-free

DNase I (Qiagen) were then used for total RNA purification. The concentration and purity of total RNA was assessed by spectrophotometry at 260/280/230 nm with a Thermo NanoDrop 2000 (Thermo Fisher Scientific Inc., West Palm Beach, FL, USA). RNA integrity was verified using Biorad's automated electrophoresis system Experion (RNA StdSens analysis kit), according to the manufacturer's instructions.

First-strand cDNA was synthesized in 20 µl reactions from 200 ng of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's instructions. Reactions were performed under the following conditions: 25 ℃ for 10 minutes, 37 ℃ for 120 minutes and 85 ℃ for 5 minutes. Primers for real-time PCR were designed with Primer Express 3.0 software (Applied Biosystems) (Table 4.1) and validated for amplification efficiency before use (95–105%). Real-time PCR reactions were performed in 10 µl reactions which contained: 5 µl Fast SYBR Green Master Mix (Applied Biosystems), 0.5 µl primers (0.25 µl of 5 µM forward primer and 0.25 µl of 5 µM reverse primer), 1.5 µl nuclease-free water, and 3 µl 10-fold diluted cDNA using a 7500 Fast Real-Time PCR System (Applied Biosystems). The real-time PCR was performed under the following conditions: 95 ℃ for 20 seconds and 40 cycles of 90 ℃ for 3 seconds plus 60 ℃ for 30 seconds. A dissociation step consisting of 95 ℃ for 15 seconds, 60 ℃ for 1 minute, 95 ℃ for 15 seconds and 60 ℃ for 15 seconds was performed at the end of each PCR reaction to ensure amplicon specificity.

Real-time PCR data was analyzed using the ΔΔCT method with β-actin as the endogenous control and the average of the quail in the vehicle group as the calibrator

sample. Relative quantities calculated as $2^{-\Delta\Delta CT}$ were used for statistical analysis (Livak and Schmittgen, 2001). The statistical model included the main effect of the treatment, sex, and their interactions. The mRNA abundance was not affected by sex for any gene measured, thus we removed sex from the model. The data were analyzed by analysis of variance (ANOVA) using JMP 11 Pro (SAS institute, Cary, NC, USA). Differences were considered significant at $P < 0.05$. Data are presented as means ± SE.

2.5. Experiment 3: c-Fos immunohistochemistry

The quail were fasted for 6 hours prior to ICV injection of CRF. Quail were randomly assigned to receive either vehicle or 22 pmol of CRF (based on the results of Experiment 1) by ICV injection (n=12 for each group). After injection, food was withheld to prevent c-Fos immunoreactivity associated with food consumption. Sixty minutes post-injection, as this is the time expected for the most robust c-Fos expression (Müller et al., 1984), quail were deeply anesthetized with sodium pentobarbital via cardiopuncture, then perfused via the carotid artery with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) containing 0.2% picric acid at pH 7.4. Brains were removed from skulls and post-fixed for 60 minutes in the same solution, after which they were blocked and placed in a series of graded sucrose incubations, consisting of 20%, 30% and 40% sucrose concentration in 0.1 M PB, until they sank in each. Several 60 µm coronal sections that contained appetite-related nuclei based on anatomies described by Kuenzel (Kuenzel and Masson, 1988) were collected using a cryostat at -15 °C, and then deposited in 0.02 M phosphate

buffered saline (PBS) containing 0.1% sodium azide (Kuenzel et al., 1987). The VMH, PVN, and LHA were collected corresponding to 6.6 interaural mm, and the DMN and the ARC at interaural 5.4 mm. Sections were processed immediately after collection. Procedures for the c-Fos immunohistochemistry assay were performed as we described (Newmyer et al., 2013) using rabbit polyclonal anti-c-Fos at a dilution of 1:20,000 (K-25, Santa Cruz, Santa Cruz, CA, USA) (Zhao and Li, 2010; Newmyer et al., 2013). Anatomies were confirmed and a digital micrograph taken of each section. Overlays containing the respective nuclei boundaries were digitally merged with micrographs and the number of c-Fos immunoreactive cells within each respective nucleus counted by a technician blind to treatment. Data were analyzed via ANOVA using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC), with the model including the effect of treatment within each nucleus.

2.6. Experiment 4: Behavior analysis

The quail were kept in individual cages with auditory but not visual contact with each other (to reduce isolation stress during the observational period), and were randomly assigned to receive either vehicle or 22 pmol of CRF (based on results of Experiment 1) by ICV injection. Following 6 hours of fasting, injections were performed and the quail were immediately placed in a 290 mm × 290 mm acrylic recording arena with food and water containers in diagonal corners. Quail were simultaneously and automatically recorded from three angles for 30 minutes post-injection on DVD and the data were analyzed in 5 minute intervals using ANY-maze behavioral analysis software (Stoelting, Wood Dale, IL). At 30 minutes post-injection, food intake was

measured. Locomotion (m traveled), the amount of time spent standing, sitting, preening, perching, or in deep rest, as well as the number of jumps, steps, feeding, exploratory pecks, and escape attempts were quantified. Food pecks were defined as pecks within the food container, whereas any other pecks were counted as exploratory. Deep rest was defined as having the eyes closed for greater than 3 seconds (and its timing starting 3 seconds after eye closure, ending when eyes reopened). Preening was defined as trimming or dressing down with the beak. Due to non-heterogeneous variance, behavioral data were analyzed by the Mann–Whitney U test. Pecking efficiency at 30 minutes post-injection was calculated by dividing the amount of food consumed by the number of food pecks for each quail. Pecking efficiency and food intake data were analyzed by ANOVA.

3. Results

3.1. Food and water intake

At 0.5 through 3 hours post-injection, food intake was lowest in quail that were injected with 222 pmol of CRF, intermediate in quail that received 22 pmol, and greatest in vehicle-injected birds (Fig. 4.1A). At 6 hours, the 22 pmol-injected group had recovered from the anorectic effect of CRF, whereas the anorectic effect of the 222 pmol-dose was sustained to 24 hours post-injection. There was no significant difference in food intake between the 2 pmol- and vehicle-injected group for the entire duration. For non-cumulative food intake, differences were similar to cumulative food intake (Fig. 4.1B).

The inhibitory effect of 22 and 222 pmol of CRF on cumulative water intake lasted 2.5 and 24 hours post-injection, respectively (Fig. 4.2A). For non-cumulative water intake, the decrease in water intake induced by 22 pmol of CRF lasted for 1 hour post-injection while inhibitory effects were observed in the 222 pmol-injected group over two time periods: up to 1.5 hours and between 6 and 16 hours (dark period) post-injection. There was no effect of 2 pmol of CRF on water intake.

3.2 Hypothalamic mRNA abundance

The 22 pmol-injected quail had greater hypothalamic mRNA expression of CRFR2 ($P = 0.0134$), CRF ($P = 0.0268$), POMC ($P = 0.0275$), melanocortin receptor 4 (MC4R; $P = 0.0316$), and c-Fos ($P = 0.0415$) at 1 hour post-injection (Fig. 4.3). The ICV injection of CRF had no effect on the mRNA abundance of CART, AGRP, CRFR1, NPY, NPY receptor sub-types 1 and 5 (NPYR1 and NPYR5), and urocortin 3 (UCN3).

3.3 c-Fos immunohistochemistry

There were more c-Fos immunoreactive cells in the PVN ($P = 0.0335$) and LHA ($P = 0.0272$) of CRF- than vehicle-injected quail at 1 hour post-injection (Fig. 4.4). Immunoreactivity was increased by a magnitude of 250% and 120% over the vehicle-injected quail in the PVN and LHA, respectively. The VMH, DMN, and ARC were not affected by CRF injection.

3.4 Behavioral observations

The CRF-treated quail displayed fewer feeding pecks at 15 through 30 minutes post-injection compared to vehicle-injected birds (Table 4.2). The CRF-injected quail also

displayed fewer jumps at 10 through 30 minutes post-injection. The numbers of exploratory pecks, defecations, steps, and distance traveled were not affected by CRF injection. From 15 minutes onward, quail treated with CRF spent more time standing than those from the vehicle-injected group (Table 4.3). Other timed-type behaviors were not influenced by CRF treatment.

4. Discussion

The potent anorexigenic effects of CRF observed in the present study are consistent with those reported in chickens (Furuse et al., 1997; Denbow et al., 1999) and white-crowned sparrows (Richardson et al., 2000). CRF exerts an inhibitory effect on food intake within a dose range of 5-20 μg in Leghorn and broiler cockerels, (Denbow et al., 1999), and 0.01-1 μg in two day-old chicks (Furuse et al., 1997). After ICV injection of 0.1, 0.4, and 0.8 μg of CRF into the 3rd ventricle, the food intake of white-crowned sparrows was reduced for 180 minutes post-injection (Richardson et al., 2000). In the present experiment, the 2 pmol dose of CRF was not associated with a change in food intake, whereas the suppressive effect of the 22 pmol dose lasted for at least 3 hours and food intake returned to baseline at 6 hours post-injection. The 222 pmol dose was even more potent, with the anorexic effect persisting for at least 24 hours post-injection.

Thus, our effective dose range overlapped with studies involving neonatal chicks (Furuse et al., 1997) and white-crowned sparrows (Richardson et al., 2000). From lowest to highest, respectively, white-crowned sparrows, Japanese quail, and neonatal chicks, have distinct anorexigenic dose thresholds of CRF. This may be a result of the

varying degree of artificial selection in these species, in which the chicken has undergone much more intense selection for growth-related traits than the Japanese quail or white-crowned sparrow. Moreover, due to the close relationship between environment and dosage-dependent responses of appetite associated factors, the varying experimental environments may be an additional factor contributing to the variance of the dose threshold among species (Woods and Langhans, 2012). In addition to the inhibitory effect on food intake, CRF injection was also associated with a reduction in water intake, similar to rats (Morley and Levine, 1982; Woods and Langhans, 2012). CRF was associated with less water intake in Leghorns but not broilers, with the suppressive effect on water intake in Leghorns likely prandial (Denbow et al., 1999).

On a non-cumulative basis, food intake results show that the anorexigenic effects of the 22 pmol dose began to dissipate after 3 hours post-injection. Based on these results, we used the 22 pmol dose for subsequent Experiments. The primary objective of these experiments was to elucidate the hypothalamic mechanism of the anorexigenic effect of CRF.

The hypothalamus is a critical area in the central nervous system for the control of both food and water intake (Grossman, 1975; Leibowitz, 1978). Thus, we measured hypothalamic c-Fos immunoreactivity, a marker for neuronal activity, at 1 hour following ICV injection. Among the five nuclei in which cells were counted, c-Fos immunoreactivity was increased in the PVN and LHA in response to CRF injection. The PVN is involved in appetite regulation (Bernardis, 1975; Kalra et al., 1999a), is

the primary site of CRF synthesis and release, and is the area with the great amount of CRF receptor expression in mammals (Konishi et al., 2003). Immunohistochemical studies have revealed that the hypothalamic distribution of CRF and its receptors in chickens and Japanese quail is similar to mammals (Hirnforsch, 1986; Richard et al., 2004), suggesting conservation of CRF system function between mammals and avian species.

We also measured the hypothalamic mRNA abundance of appetite-associated factors and found that CRF and CRFR2 mRNAs were increased at 1 hour post-injection. Thus, c-Fos and gene expression results collectively suggest that exogenous CRF may activate the CRF-signaling system in the PVN. The anorexigenic effect of CRF in other species is preferentially mediated by CRFR2 (Stengel et al., 2015). For example, selective CRFR2 but not CRFR1 antagonists, block the anorexigenic effect of exogenous CRF (Pelleymounter et al., 2000; Cullen et al., 2001; Sekino et al., 2004). Neither gene deletion (Contarino et al., 2000) nor pharmacologic antagonism of CRFR1 (Bradbury et al., 2000) abolishes the anorexigenic effect of CRF. Hence, the activation of CRF-CRFR2 signaling in the PVN may contribute to the anorexigenic effect of CRF in Japanese quail.

In the present study, CRF injection was also associated with an increase in POMC and MC4R mRNA. POMC is the precursor protein to the cleavage product, α-MSH, which plays an important role in regulating food intake in chickens (Takeuchi et al., 1999; Gerets et al., 2000). The anorexigenic effects of α-MSH are mediated primarily through MC4R (Forbes et al., 2001). POMC and MC4R are most abundance in the

ARC (Pelletier et al., 1980; Saneyasu et al., 2013) and PVN (Kishi et al., 2003), respectively. Within the hypothalamus, the activation of ARC POMC-containing neurons initiates the synthesis and release of α-MSH from axon terminals, which in turn activates MC4R-expressing cell bodies elsewhere in the hypothalamus, such as in the PVN, leading to the inhibition of food intake and promotion of energy expenditure (Cowley et al., 2001). Although POMC mRNA was elevated in the whole hypothalamus, c-Fos immunoreactivity was not increased in the ARC, suggesting that the increased POMC mRNA induced by exogenous CRF may not originate from the ARC. Due to the widespread distribution of POMC in the brain, especially in regions that are involved in general homeostasis including the brainstem and other hypothalamic nuclei (King and Hentges, 2011), the other hypothalamic nuclei not measured in the present study may be the major source of increased POMC mRNA. Moreover, the increase in MC4R mRNA coupled to increased expression of c-Fos in the PVN, suggests that MC4R may play important roles in mediating the anorexigenic effects of both melanocortin peptides and CRF.

α-MSH is a well-studied anorexic peptide in birds (Kawakami et al., 2000; Tachibana et al., 2001; Strader et al., 2003). The anorexigenic effect of α-MSH is mediated by MC4R, where such an effect could be attenuated by antagonism of the MC4R (Tachibana et al., 2001; Strader et al., 2003), indicating MC4R is an important mediator of the anorexic effect of α-MSH. To date, there is a growing body of evidence that suggests there is an interaction between the melanocortin and CRF systems. For example, anatomically, MC4R mRNA is co-expressed with CRF mRNA

in the PVN (Lu et al., 2003) and CRF-containing neurons in the PVN interact with neuronal terminals of releasing α-MSH (Mihály et al., 2002). Functionally, CRF may act as a downstream mediator of the melanocortin signaling (Lu et al., 2003; Kawashima et al., 2008) and thus play important roles in mediating the anorexic effect of α-MSH (Tachibana et al., 2007). In the present study we found both MC4R and CRF mRNA having increased expression in the hypothalamus, coinciding with the activation of c-Fos in the PVN, simultaneously. These results suggest that the anatomical connections and the functional similarities between melanocortin and CRF systems may be the primary contribution to the anorexigenic effect of CRF in Japanese quail.

There was also increased c-Fos immunoreactivity in the LHA, another hypothalamic nucleus that controls energy metabolism and feeding behavior (Elmquist et al., 1999). The LHA is suggested to be the "hunger center" since electrical stimulation of LHA leads to voracious feeding behavior (Delgado and Anand, 1953; Stuber and Wise, 2016). Numerous orexigenic peptides are produced in the LHA, such as melanin-concentrating hormone (MCH) (Bittencourt et al., 1992) and orexins (Sakurai et al., 1998). It is unclear why this nucleus was activated in response to CRF injection, although the axons of CRF neurons in the PVN terminate on the LHA (Rho and Swanson, 1987; Füzesi et al., 2016), indicating a possible neural pathway that bridges the signaling between the PVN and LHA.

A comprehensive behavior analysis was conducted to determine the behavioral effects of exogenous CRF injection in Japanese quail. The number of feeding pecks

decreased in quail treated with CRF, consistent with the food intake data. In addition to decreased feeding behavior, we also observed that the quail treated with exogenous CRF exhibited a "calmness", with decreased a number of jumps and increased time spent standing. These results agree with a previous study where CRF-injected chicks displayed freezing behavior, characterized by a lack of movement and feeding during the first 15 minutes (Ohgushi et al., 2001). However, the opposite types of behaviors have also been reported, where chicks showed excited behaviors following central injection of CRF, including loud vocalizations (Furuse et al., 1997). In the rodent studies, environmental context is a critical factor that determines the behavioral response following CRF administration (Lowry and Moore, 2006).

In conclusion, we demonstrated that ICV injection of CRF is associated with decreased food and water intake in 7-day old Japanese quail, with associated changes in feeding-related behaviors. The hypothalamic mechanisms likely involves activation of CRFR2 and melanocortin signaling pathways in the PVN. Measurement of gene expression and pharmacological targeting of receptors in individual nuclei should provide further insights.

Table 4.1 Primers for real-time PCR.

Gene	Accession No.	Sequences (forward/reverse)
β-actin	NM_205518.1	GTCCACCGCAAATGCTTCTAA/TGCGCATTTATGGGTTTTGTT
AgRP	XM_004950992.1	GGTTCTTCAACGCCTTCTGCTA/TTCTTGCCACATGGGAAGGT
AVT	NM_205185.2	TCCGGGCACACTCAGCAT / ATGTAGCAGGCGGAGGACAA
CART	XM_003643097.2	GCTGGAGAAGCTGAAGAGCAA/GGCACCTGCCCGAACTT
CRF	XM_015855730.1	TCAGCACCAGAGCCATCACA/GCTCTATAAAAATAAAGAGATGACATCAGA
CRFR1	XM_015885973.1	GGCTGGCTTTGTGGGAAA/CTGTCTTCTGCTTGCGTTTTGT
CRFR2	NM_204454.1	GGATCAAATACAACACCACAAAAAAT/GGCCCATGTCCCATTGC
MC4R	XM_015854466.1	CATCAGCTTGCTGGAGAACGT/GCGAATGGAGGTTCTTGTTCTT
NPY	XM_015853870.1	CATGCAGGGCACCATGAG/CAGCGACAGGGCGAAAGTC
NPYR1	NM_001031535.1	TAGCCATGTCCACCATGCA/GGGCTTGCCTGCTTTAGAGA
NPYR5	XM_015861003.1	GGCTGGCTTTGTGGGAAA/CTGTCTTCTGCTTGCGTTTTGT
POMC	XM_015859667.1	GCCAGACCCCGCTGATG/CTTGTAGGCGCTTTTGATGAT
UCN3	XM_001231710.2	GGGCCTTCCGTCTCTACAATG/GGTGAGGGCCGTGTTGAG
c-Fos	NM_205508.1	TGTTCCTGGCAATATCGTGTTC/CTTTCCCCCCCACGTAAGA

Primers were designed with Primer Express 3.0 (Applied Biosystems). Abbreviations: agouti-related peptide (AgRP), arginine vasotocin (AVT), cocaine and amphetamine-regulated transcript (CART), corticotropin-releasing factor (CRF), corticotropin-releasing factor receptor sub-types 1 and 2 (CRFR1 and CRFR2, respectively), melanocortin receptor 4 (MC4R), neuropeptide Y (NPY), neuropeptide Y receptor sub-types 1 and 5 (NPYR1 and NPYR5, respectively), proopiomelanocortin (POMC), urocortin 3 (UCN3).

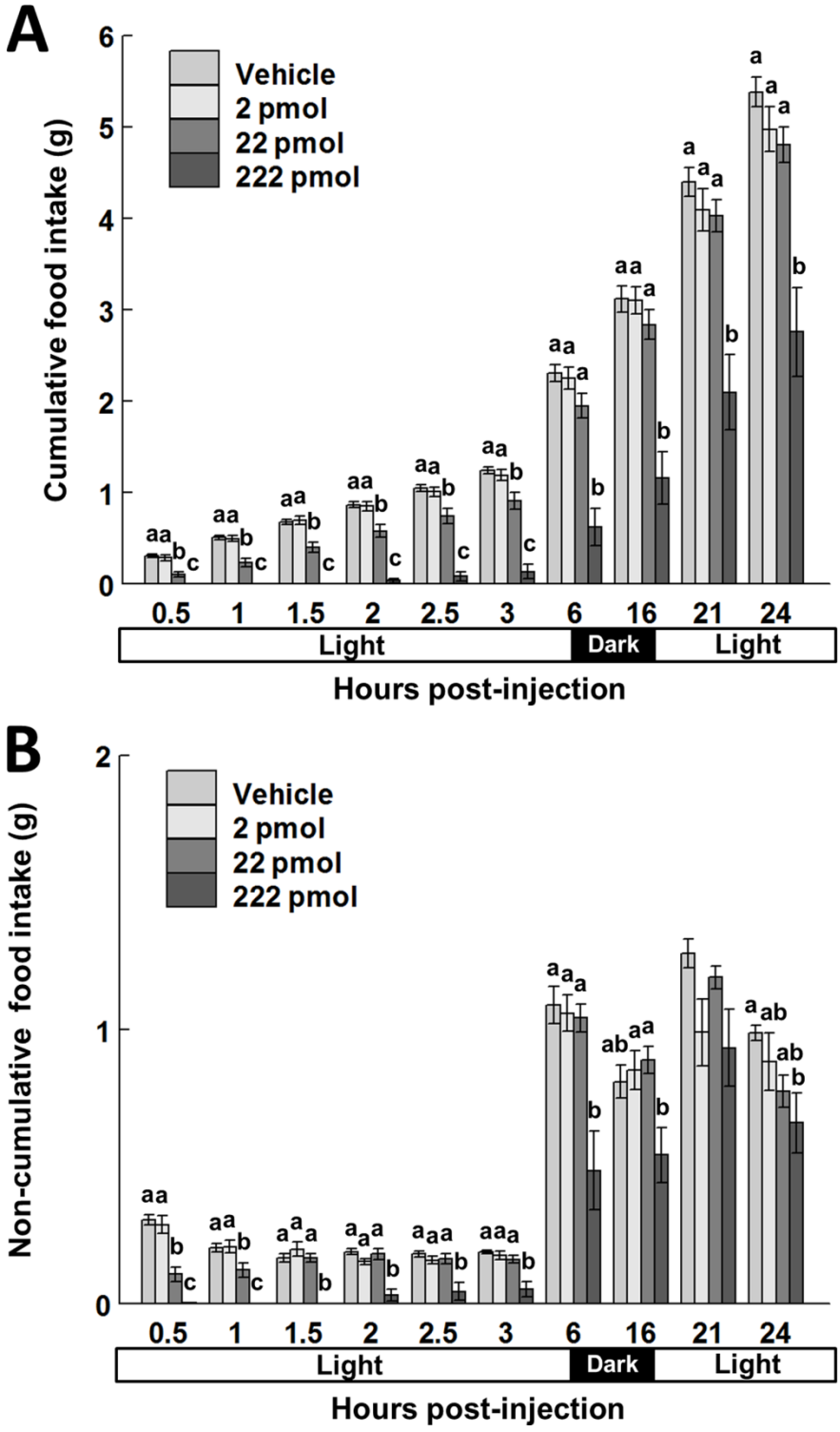

Fig. 4.1 Cumulative (A) and non-cumulative (B) food intake expressed as a percentage of body weight of Japanese quail at 7 days post-hatch (Experiment 1). Values are means ± S.E.M.; bars with different superscripts are different from each

other within a time point ($P < 0.05$). There were 12 quail per group available for analysis.

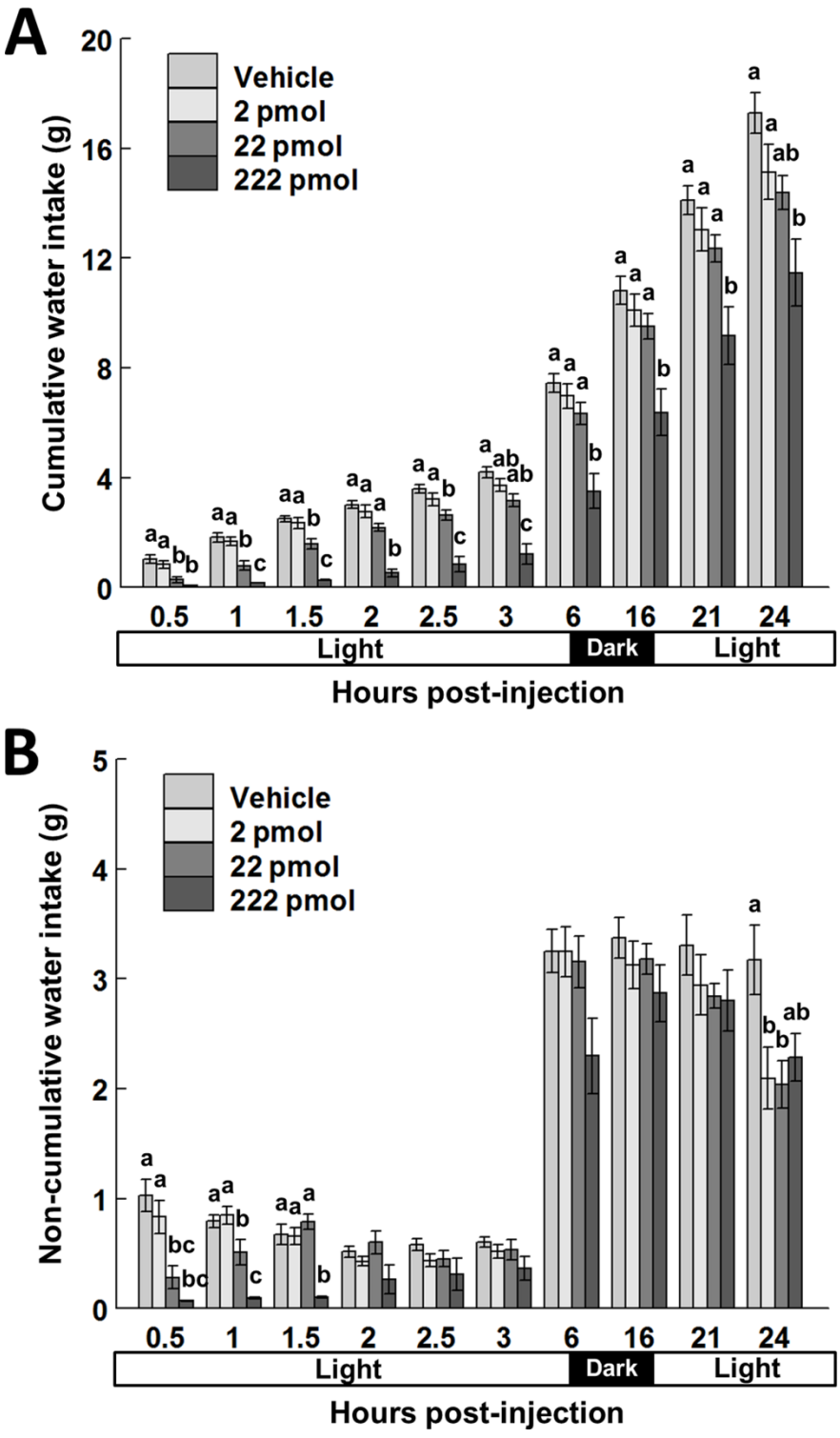

Fig. 4.2 Cumulative (A) and non-cumulative (B) water intake expressed as a percentage of body weight of Japanese quail at 7 days post-hatch (Experiment 1). Values are means ± S.E.M.; bars with different superscripts are different from each other within a time point ($P < 0.05$). There were 12 quail per group available for analysis.

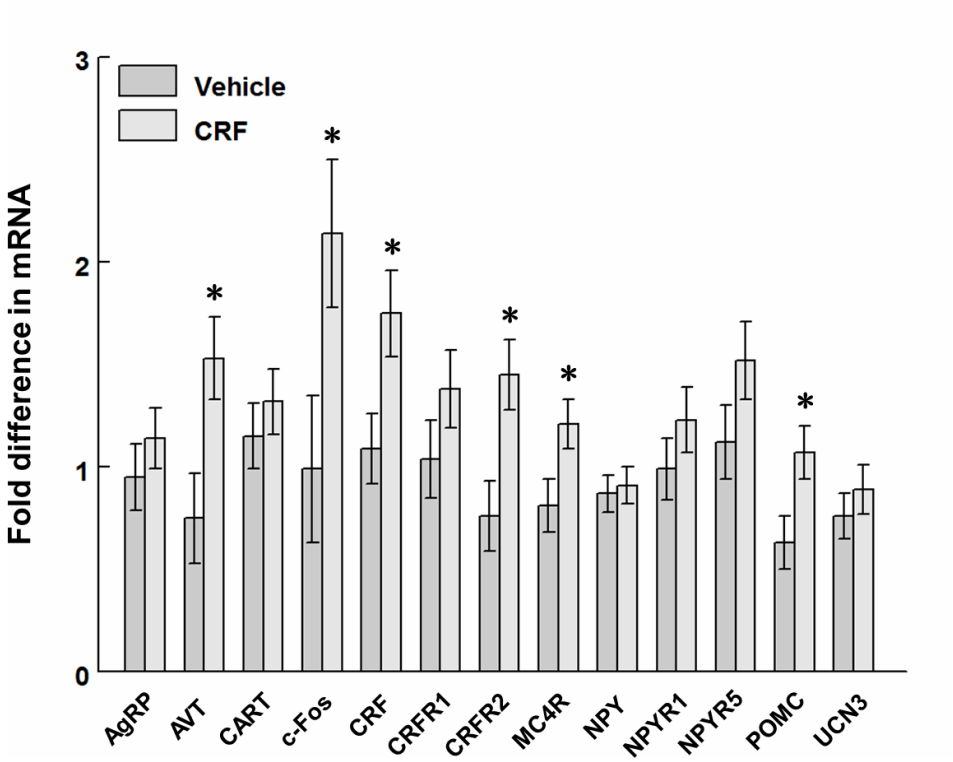

Fig. 4.3 Effect of ICV administration of CRF (22 pmol) in Japanese quail at 7 days post-hatch on hypothalamic expression of appetite-associated factor mRNA. (*) denotes difference from control ($P < 0.05$). Values are means ± S.E.M. For this experiment, 11 vehicle and 11 CRF-treated Japanese quail were available for the analysis. Abbreviations: agouti-related peptide (AgRP), arginine vasotocin (AVT), cocaine and amphetamine-regulated transcript (CART), corticotropin-releasing factor (CRF), corticotropin-releasing factor receptor sub-types 1 and 2 (CRFR1 and CRFR2, respectively), melanocortin receptor 4 (MC4R), neuropeptide Y (NPY), neuropeptide Y receptor sub-

types 1 and 5 (NPYR1 and NPYR5, respectively), pro-opiomelanocortin (POMC), urocortin 3 (UCN3).

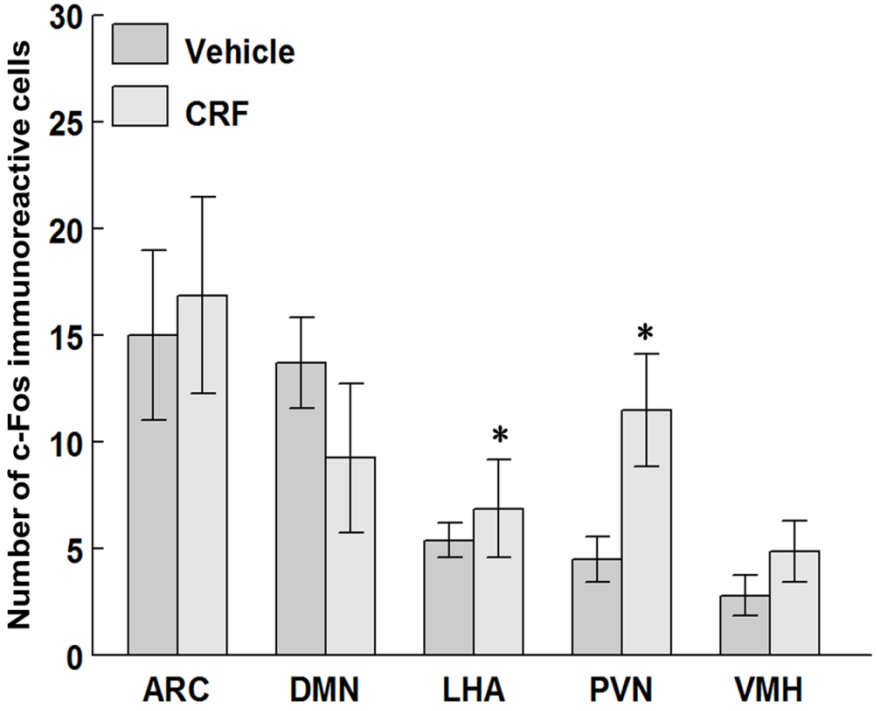

Fig. 4.4 Effect of ICV administration of CRF (22 pmol) in Japanese quail at 7 days post-hatch on the number of c-Fos immunoreactive cells in the arcuate (ARC), dorsomedial hypothalamic nucleus (DMN), lateral hypothalamic area (LHA), paraventricular nucleus (PVN), and ventromedial nucleus of the hypothalamus (VMH). (*) denotes difference from control ($P < 0.05$). Values are means ± S.E.M. For this experiment, 7 vehicle and 9 CRF-treated Japanese quail were available for the analysis.

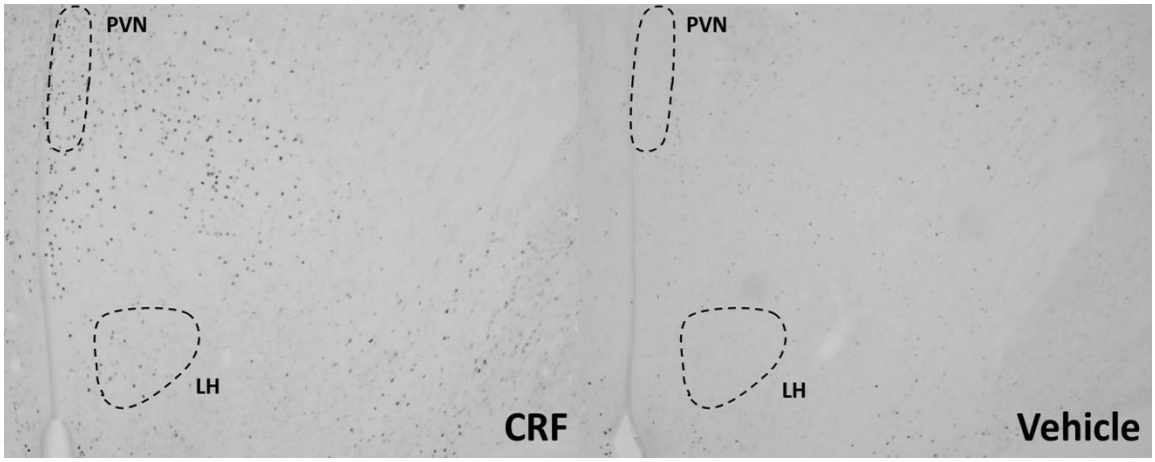

Fig. 4.5 Photomicrographs of c-Fos immunohistochemistry in the lateral hypothalamus (LH) and paraventricular nucleus (PVN) of the hypothalamus. Treatment is indicated on each photomicrograph.

Table 4.2 Count-type behaviors after ICV injection of CRF (22 pmol) in Japanese quail at 7 days post-hatch[1].

Count-type Behavior	Treatment	Time after injection (min)					
		5	10	15	20	25	30
Feeding pecking (n)	Vehicle	129.75 ±56.14	365.50 ±106.44	776.00 ±34.60*	1008.25 ±104.39*	1223.25 ±126.42*	1375.92 ±163.34*
	CRF	86.27 ±45.08	160.36 ±64.35	212.73 ±70.40	251.27 ±79.73	266.09 ±85.44	326.00 ±114.70
Exploratory pecking (n)	Vehicle	60.50 ±14.95	157.08 ±33.37	225.58 ±53.39	274.41 ±56.07	322.97 ±61.63	383.83 ±56.26
	CRF	58.55 ±16.48	130.73 ±26.74	226.00 ±37.43	318.00 ±51.86	393.82 ±62.54	474.09 ±72.65
Jumps (n)	Vehicle	4.00 ±1.04	4.58 ±0.96*	5.42 ±1.17*	4.58 ±0.96*	4.83 ±0.93*	5.17 ±1.08*
	CRF	2.36 ±1.19	2.55 ±1.16	2.63 ±1.19	2.64 ±1.19	2.73 ±1.18	2.73 ±1.18
Defecations(n)	Vehicle	0	0	0.08 ±0.08	0.17 ±0.11	0.17 ±0.11	0.25 ±0.13
	CRF	0	0	0	0	0.09 ±0.09	0.09 ±0.09
Steps (n)	Vehicle	279.08 ±30.16	536.08 ±57.77	776.00 ±56.21	988.17 ±74.46	1238.75 ±104.16	1594.00 ±121.09
	CRF	273.00 ±30.37	551.91 ±61.74	863.36 ±111.98	1142.45 ±150.55	1478.73 ±191.93	1853.73 ±238.68
Distance moved (m)	Vehicle	6.23 ±1.34	11.35 ±2.52	16.55 ±3.38	20.94 ±4.35	26.44 ±5.48	34.06 ±6.50
	CRF	5.19 ±0.76	10.15 ±1.97	15.58 ±3.32	19.79 ±5.25	25.59 ±5.62	32.40 ±7.31

[1]Twelve vehicle- and 12 CRF-treated Japanese quail per treatment group. Values are the means ± S.E.M. (*) denotes difference from control ($P < 0.05$).

Table 4.3 Timed-type behaviors after ICV injection of CRF (22 pmol) in Japanese quail at 7 days post-hatch[1]

Timed-type Behavior	Treatment	Time after injection (min)					
		5	10	15	20	25	30
Standing (s)	Vehicle	253.12 ±16.45	477.90 ±31.25	651.83 ±38.25	865.45 ±29.95	1062.73 ±30.95	1324.41 ±48.64
	CRF	265.90 ±13.46	536.26 ±19.81	807.60 ±20.77*	1070.74 ±25.28*	1341.58 ±34.13*	1607.11 ±44.69*
Sitting (s)	Vehicle	0.53 ±0.47	1.96 ±1.27	2.36 ±1.25	5.21 ±2.83	6.67 ±2.93	13.19 ±7.42
	CRF	2.65 ±1.72	5.29 ±2.37	12.01 ±7.67	33.23 ±18.15	56.02 ±32.57	66.26 ±39.35
Deep rest (s)	Vehicle	0	0	0	0	0	0
	CRF	0	0	0	0	0	0
Preening(s)	Vehicle	1.60 ±0.79	1.85 ±0.93	1.92 ±0.86	3.64 ±1.20	4.94 ±1.94	7.06 ±3.00
	CRF	0.334 ±0.33	1.22 ±0.75	4.12 ±2.23	4.91 ±2.51	6.32 ±3.27	7.44 ±4.00

[1]Twelve vehicle- and 12 CRF-treated Japanese quail per treatment group. Values are the means ± S.E.M. (*) denotes difference from control ($P < 0.05$).

Chapter 5: Hypothalamic mechanisms associated with neuropeptide K-induced anorexia in Japanese quail (*Coturnix japonica*)

Abstract

Central administration of neuropeptide K (NPK), a 36-amino acid peptide, is associated with anorexigenic effects in rodents and chickens. The mechanisms underlying the potent anorexigenic effects of NPK are still poorly understood. Thus, the aim of the present study was to identify the hypothalamic nuclei and neuropeptides that mediate anorexic effects of NPK in 7 day-old Japanese quail (*Coturnix japonica*). After a 6 hour fast, intracerebroventricular (ICV) injection of NPK decreased food and water intake for 180 minutes post-injection. Quail injected with NPK had more c-Fos immunoreactive cells in the arcuate nucleus (ARC), lateral hypothalamus, and paraventricular nucleus (PVN) compared to the birds that were injected with the vehicle. NPK injection decreased neuropeptide Y (NPY), NPY receptor sub-type 1, and agouti-related peptide mRNAs in the ARC whereas there was increased CART, POMC, and neurokinin receptor 1. NPK-injected quail expressed greater corticotropin-releasing factor (CRF), CRF receptor sub-type 2, melanocortin receptors 3 and 4, and urocortin 3 mRNA in the PVN. In conclusion, the results provide insights into understanding NPK-induced hypothalamic physiology and feeding behavior and suggest that the anorexigenic effects of NPK involve the ARC and PVN and increased CRF and melanocortin and reduced NPY signaling.

Key words: Neuropeptide K; Quail; Neurokinin Receptor 1; Arcuate Nucleus;

Paraventricular Nucleus

1. Introduction

Neuropeptide K (NPK), a 36-amino acid peptide, was first isolated from the porcine brain (Tatemoto et al., 1985) and is a member of the tachykinin family which includes a series of structurally and functionally related peptides, such as substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) (Steinhoff et al., 2014). In general, the tachykinins participate in a number of essential physiological processes in the nervous, respiratory, dermal, and immune systems, for example, smooth muscle contraction, epithelial secretion, and inflammation (Steinhoff et al., 2014). The biological functions of tachykinins are mediated by three types of neurokinin receptors (NKRs) named NK_1R, NK_2R, and NK_3R that all belong to the G protein-coupled receptor family (Almeida et al., 2004). NPK is distributed throughout the central nervous system in rats, in regions including the hypothalamus, amygdala, and the nucleus of the solitary tract (Valentino et al., 1986).

In rats, intracerebroventricular (ICV) administration of NPK results in tachycardia, elevation of arterial blood pressure and causes a series of behavioral alterations, such as elevated frequency of head scratching, grooming, and face-washing (Prat et al., 1994). Meanwhile, these NPK-induced responses are attenuated by NK_1R but not NK_2R, and NK_3R antagonists (CP96345 or RP67580), implying that NK_1R may be the primary receptor mediating those effects induced by exogenous NPK. ICV and intraperitoneal (IP) injection of NPK result in decreased food and water intake (Sahu et al., 1988b; Achapu et al., 1992) and also increased grooming behaviors (Achapu et

al., 1992). In chicks, NPK decreases food intake but does not affect water intake, and in NPK-treated chicks there was increased c-Fos immunoreactivity in several appetite-associated hypothalamic nuclei, such as the arcuate nucleus (ARC) and paraventricular nucleus (PVN) (Prall and Cline, 2008). In addition, chicks injected with NPK display a decreased number of feed pecks, exploratory pecks, and jumps whereas they spend more time standing (Prall and Cline, 2008).

In general, the hypothalamus is an essential center for regulation of feeding behavior and energy homeostasis, and contains several nuclei and neuropeptides that are important for regulating feeding behavior (Maniam and Morris, 2012; Parker and Bloom, 2012; Sinha and Jastreboff, 2013; Tachibana and Tsutsui, 2016). The chicken is the most common avian model for studying appetite regulation; however, the chicken has undergone intensive artificial selection for many growth and reproduction-associated traits, likely giving rise to changes in central mechanisms of feeding behavior. Compared with chickens, the Japanese quail (*Coturnix japonica*) is less artificially-selected and better adapted to the lab environment than a wild bird, thus may be more representative as a model for understanding feeding behavior regulation in birds. To date, the central mechanisms underlying NPK's anorexigenic effects are still unclear. Thus, the purpose of this study was to determine the effect of ICV injection of NPK on food intake and hypothalamic physiology in Japanese quail.

2. Materials and Methods

2.1. Animals

Japanese quail were bred and hatched in our vivarium. The breeder flock was

established with eggs donated by Mike Lacy at the University of Georgia. Upon removal from the hatcher, quail were group-caged in a brooder for 4 days, then individually caged in galvanized wire cages (8 cm wide, 7 cm deep, and 8 cm high) in a room maintained at a constant temperature of 35 ± 1 °C and a relative humidity of 50 ± 5% with a 14-hour light/10-hour dark period (lights on at 05:00 hours). At all times, unless otherwise noted, quail had ad libitum access to a mash diet (energy: 2,900 kcal ME/kg and 24% crude protein) and tap water. The individual cages allowed visual and auditory contact with other quail. After the quail were individually caged, they were handled twice daily to reduce the effects of stress on the day of data collection. The handling procedure was conducted as we described (Lear et al., 2017). All quail in this experiment were 7 days-old and there were 12 chicks in each treatment group. All procedures were performed according to the National Research Council publication, Guide for Care and Use of Laboratory Animals and were approved by the Virginia Tech Animal Care and Use Committee.

2.2. ICV injection procedure

On the day of the experiment, quail were injected using an adapted method that does not appear to induce physiological stress (Lear et al., 2017; Yuan et al., 2017). The head of the quail was briefly inserted into the restraining device. Injection coordinates were 2 mm anterior to the coronal suture, .75 mm lateral from the sagittal suture, and 1.5 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 base of the needle. The needle remained at injection

depth in the un-anesthetized quail for 5 seconds post-injection to reduce backflow. Quail were assigned to treatments at random. Porcine NPK (American Peptide Co., Sunnyvale, CA, USA) was dissolved in artificial cerebrospinal fluid (Anderson and Heisley, 1972) as a vehicle for a total injection volume of 5 µl with 0.1% Evans Blue dye to facilitate injection site localization. After data collection, the quail was decapitated and its head sectioned along the frontal plane to determine the site of injection. Any quail without dye present in the lateral ventricle system was eliminated from analysis. The sex of the quail was determined visually by dissection at the time of decapitation.

2.3. Experiment 1: food and water intake: higher doses

The quail were fasted for 6 hours prior to ICV injection of NPK. Quail were randomly assigned to receive either 0 (vehicle only), 0.01, 0.1, or 1.0 nmol of NPK by ICV injection (n=12 for each group). After injection, quail were returned to their individual home cages and given ad libitum access to food and water. Food and water intake were monitored (0.01 g) every 30 min for 180 min. Water weight (g) was converted to volume (ml; 1 g = 1 ml). Data were analyzed using analysis of variance (ANOVA) within each time point, and the statistical model included the main effect of dose. When dose effects were significant, Tukey's method of multiple comparisons was used to separate the means within each time point. Statistical significance was set at $P < 0.05$ for all experiments. Food and water intake results are shown on a cumulative and non-cumulative basis.

2.4 Experiment 2: food and water intake: lower doses

Procedures were identical to Experiment 1, except that doses were 0, 0.63, 2.5, or 10 pmol of NPK.

2.5 Experiment 3: water intake only

Procedures were identical to Experiment 1, except that quail were given access only to water.

2.6. Experiment 4: c-Fos immunohistochemistry

The quail were fasted for 6 hours prior to ICV injection of NPK. Quail were randomly assigned to receive either vehicle or 0.01 nmol of NPK (based on results of Experiment 1) by ICV injection (n=12 for each group). After injection, food was withheld to prevent c-Fos immunoreactivity associated with food consumption. Sixty minutes post-injection, as this is the time expected for the most robust c-Fos expression (Müller et al., 1984), quail were deeply anesthetized with sodium pentobarbital via cardiopuncture, then perfused via the carotid artery with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) containing 0.2% picric acid at pH 7.4. Brains were removed from skulls and post-fixed for 60 min in the same solution, after which they were blocked and placed through a series of graded sucrose incubations, consisting of 20, 30 and 40% in 0.1 M PB, until they sank in each. Several 60 μm coronal sections that contained appetite-related nuclei based on anatomies described by Kuenzel (Kuenzel and Masson, 1988) were collected using a cryostat at -15 °C, and then deposited in 0.02 M phosphate buffered saline (PBS) containing 0.1% sodium azide (Kuenzel et al., 1987). The PVN and lateral hypothalamus (LH) were collected corresponding to 7.8 interaural, the ventromedial

hypothalamus (VMH) was collected corresponding to 6.8 interaural and the dorsomedial nucleus (DMN) and the ARC were collected corresponding to 5.4 interaural. Sections were processed immediately after collection. Procedures for the c-Fos immunohistochemistry assay were performed as we described (Newmyer et al., 2013) using rabbit polyclonal anti-c-Fos at a dilution of 1:20,000 (K-25, Santa Cruz, CA, USA) (Zhao and Li, 2010; Newmyer et al., 2013). Anatomies were confirmed and a digital micrograph taken of each section. Overlays containing the respective nuclei boundaries were digitally merged with micrographs and the number of c-Fos immunoreactive cells within each respective nucleus counted by a technician blind to treatment. Data were analyzed via ANOVA using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC), with the model including the effect of treatment within each nucleus.

2.7 Experiment 5: mRNA abundance of appetite-associated factors in hypothalamic nuclei

The quail were fasted for 6 hours prior to ICV injection of NPK. Quail were randomly assigned to receive either 0 (vehicle) or 0.01 nmol of NPK via ICV injection (n=12 in each group). At one hour post-injection, each quail was deeply anesthetized with sodium pentobarbital via cardiopuncture and then perfused via the carotid artery with 1.0 mL of RNA stabilizing buffer (16.7 mM sodium citrate, 13.3 mM EDTA, and 3.5 M ammonium sulfate; pH = 5.2). Within 30 minutes of perfusion, brains were sectioned in a cryostat at -10°C into 500 µm thick coronal sections in the direction from rostral to caudal: LH and PVN were collected at plate 7.8, and ARC was

collected at plate 5.4 based on anatomy described (Kuenzel and Masson, 1988) (based on results of Experiments 4). Punches were collected using sterile disposable biopsy punch instruments (1 mm, Braintree Scientific Inc., Braintree, MA) and were immediately transferred to sterile microcentrifuge tubes containing RNA lysis buffer with 1% beta-mercaptoethanol (Norgen Biotek, Thorold, ON, Canada), vortexed, snap-frozen in liquid nitrogen, and stored at -80°C. The remaining brain section was photographed and punch accuracy verified via the overlays containing the respective nuclei boundaries.

The punches were thawed, vortexed vigorously for 30 seconds, and incubated at room temperature for 5 minutes before adding 70% molecular biology-grade ethanol, and total RNA was isolated following the manufacturer's instructions for the Total RNA Purification Micro Kit (Norgen Biotek). The concentration and purity of total RNA was assessed by spectrophotometry at 260/280/230 nm with a NanoDrop 2000 (Thermo Fisher Scientific Inc., West Palm Beach, FL, USA). Total RNA integrity was verified using an Experion (Bio-Rad, Hercules, CA) and RNA StdSens analysis kit (Bio-Rad), according to the manufacturer's instructions.

First-strand cDNA was synthesized in 20 µl reactions from 100 ng of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's instructions. Reactions were performed under the following conditions: 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 minutes. Primers for real time PCR were designed with Primer Express 3.0 software (Applied Biosystems) (Table 1) and validated for amplification efficiency

before use (95-105%). Real-time PCR reactions were performed in 10 µl reactions that contained 5 µl Fast SYBR Green Master Mix (Applied Biosystems), 0.5 µl primers (0.25 µl of 5 µM forward primer and 0.25 µl of 5 µM reverse primer), 1.5 µl nuclease-free water, and 3 µl 5-fold diluted cDNA using a 7500 Fast Real-Time PCR System (Applied Biosystems). Real-time PCR was performed under the following conditions: 95°C for 20 seconds and 40 cycles of 90°C for 3 seconds plus 60°C for 30 seconds. A dissociation step consisting of 95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds and 60°C for 15 seconds was performed at the end of each PCR reaction to ensure amplicon specificity.

Real-time PCR data were analyzed using the ΔΔCT method (Livak and Schmittgen, 2001) with β-actin as the reference gene. Due to the limited amount of template, not every factor was measured in all three nuclei. Thus, the analysis of each gene employed a different calibrator sample. The average of the vehicle quail in the LH was used as the calibrator sample for genes that were measured in all nuclei. The average of the vehicle quail within a nucleus was used as the calibrator sample for genes that were measured in only one nucleus. Relative quantities calculated as $2^{-\Delta\Delta CT}$ were used for statistical analysis. The statistical model included the main effect of treatment (vehicle and NPK), hypothalamic nuclei (ARC, LH, and PVN; not all genes were measured in all nuclei) and their interactions. Data were analyzed by ANOVA using JMP Pro 11 (SAS institute, Cary, NC, USA). Means were separated using Tukey's test when the interaction or effect of nucleus was significant. For all experiments, differences were considered to be significant at $P < 0.05$.

3. Results

3.1 Food and water intake

In Experiment 1, during the first 60 minutes post-injection, all treatment doses decreased food intake at same the magnitude (Fig. 5.1A). During 90-150 minutes post-injection, quail injected with 0.01 nmol of NPK ate less than those injected with the two higher doses but ate more than the vehicle group. The inhibitory effect of the 0.01 nmol dose of NPK eventually diminished at 180 minutes post-injection. Doses of 0.1 and 1.0 nmol of NPK decreased food intake through 180 minutes post-injection relative to the vehicle-injected quail. On a non-cumulative basis, differences were similar during the first 60 minutes post-injection (Fig. 5.1B). At 90 minutes post-injection, the two higher doses of NPK continued to decrease food intake, and there was not a difference between the lowest dose and vehicle-injected birds. At 120 minutes post-injection, only the quail injected with 1.0 nmol of NPK ate less than vehicle-injected group. At 150 minutes post-injection, quail injected with 0.01 nmol of NPK ate more than the 1.0 nmol dose-treated group, which is the only difference that was observed at that time point.

At 30, 60, and 90 minutes post-injection, all treatment doses decreased water intake to the same magnitude (Fig. 5.2A). During 120-180 minutes post-injection, the inhibitory effect of the lowest dose was weaker than the two higher doses but continued to decrease water intake compared to the vehicle group. Except for the 0.1 nmol dose at 120 minutes post-injection, quail treated with 0.1 and 1.0 nmol of NPK drank the least during this period of time. On a non-cumulative basis, water intake

responses were similar to food intake (Fig. 5.2B).

In Experiment 2, among the doses tested, only quail injected with 10 pmol of NPK ate less than the vehicle group during the entire observation period. The magnitude of this inhibition began to diminish at 90 minutes post-injection although the inhibition continued (Fig. 5.3A). For non-cumulative food intake, at 30 minutes post-injection, only the quail injected with 10 pmol of NPK ate less than the vehicle-treated group (Fig. 5.3B). At 180 minutes post-injection, the group injected with 0.63 pmol of NPK ate less than the vehicle-injected group, which was the only difference observed at that time point. No difference was observed at other time points. Water intake was similar, the only difference being that the magnitude of inhibition was reduced at 120 minutes post-injection (Fig. 5.4A). On a non-cumulative basis, only the highest dose of NPK decreased water intake at 30 minutes post-injection (Fig. 5.4B).

In Experiment 3, there was no difference in water intake among groups at any of the time points (Fig. 5.5 A and 5B).

3.2 Hypothalamic c-Fos immunoreactivity

The number of c-Fos immunoreactive cells in NPK-injected quail increased by about 260% ($P = 0.0329$), 210% ($P = 0.0186$), and 280% ($P = 0.0186$) over the numbers in vehicle-injected quail in the PVN, LH, and ARC, respectively (Fig. 5.6). The c-Fos immunoreactivity was similar between groups in the DMN and VMH.

3.3 Hypothalamic mRNA abundance

Results for the mRNA abundance of anorexigenic and orexigenic factors are summarized in Tables 5.2 and 5.3, respectively. Results will be described in rank

order from interactions to main effects.

Interactions of treatment and nucleus. There were interactions of treatment and nucleus for neuropeptide Y (NPY; P <.0001), NPY receptor sub-type 1 (NPYR1; P <.0001), corticotropin-releasing factor (CRF; P <.0001), CRF receptor sub-type 2 (CRFR2; P = 0.0081) and NK_1R (P = 0.0014). NPK injection decreased NPY and NPYR1 and increased NK_1R mRNA in the ARC but did not affect expression in other nuclei (Figures 5.8 A, B, and C, respectively). NPK-injected quail expressed greater amounts of CRF (about 200-fold) and CRFR2 mRNA in the PVN than vehicle-treated birds with no difference between groups in the other nuclei (Figures 5.8 D and E, respectively).

Main effect of treatment. ICV injection of NPK increased pro-opiomelanocortin (POMC; P = 0.0008) and cocaine and amphetamine-regulated transcript (CART; P = 0.002) mRNAs in the ARC and melanocortin receptors 3 and 4 (MC3R and MC4R; P = 0.0055 and 0.004, respectively) and urocortin 3 (UCN3; P < .0001) in the PVN. NPK injection decreased mRNA abundance of agouti-related peptide (AgRP; P = 0.0008). The CRF receptor sub-type 1 (CRFR1; P = 0.0143) and NK_3R (P = 0.0113) mRNAs were greater in vehicle- than NPK-injected quail.

Main effect of nucleus. NPY receptor sub-type 5 (NPYR5; P <.0001) and NK_3R (P <.0001) mRNAs were differentially distributed among nuclei as follows: PVN > LH = ARC. NK_2R (P <.0001) and CRFR1 (P <.0001) mRNAs were distributed as follows: ARC = LH > PVN and LH > ARC > PVN, respectively.

4. Discussion

The anorexigenic effects of NPK in 7 day-old Japanese quail are consistent with effects reported for Cobb-500 chicks (Prall and Cline, 2008) and rats (Sahu et al., 1988b; Achapu et al., 1992; Trivedi et al., 2015). In chicks and rats, the lowest effective doses of NPK are 0.3 nmol (Prall and Cline, 2008) and 0.13 nmol (Achapu et al., 1992), causing a 43% and 80% reduction in food intake at 30 minutes post-injection, respectively. In the present study, the lowest effective dose of NPK (0.01 nmol) is much lower than the lowest effective dose in chicks and rats; however, it had a much greater anorexic effect, about a 450% reduction in food intake. Thus, there are differential dose thresholds for NPK's anorexic effects across species. Differences in experimental design, diet, age, body weight, and genetic background may also contribute to such differences, although such a large difference in efficacy for the quail would suggest that the difference has an evolutionary basis.

On a non-cumulative basis, NPK exerted its anorexigenic effect within 30 minutes post-injection and the anorexigenic effects of 0.01, 0.1, and 1.0 nmol NPK began to dissipate at 90, 120, and 150 minutes post-injection, respectively, demonstrating that it is a fast-acting satiety-associated factor in quail. Moreover, we did not observe compensatory food intake, which is consistent with previous studies (Achapu et al., 1992; Prall and Cline, 2008).

Unlike food intake, the effect of NPK on water intake varies across species. The inhibitory effect on water intake was observed in rats (Achapu et al., 1992) but not in chicks (Prall and Cline, 2008). In the present study, NPK had similar effects on food

and water intake in quail, although injection of NPK did not decrease water intake in the absence of food, indicating that NPK-induced effects on water intake were prandial.

To understand the hypothalamic mechanism underlying the anorexigenic effect of NPK in Japanese quail, we measured c-Fos immunoreactivity at 1 hour post-injection. In general, increased c-Fos immunoreactivity is indicative of recent activation of a brain region (Kovács, 1998). We observed increased c-Fos immunoreactivity in the ARC, LH and PVN in response to ICV injection of NPK. These three activated hypothalamic nuclei are important in the regulation of feeding behavior and energy balance. For example, the ARC is thought to contain the 'first order neurons' integrating satiety and hunger signals from the central and peripheral nervous systems (Funahashi et al., 2000; Hillebrand et al., 2002). The primary role of the PVN is to integrate signals from appetite-associated neuronal pathways, such as orexin neurons from the LH or NPY/AgRP neurons from the ARC (Elmquist et al., 1998b; Elmquist et al., 1999). In chicks, NPK injection is associated with increased c-Fos immunoreactivity in the ARC and PVN, but not the LH (Prall and Cline, 2008), indicating there might be species-specific physiological effects.

We then measured mRNA abundance of several appetite-associated factors in the nuclei activated in response to NPK injection. ICV injection of NPK increased mRNA expression of NK_1R in the ARC. This was the only effect that we observed for neurokinin receptors, suggesting that NK_1R may mediate the hypothalamic actions of NPK in quail. As one of the receptors for the tachykinin family, the NK_1R is widely

distributed throughout the brain, with high expression in regions such as the hypothalamus, thalamus, and amygdala (Yip and Chahl, 2001; Halasz et al., 2009). This receptor distribution in the brain is conserved across species (Rigby et al., 2005). In general, SP displays a high affinity for NK_1R, NKA and NPK prefer NK_2R, and NKB preferentially binds to NK_3R (Debeljuk and Lasaga, 1999). Our results revealed that NK_1R mRNA was more highly expressed in the PVN than in the LH and ARC, which is in agreement with what has been reported in rats (Maeno et al., 1993). NK_2R and NK_3R mRNA abundance displayed nucleus-specific distributions, which are similar to the findings in other species (Ding et al., 1999; Pennefather et al., 2004). In addition to NK_1R, ICV injection of NPK increased CART and POMC whereas injection was associated with decreased NPY, NPYR1 and AgRP mRNA expression in the ARC. It is well known that two functionally discrete neuronal populations in the ARC exert opposite effects on appetite regulation including the anorexigenic, POMC/CART and the orexigenic, NPY/AgRP neurons (Hillebrand et al., 2002; Parkinson et al., 2008). To date, the functions of each neuropeptide on feeding behavior have been extensively documented and thus will not be discussed here in detail. However, it should be noted that the neurons expressing those appetite-associated neuropeptides have extensive reciprocal connections, forming a complex network to regulate feeding behavior. In regards to the results in the ARC, increased anorexigenic factor and decreased orexigenic factor gene expression may collectively generate an net inhibitory effect on food intake. There are neuroanatomical connections between tachykinergic terminals and POMC neurons in the ARC

(Magoul et al., 1994). Because of a lack of accompanying protein/peptide data, mRNA results herein should be interpreted with caution as they may not reflect changes that are occurring on a functional level, although transcriptional changes hint at the pathways involved.

In the PVN, ICV injection of NPK was associated with an increase in the mRNA abundance of a group of anorexigenic factors, including MC3R, MC4R, UCN3, CRF and CRFR2. CRF and UCN3, belonging to the CRF family, have potent anorexigenic effects across species (Stengel and Taché, 2014) and CRFR2 is the primary receptor mediating the anorexigenic effect of CRF (Bradbury et al., 2000; Pelleymounter et al., 2000; Cullen et al., 2001). In chicks, blockage of CRF receptors via the antagonist astressin does not influence the anorexigenic effect of NPK, suggesting that the CRF system may not be involved in NPK-associated anorexia (Prall and Cline, 2008). In the present study, however, the NPK-induced increases in gene expression of CRF receptors and endogenous ligands provide evidence that mechanisms might be different between chicks and quail and that in quail NPK-induced anorexia may involve CRF signaling, a hypothesis that requires further evaluation.

MC3R and MC4R are two major receptors in melanocortin system signaling and play essential roles in the regulation of feeding behavior, namely, inhibition of food intake (Cone, 2005). There are reciprocal connections between the ARC and PVN through monosynaptic neuronal projections (Tóth and Palkovits, 1998). Thus, changes in gene expression of two MCRs suggest involvement of the melanocortin system in mediating NPK's effects in the both ARC and PVN.

In our study, ICV injection of NPK did not affect mRNA abundance of appetite-associated factors in the LH, although there was increased c-Fos immunoreactivity in this nucleus. This may be interpreted that other appetite-associated factors were influenced by NPK or that changes induced were occurring at the post-translational level during this timeframe. Another explanation is that the LH may be involved in other physiological activities induced by NPK. For instance, NPK is associated with hypotension and gallbladder contraction (Tatemoto et al., 1985), and the LH is implicated in hypotensive responses (Pajolla et al., 2001) and regulation of gallbladder pressure (Furukawa and Okada, 1991).

In conclusion, ICV injection of NPK induced anorexigenic effects in 7-day old Japanese quail. The hypothalamic mechanisms likely involve NK_1R and the ARC and PVN, and also possibly CRF and melanocortin signaling. These results thus provide targets for future studies aimed at further understanding anorexigenic mechanisms and provide evolutionary perspective.

Table 5.1 Primers for real-time PCR[1]

Gene	Accession No.	Sequences (forward/reverse)
AgRP	XM_015873899.1	GGTTCTTCAACGCCTTCTGCTA/TTCTTGCCACATGGGAAGGT
CART	XM_003643097.3	GCTGGAGAAGCTGAAGAGCAA/GGCACCTGCCCGAACTT
CRF	XM_015855730.1	TCAGCACCAGAGCCATCACA/GCTCTATAAAAATAAAGAGATGACATCAGA
CRFR1	XM_015885973.1	CTGCTGCCCTTGCTGGGAAT/ATCCTCCCCTGGATTGAC
CRFR2	XM_015852926.1	GGATCAAATACAACACCACAAGAAAT/AGCCCACGTCCCATTGC
MC3R	XM_015881722.1	GCCTCCCTTTATGTTCACATGT/GCTGCGATGCGCTTCAC
MC4R	XM_015854466.1	CATCAGCTTGCTGGAGAACGT/GCGAATGGAGGTTCTTGTTCTT
NPY	XM_015853870.1	CATGCAGGGCACCATGAG/CAGCGACAGGGCGAAAGTC
NPYR1	XM_015861016.1	TAGCCATGTCCACCATGCA/GGGCTTGCCTGCTTTAGAGA
NPYR5	XM_015861003.1	GGCTGGCTTTGTGGGAAA/CTGTCTTCTGCTTGCGTTTTGT
NK_1R	XM_015883061.1	CTGCTCACCTGCGATGCTT/GGAACACGTGAAATCAGTCTGAAG
NK_2R	XM_015866389.1	GGCCACAGGCAAGTATGAATTC/AACTGGAGTGATTTTTGCAAAGG
NK_3R	XM_015861456.1	CGCATGAGGACTGTGACCAAT/GCCATGGAGGCATCAGAGAA
POMC	NM_001323229.1	GCCAGACCCCGCTGATG/CTTGTAGGCGCTTTTGATGAT
UCN3	XM_015859981.1	GGGCCTTCCGCCTCTACAACG/GGTGAGGGCTGCGTTGAG
β-actin	XM_015876619.1	GTCCACCGCAAATGCTTCTAA/TGCGCATTTATGGGTTTTGTT

[1]Primers were designed with Primer Express 3.0 (Applied Biosystems).

Abbreviations: agouti-related peptide (AgRP), cocaine and amphetamine-regulated transcript (CART), corticotropin-releasing factor (CRF), CRF receptor sub-types 1 and 2 (CRFR1 and CRFR2, respectively), melanocortin receptors 3 and 4 (MC3R and MC4R, respectively), neuropeptide Y (NPY), NPY receptor sub-types 1 and 5 (NPYR1 and NPYR5, respectively), neurokinin receptors 1, 2 and 3 (NK_1R, NK_2R, and NK_3R), pro-opiomelanocortin (POMC), and urocortin 3 (UCN3).

Table 5.2 Anorexigenic neuropeptides and receptors mRNA in hypothalamic nuclei[1]

Effect	CRF	CRFR1	CRFR2	NK_1R	NK_2R	NK_3R	POMC	CART	MC3R	MC4R	UCN3
Treatment											
Vehicle	0.12±0.85	0.31±0.03	8.60±1.27	1.42±0.16	0.15±0.03	1.95±0.12	3.67±5.07	0.56±0.94	0.002±0.89	2.13±2.06	1.14±0.46
NPK	5.38±0.72	0.20±0.03	10.69±1.20	1.39±0.15	0.26±0.03	1.44±0.11	30.57±3.58	4.79±0.56	3.77±0.66	11.63±1.82	4.90±0.43
P-value	<.0001	0.0143	0.2091	0.7688	0.1602	0.0113	0.0008	0.002	0.0055	0.004	<.0001
Nucleus											
ARC	0.01±0.89[b]	0.12±0.03[c]	14.23±1.39[a]	1.10±0.18[b]	0.33±0.04[a]	0.61±0.15[b]	17.12±3.10	2.67±0.54	NM	NM	NM
LH	0.13±0.94[b]	0.51±0.05[a]	1.70±1.57[b]	0.82±0.19[b]	0.35±0.05[a]	0.70±0.14[b]	NM	NM	NM	NM	NM
PVN	10.79±1.06[a]	0.29±0.03[b]	11.76±1.57[a]	2.49±0.21[a]	0.03±0.03[b]	3.33±0.13[a]	NM	NM	1.88±0.55	6.88±1.37	3.02±0.31
P-value	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001					
Interactions											
Treatment*Nucleus	<.0001	0.0639	0.0081	0.0014	0.2396	0.9508					

[1] Relative quantity values were analyzed by ANOVA and the model included the main effect of treatment (vehicle and NPK), hypothalamic nuclei (ARC, LH, and PVN) and their interactions. Data are expressed as means ± standard error with corresponding *P*-values for main effects and interactions (n=12 per group). Unique letters denote a difference within the gene and effect, $P < 0.05$ (Tukey's test). Abbreviations: arcuate nucleus (ARC), lateral hypothalamus (LH) and paraventricular nucleus (PVN), corticotropin-releasing factor (CRF), CRF receptor sub-type 1 and 2 (CRFR1 and CRFR2, respectively), neurokinin receptors 1, 2 and 3 (NK_1R, NK_2R, and NK_3R), pro-opiomelanocortin (POMC), cocaine and amphetamine-regulated transcript (CART), melanocortin receptors 3 and 4 (MC3R and MC4R, respectively), and urocortin 3 (UCN3). NM

indicates that the respective mRNA was not measured in those nuclei.

Table 5.3 Orexigenic neuropeptides and receptors mRNA in hypothalamic nuclei[1]

Effect	NPY	NPYR1	NPYR5	AGRP
Treatment				
Vehicle	1.67 ±0.16	8.80 ±1.05	5.87 ±0.81	6.34 ±0.71
NPK	0.91 ±0.17	3.25 ±0.93	5.84 ±0.76	1.95 ±0.65
P-value	0.0004	0.0011	0.5793	0.0008
Nucleus				
ARC	2.84 ±0.12[a]	15.91 ±1.11[a]	2.76 ±0.87[b]	4.14 ±0.48
LH	0.52 ±0.23[b]	0.95 ±1.14[b]	2.19 ±1.02[b]	NM
PVN	0.66 ±0.19[b]	0.81 ±1.37[b]	13.34 ±0.98[a]	NM
P-value	<.0001	<.0001	<.0001	
Interactions				
Treatment*Nucleus	<.0001	<.0001	0.1228	

[1]Relative quantity values were analyzed by ANOVA and the model included the main effect of treatment (vehicle and NPK), hypothalamic nuclei (ARC, LH, and PVN) and their interactions. Data are expressed as means ± standard error with corresponding *P*-values for main effects and interactions (n=12 per group). Unique letters denote a difference within the gene and effect, $P < 0.05$ (Tukey's test). Abbreviations: arcuate nucleus (ARC), lateral hypothalamus (LH) and paraventricular nucleus (PVN), agouti-related peptide (AgRP), neuropeptide Y (NPY), and NPY

receptor sub-types 1 and 5 (NPYR1 and NPYR5, respectively). NM indicates that the respective mRNA was not measured in those nuclei.

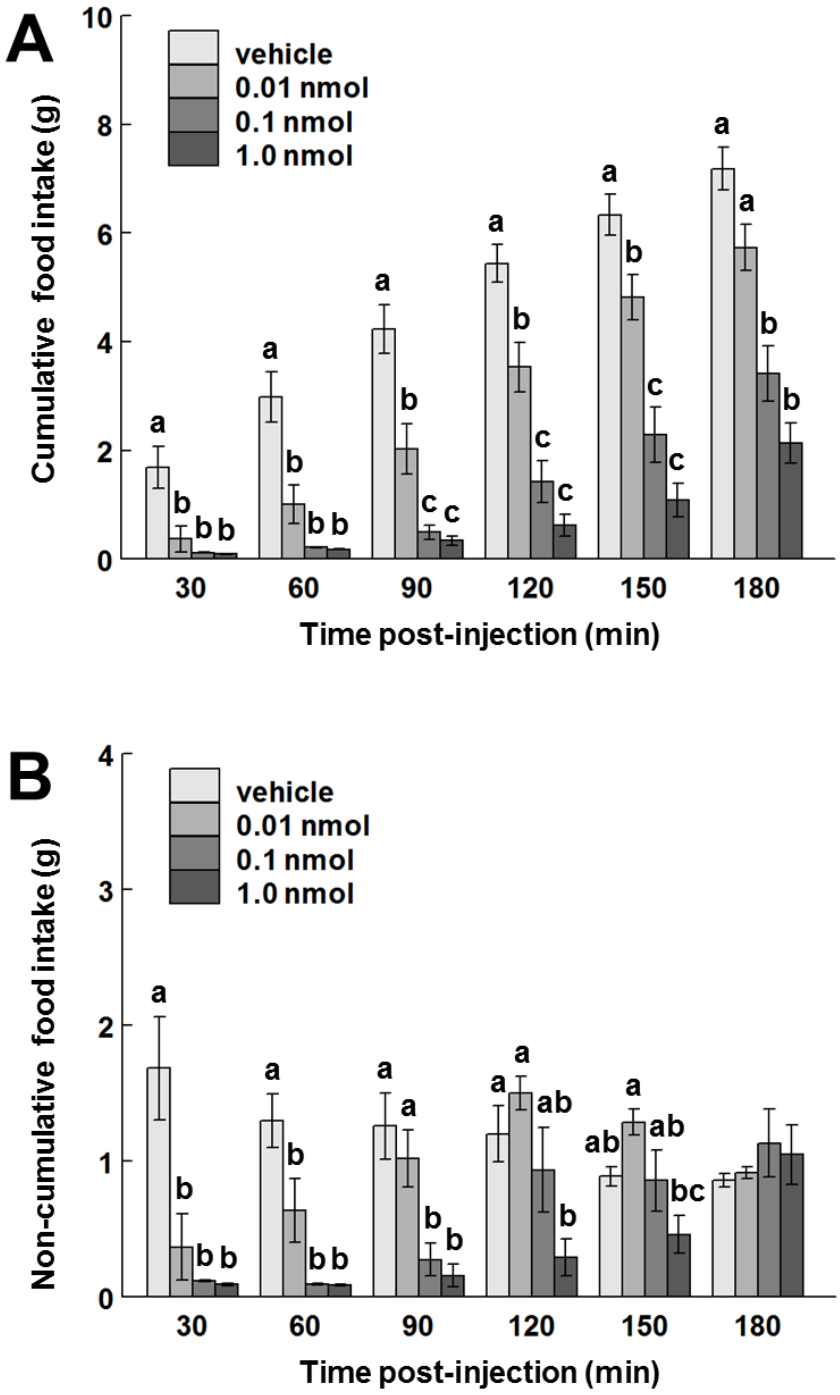

Fig. 5.1 Cumulative food intake (A) and non-cumulative food intake (B) expressed as a percentage of body weight of Japanese quail at 7 days post-hatch (Experiment 1). Values are means ± standard error; bars with different superscripts are different from each other

within a time point ($P < 0.05$). There were 12 quail per group available for analysis.

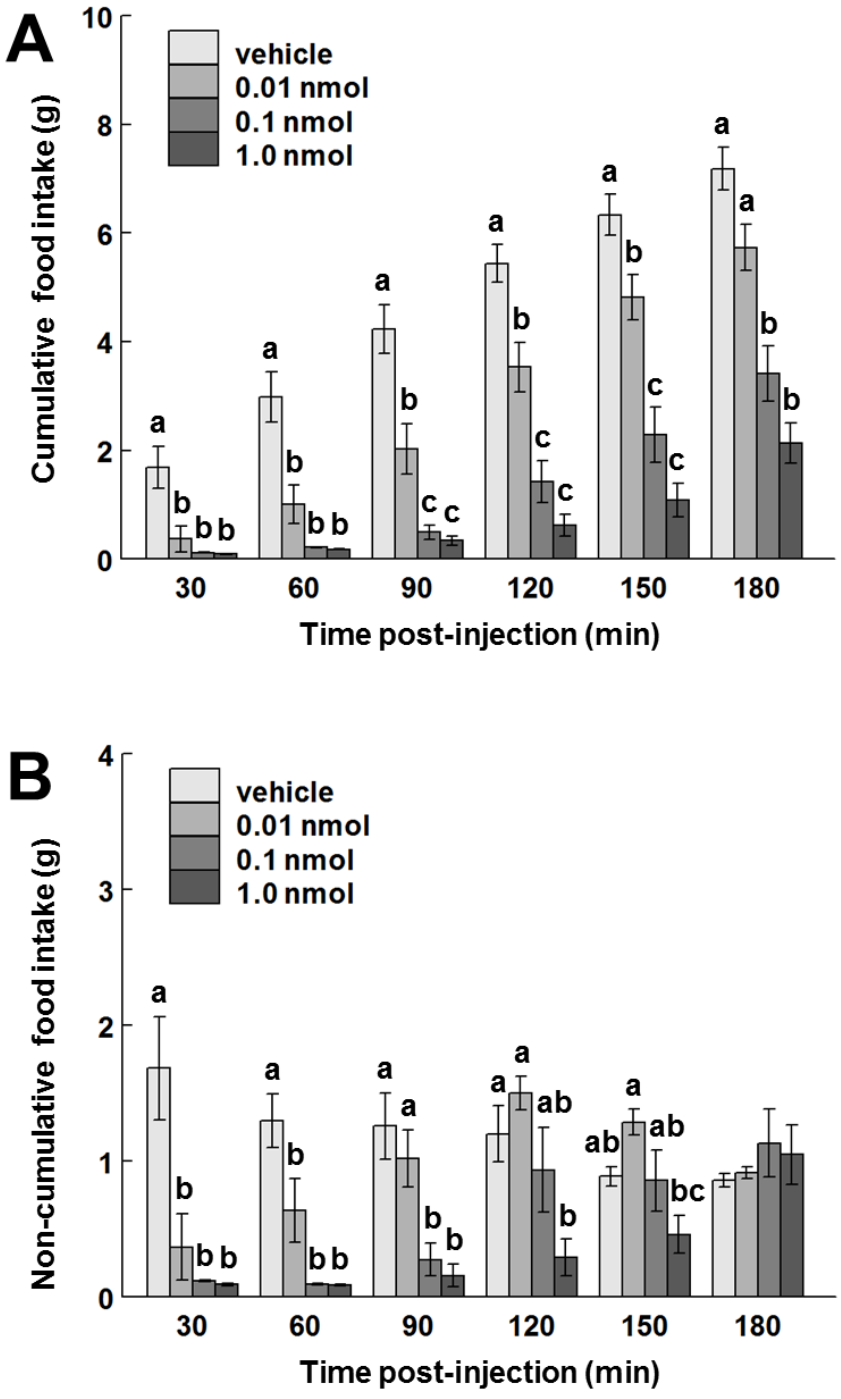

Fig. 5.2 Cumulative water intake (A) and non-cumulative water intake (B) expressed as a

percentage of body weight of Japanese quail at 7 days post-hatch (Experiment 1). Values are means ± standard error; bars with different superscripts are different from each other within a time point ($P < 0.05$). There were 12 quail per group available for analysis.

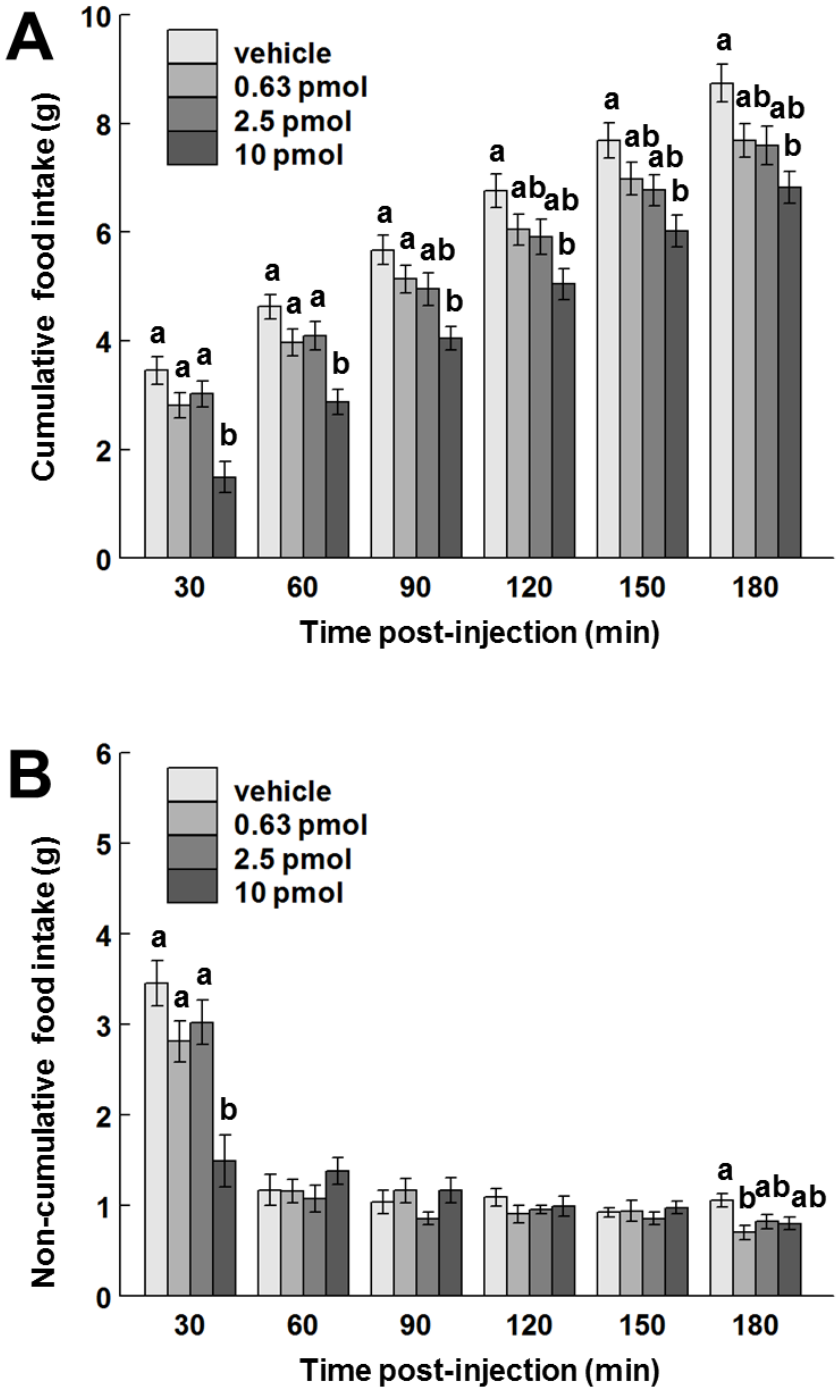

Fig. 5.3 Cumulative food intake (A) and non-cumulative food intake (B) expressed as a percentage of body weight of Japanese quail at 7 days post-hatch (Experiment 2). Values are means ± standard error; bars with different superscripts are different from each other within a time point ($P < 0.05$). There were 12 quail per group available for analysis.

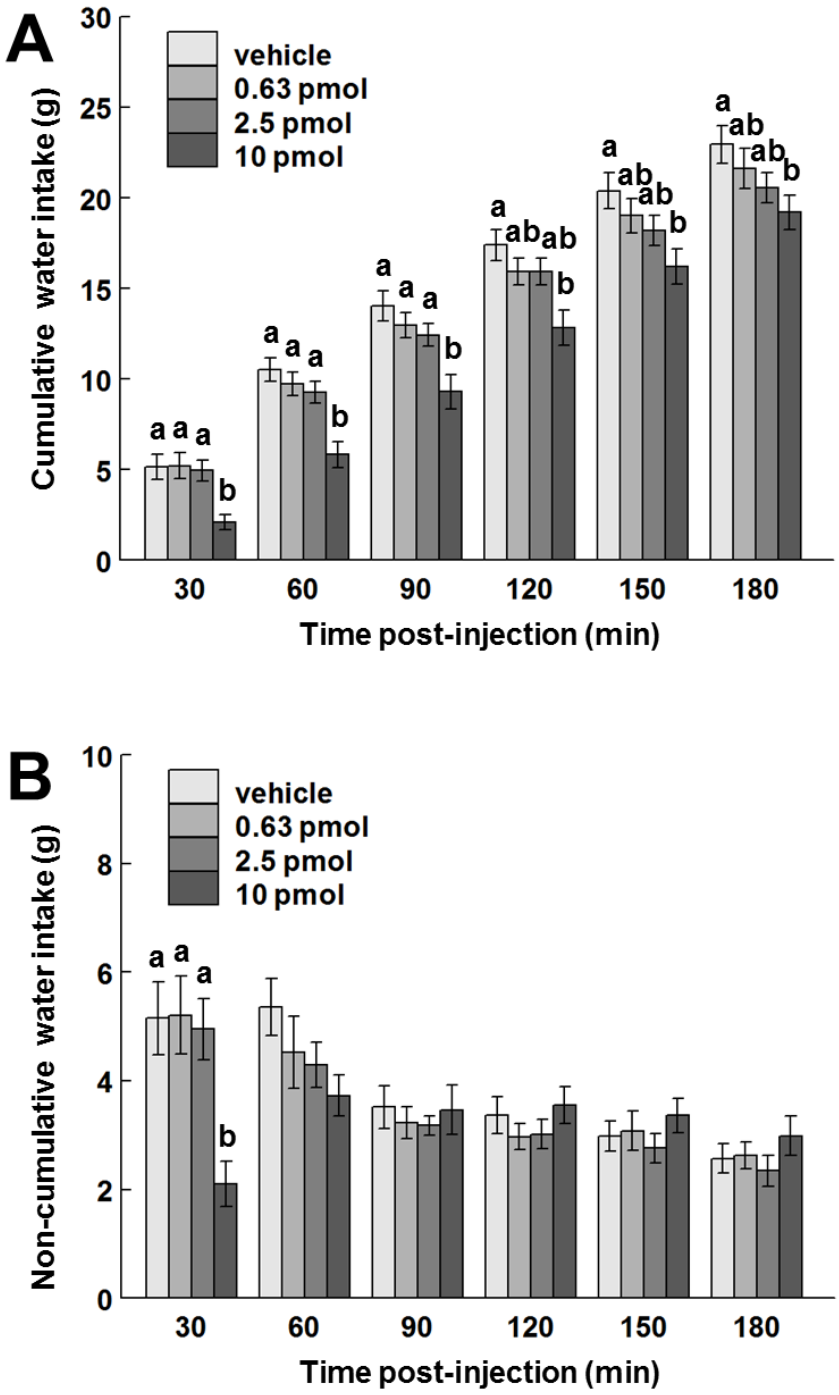

Fig. 5.4 Cumulative water intake (A) and non-cumulative water intake (B) expressed as a percentage of body weight of Japanese quail at 7 days post-hatch (Experiment 2). Values are means ± standard error; bars with different superscripts are different from each other

within a time point ($P < 0.05$). There were 12 quail per group available for analysis.

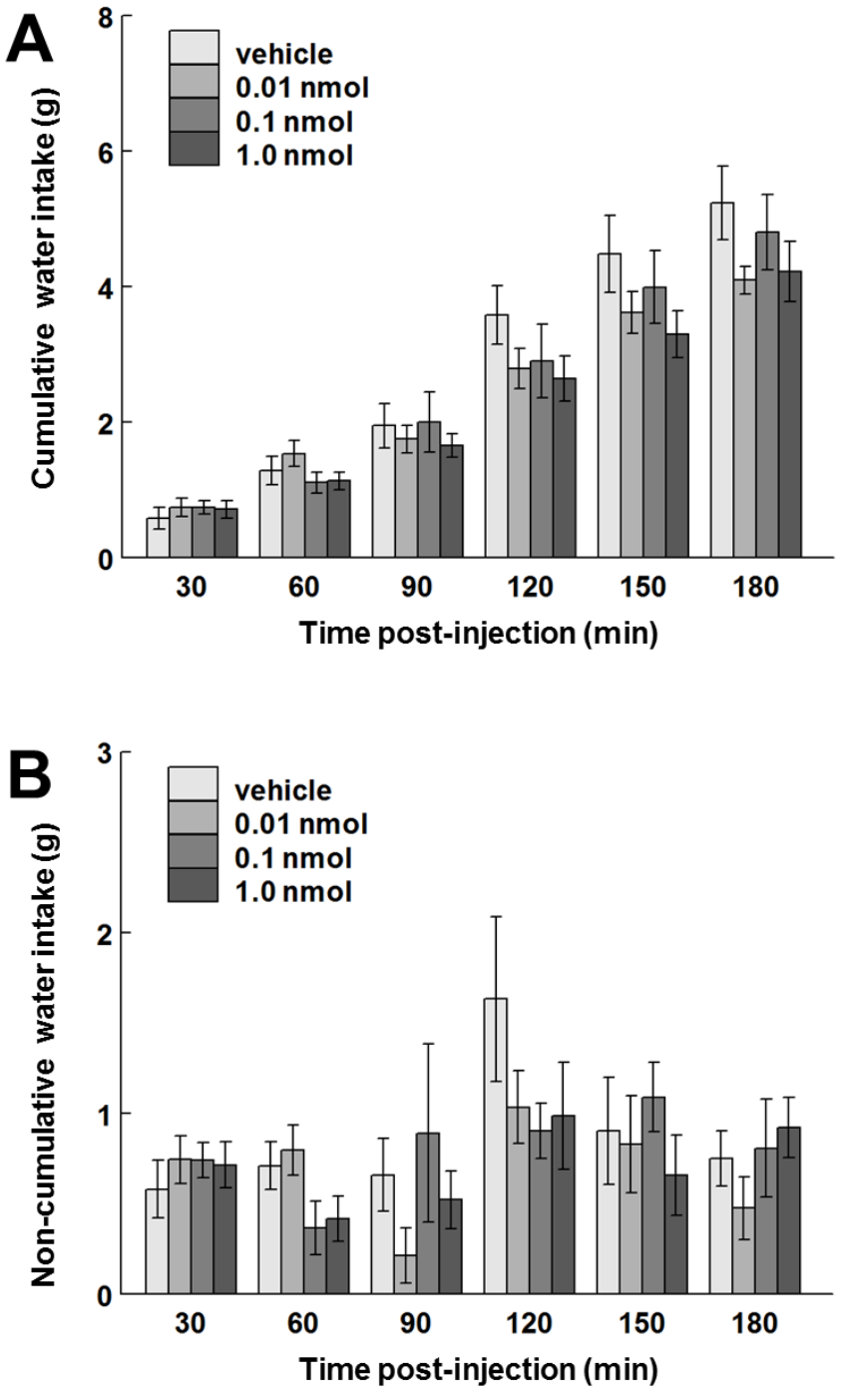

Fig. 5.5 Cumulative water intake (A) and non-cumulative water intake (B) expressed as a

percentage of body weight of Japanese quail at 7 days post-hatch (Experiment 3). Values are means ± standard error; bars with different superscripts are different from each other within a time point ($P < 0.05$). There were 12 quail per group available for analysis.

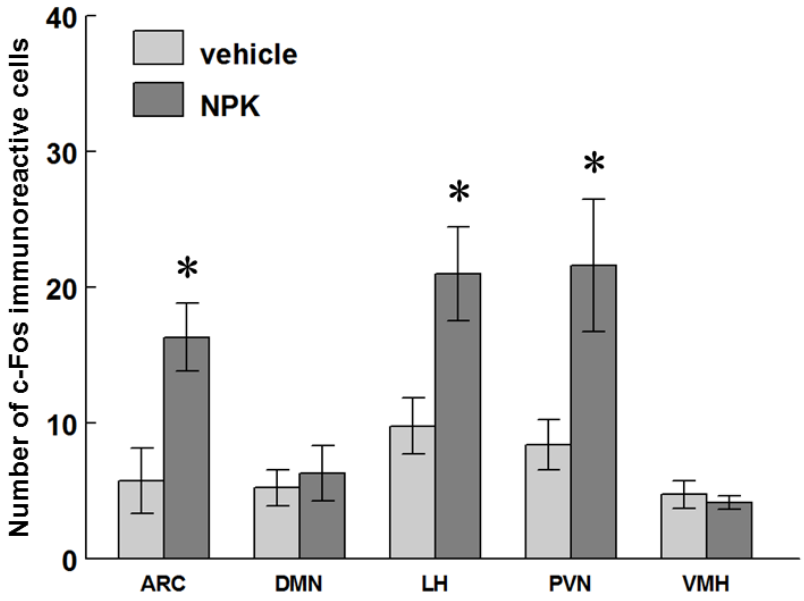

Fig. 5.6 Effect of ICV administration of NPK (0.01 nmol) in Japanese quail at 7 days post-hatch on the number of c-Fos immunoreactive cells in the arcuate nucleus (ARC), dorsomedial nucleus (DMN), lateral hypothalamus (LH), paraventricular nucleus (PVN), and ventromedial hypothalamus (VMH) (Experiment 4). (*) denotes difference from vehicle-injected group ($P < 0.05$). Values are means ± S.E.M. For this experiment, 7 vehicle and 8 NPK-treated Japanese quail were available for the analysis.

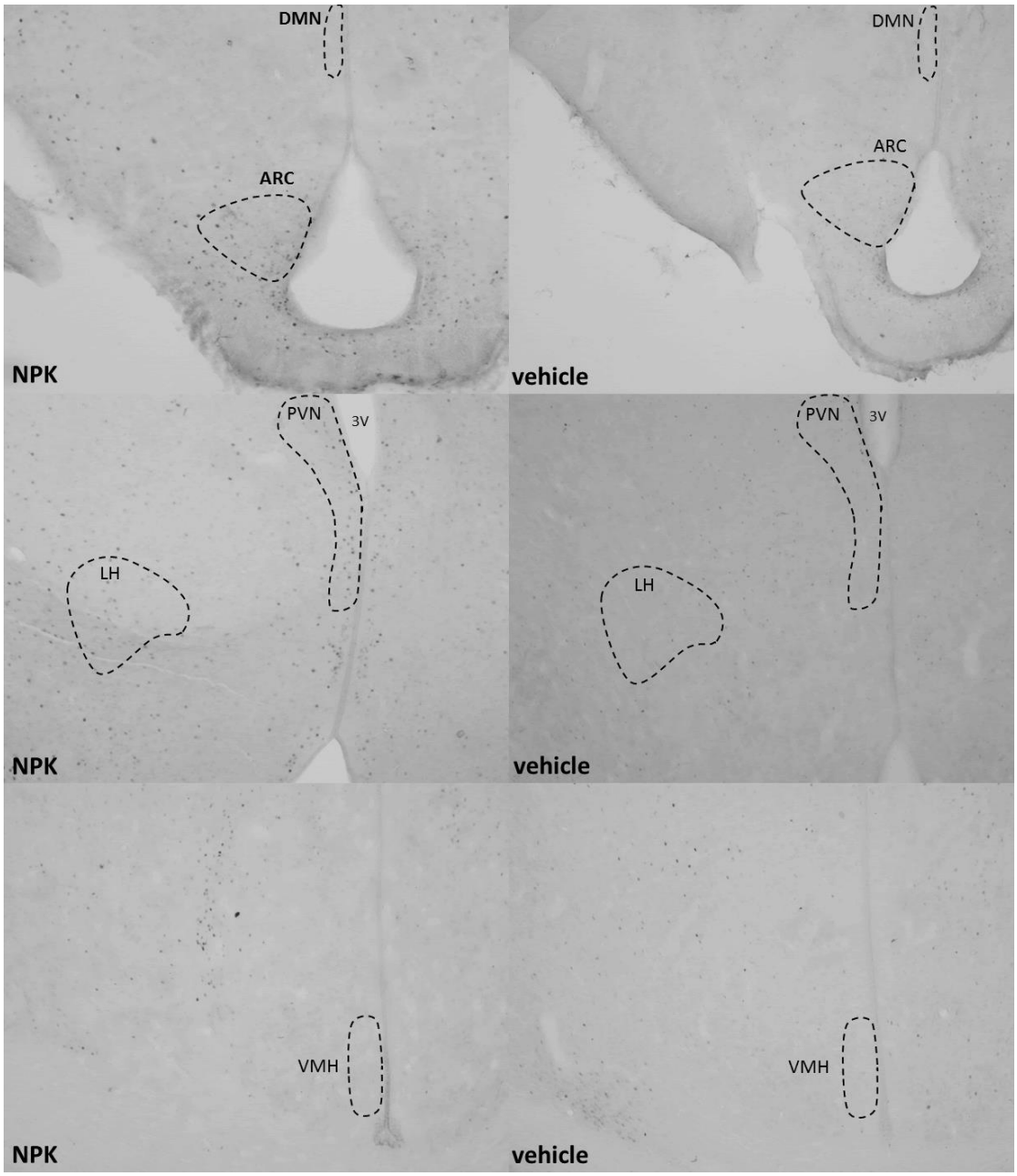

Fig. 5.7 Photomicrographs after immunohistochemical detection of c-Fos in the arcuate nucleus (ARC), dorsomedial nucleus (DMN), lateral hypothalamus (LH), paraventricular nucleus (PVN), and ventromedial hypothalamus (VMH) (Experiment 4). Treatment is indicated on each photomicrograph. “3v” denotes the third ventricle.

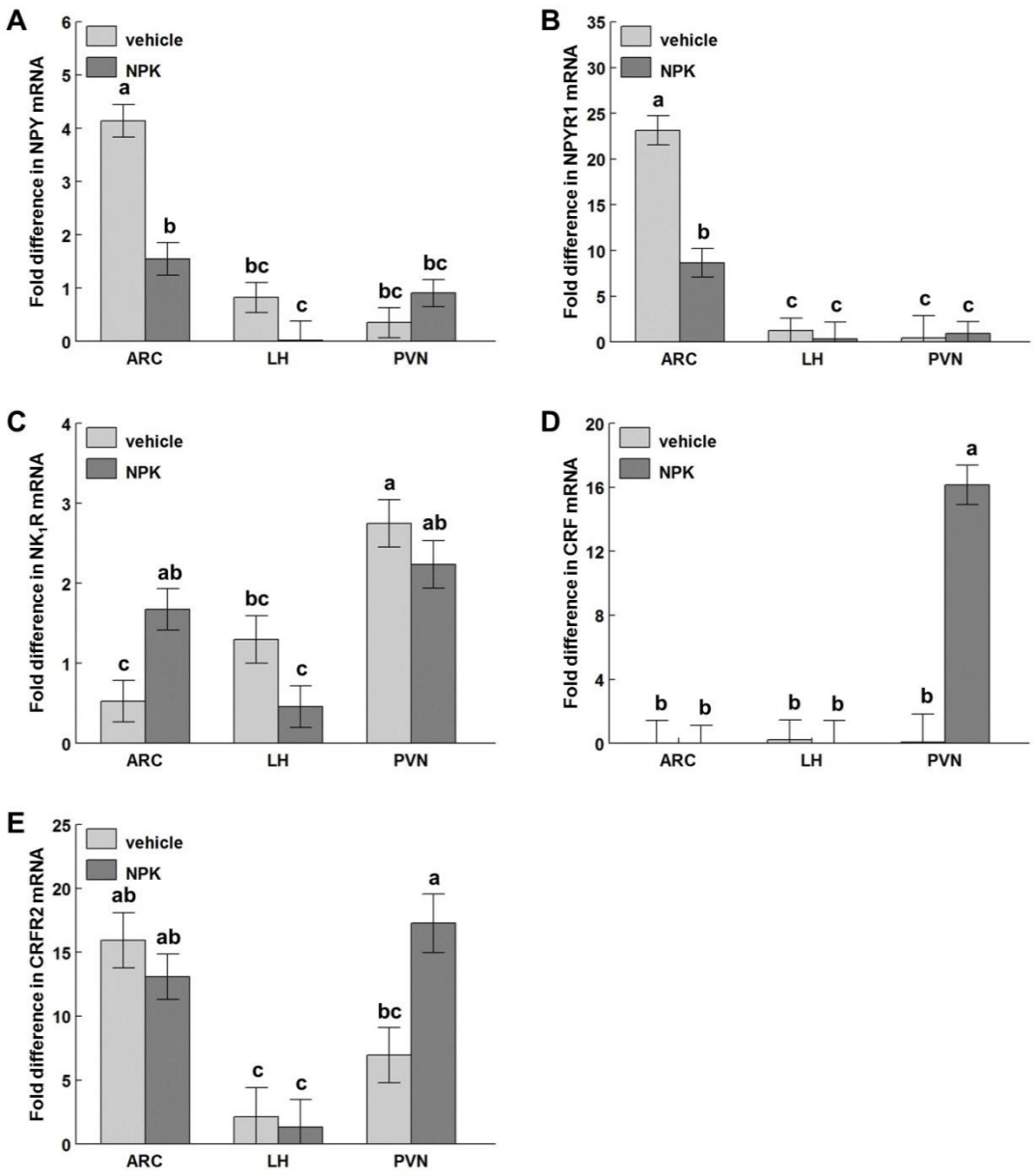

Fig. 5.8 Interactions of treatment and hypothalamic nuclei for A) neuropeptide Y (NPY), B) NPY receptor sub-type 1 (NPYR1), C) neurokinin receptor 1 (NK_1R), D) corticotropin-releasing factor (CRF) and E) CRF receptor sub-type 2 (CRFR2) mRNAs. Values represent means ± SEM (n=12). The interactions were separated using Tukey's test; bars with unique letters indicate a difference at $P < 0.05$.

Chapter 6: The anorexigenic effect of adrenomedullin in Japanese quail (*Coturnix japonica*) involves increased pro-opiomelanocortin and cocaine and amphetamine-regulated transcript mRNAs in the arcuate nucleus of the hypothalamus

Abstract

Central administration of adrenomedullin (AM), a 52-amino acid peptide, is associated with anorexigenic effects across various species, including rodents and chickens. However, the hypothalamic mechanisms underlying this effect remain unknown. The objective of this study was to investigate AM-induced anorexigenic effects in 7 day-old Japanese quail (*Coturnix japonica*), which have undergone less artificial selection than chickens. After intracerebroventricular (ICV) injection of 0.3, 1.0, or 3.0 nmol of AM, quail injected with 3.0 nmol of AM ate and drank less than vehicle-injected quail at 180 minutes post-injection. Except for the 1.0 nmol dose of AM exerting an anorexic effect at 90 minutes post-injection, no other inhibitory effects on food or water intake were observed. At 60 minutes post-injection, the AM-injected quail had more c-Fos immunoreactive cells in the arcuate nucleus (ARC) than vehicle-injected birds. In the ARC, AM injection was also associated with increased mRNA abundance of pro-opiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) mRNAs. In conclusion, the results suggest that the anorexigenic effect of AM is possibly influenced by the synergistic effect of POMC and CART in the ARC.

Key words: Adrenomedullin; Quail; Arcuate Nucleus; Pro-opiomelanocortin; Cocaine and amphetamine-regulated transcript

1. Introduction

Adrenomedullin (AM), a 52-amino acid peptide, was first discovered in the human adrenal medulla and named accordingly (Kitamura, 1993). AM is a member of the calcitonin gene-related peptide (CGRP) superfamily, which also includes adrenomedullin-2/intermedin (AM2/IMD), amylin, and CGRP (Chang et al., 2004; Ogoshi et al., 2006). The functions of the CGRP superfamily are mediated via calcitonin receptor-like receptor (CRLR) (Kapas et al., 1995; Oliver et al., 1998). AM is involved in diverse physiological processes, for example, cell growth and differentiation (Di Liddo et al., 2016), renal function (Saulnier et al., 2017), reproduction (Li et al., 2015), and nervous system (Zhao et al., 2016) and cardiovascular (Yuan et al., 2015) functions. In birds, AM is found in the pancreas, adrenal glands, cardiovascular system, central nervous system, lungs, kidneys, and reproductive organs (López and Cuesta, 2002)(Zudaire et al., 2005).

In rats, intracerebroventricular (ICV) administration of AM leads to decreased food intake and activates several hypothalamic nuclei, such as the paraventricular nucleus (PVN) and supraoptic nucleus (SON) (Taylor et al., 1996). AM injection was also associated with decreased water intake in rats (Murphy and Samson, 1995). In chicks, AM decreases food intake but not water intake via activating the PVN, ventromedial hypothalamus (VMH) and dorsomedial nucleus (DMN) of the hypothalamus, and is associated with less pecking in the feed but more locomotion, jumping, and time spent in deep rest (Wang et al., 2014).

The hypothalamus is the primary regulatory center for appetite control and integrates central and peripheral signals to manipulate the body's response in energy storage and

expenditure (Valassi et al., 2008). Within the hypothalamus, several nuclei play essential roles in food intake regulation, such as the PVN, VMH, DMN, arcuate nucleus (ARC) and lateral hypothalamus (LH) (Arora and Anubhuti, 2006). A large number of appetite-associated neuropeptides have been identified in the hypothalamus. There are anorexigenic factors such as cocaine and amphetamine-regulated transcript (CART), α-melanocyte stimulating hormone derived from pro-opiomelanocortin (POMC), and corticotropin-releasing factor (CRF), and orexigenic factors such as agouti-related peptide (AgRP) and neuropeptide Y (NPY) (Tachibana and Tsutsui, 2016). Collectively, these hypothalamic nuclei and neuropeptides form a complex regulatory network to manipulate feeding behavior that is evolutionarily conserved among species (Allen et al., 2005).

Compared to chickens and wild birds, the Japanese quail (*Coturnix japonica*) have undergone much less artificial selection and are better adapted to the lab environment, respectively. Thus, quail may be a more representative model in which to study feeding behavior regulation in birds. The hypothalamic mechanisms underlying the anorexigenic effects of AM are still unclear. Thus, the objective of the present study was to investigate the effect of central administration of AM on food intake and related hypothalamic physiology in 7 day-old Japanese quail.

2. Materials and Methods

2.1. Animals

Japanese quail were bred and hatched in our vivarium. The breeder flock was established with eggs donated by Mike Lacy at the University of Georgia. Upon removal from the

hatcher, quail were group caged in a brooder for 4 days, then individually caged in galvanized wire cages (8 cm wide, 7 cm deep, and 8 cm high) in a room maintained at a constant temperature of 35 ± 1 °C and a relative humidity of 50 ± 5% with a 14-hour light/10-hour dark period (lights on at 05:00 hours). At all times, unless otherwise noted, quail had ad libitum access to a mash diet (energy: 2,900 kcal ME/kg and 24% crude protein) and tap water. The individual cages allowed visual and auditory contact with other quail. After the quail were individually caged, they were handled twice daily to reduce the effects of stress on the day of data collection. The handling procedure was conducted as we described (Lear et al., 2017). All quail in this experiment were 7 days-old and there were 12 chicks in each treatment group. All procedures were performed according to the National Research Council publication, Guide for Care and Use of Laboratory Animals and were approved by the Virginia Tech Animal Care and Use Committee.

2.2. ICV injection procedure

On the day of the experiment, quail were injected using an adapted method that does not appear to induce physiological stress (Lear et al., 2017; Yuan et al., 2017). The head of the quail was briefly inserted into the restraining device. Injection coordinates were 2 mm anterior to the coronal suture, .75 mm lateral from the sagittal suture, and 1.5 mm deep, targeting the left lateral ventricle. Anatomical landmarks were determined both visually and by palpation. Injection depth was controlled by placing a plastic tubing sheath over the base of the needle. The needle remained at injection depth in the un-anesthetized quail for 5 seconds post-injection to reduce backflow. Quail were assigned to treatments at random. Human adrenomedullin (1-52) (AnaSpec, Inc., Fremont, CA, USA) was

dissolved in artificial cerebrospinal fluid (Anderson and Heisley, 1972) as a vehicle for a total injection volume of 5 µl with 0.1% Evans Blue dye to facilitate injection site localization. After data collection, the quail was decapitated and its head sectioned along the frontal plane to determine the site of injection. Any quail without dye present in the lateral ventricle system was eliminated from analysis. The sex of the quail was determined visually by dissection at the time of decapitation.

2.3. Experiment 1: food and water intake

The quail were fasted for 6 hours prior to ICV injection of AM. Quail were randomly assigned to receive either 0 (vehicle only), 0.3, 1.0, or 3.0 nmol of AM by ICV injection (n=12 for each group). After injection, quail were returned to their individual home cages and given ad libitum access to both food and water. Food and water intake were monitored (0.01 g) every 30 minutes for 180 minutes. Water weight (g) was converted to volume (ml; 1 g = 1 ml). Data were analyzed using analysis of variance (ANOVA) within each time point, and the statistical model included the main effect of dose. When dose effects were significant, Tukey's method of multiple comparisons was used to separate the means within each time point. Statistical significance was set at $P < 0.05$ for all experiments. Food and water intake results are shown on a cumulative and non-cumulative basis.

2.4. Experiment 2: c-Fos immunohistochemistry

The quail were fasted for 6 hours prior to ICV injection of AM. Quail were randomly assigned to receive either vehicle or 3.0 nmol of AM (based on results of Experiments 1) by ICV injection (n=12 for each group). After injection, food was withheld to prevent c-Fos immunoreactivity associated with food consumption. 60 minutes post-injection, as

this is the time expected for the most robust c-Fos expression (Müller et al., 1984), quail were deeply anesthetized with sodium pentobarbital via cardiopuncture, then perfused via the carotid artery with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) containing 0.2% picric acid at pH 7.4. Brains were removed from skulls and post-fixed for 60 minutes in the same solution, after which they were blocked and placed through a series of graded sucrose incubations, consisting of 20, 30 and 40% in 0.1 M PB, until they sank in each. Several 60 µm coronal sections that contained appetite-related nuclei based on anatomies described by Kuenzel (Kuenzel and Masson, 1988) were collected using a cryostat at -15 °C, and then deposited in 0.02 M phosphate buffered saline (PBS) containing 0.1% sodium azide (Kuenzel et al., 1987). The PVN and LH were collected corresponding to 7.8 interaural, the VMH was collected corresponding to 6.8 interaural, and the DMN and the ARC at interaural 5.4 mm. Sections were processed immediately after collection. Procedures for the c-Fos immunohistochemistry assay were performed as we described (Newmyer et al., 2013) using rabbit polyclonal anti-c-Fos at a dilution of 1:20,000 (K-25, Santa Cruz, Santa Cruz, CA, USA) (Zhao and Li, 2010; Newmyer et al., 2013). Anatomies were confirmed and a digital micrograph taken of each section. Overlays containing the respective nuclei boundaries were digitally merged with micrographs and the number of c-Fos immunoreactive cells within each respective nucleus counted by a technician blind to treatment. Data were analyzed via ANOVA using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC), with the model including the effect of treatment within each nucleus.

2.5 Experiment 3: mRNA abundance of appetite-associated factors in hypothalamic nuclei

The quail were fasted for 6 hours prior to ICV injection of AM. Quail were randomly assigned to receive either 0 (vehicle) or 3.0 nmol of AM via ICV injection (n=12 in each group). At one hour post-injection, each quail was deeply anesthetized with sodium pentobarbital via cardiopuncture and then perfused via the carotid artery with 1.0 mL of RNA stabilizing buffer (16.7 mM sodium citrate, 13.3 mM EDTA, and 3.5 M ammonium sulfate; pH = 5.2). Within 30 minutes of perfusion, brains were sectioned in a cryostat at -10 ℃ into 500 µm thick coronal sections in the direction from rostral to caudal: ARC was collected at plate 5.4 based on anatomy described by Kuenzel and Masson, 1988 (Kuenzel and Masson, 1988) (based on results of Experiments 2). Punches were collected using sterile disposable biopsy punch instruments (1 mm, Braintree Scientific Inc., Braintree, MA) and were immediately transferred to sterile microcentrifuge tubes containing RNA lysis buffer with 1% beta-mercaptoethanol (Norgen Biotek, Thorold, ON, Canada), vortexed, snap-frozen in liquid nitrogen, and stored at -80 ℃. The remaining brain section was photographed and punch accuracy verified via the overlays containing the respective nuclei boundaries.

The punches were thawed, vortexed vigorously for 30 seconds, and incubated at room temperature for 5 minutes before adding 70% molecular biology-grade ethanol, and total RNA was isolated following the manufacturer's instructions for the Total RNA Purification Micro Kit (Norgen Biotek). The concentration and purity of total RNA was assessed by spectrophotometry at 260/280/230 nm with a NanoDrop 2000 (Thermo Fisher Scientific Inc., West Palm Beach, FL, USA). Total RNA integrity was verified using an Experion (Bio-Rad, Hercules, CA) and RNA StdSens analysis kit (Bio-Rad), according to the manufacturer's instructions.

First-strand cDNA was synthesized in 20 µl reactions from 100 ng of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's instructions. Reactions were performed under the following conditions: 25 ℃ for 10 minutes, 37 ℃ for 120 minutes and 85 ℃ for 5 minutes. Primers for real-time PCR were designed with Primer Express 3.0 software (Applied Biosystems) (Table 1) and validated for amplification efficiency before use (95-105%). Real-time PCR reactions were performed in 10 µl reactions contained 5 µl Fast SYBR Green Master Mix (Applied Biosystems), 0.5 µl primers (0.25 µl of 5 µM forward primer and 0.25 µl of 5 µM reverse primer), 1.5 µl nuclease-free water, and 3 µl 5-fold diluted cDNA using a 7500 Fast Real-Time PCR System (Applied Biosystems). Real-time PCR was performed under the following conditions: 95 ℃ for 20 seconds and 40 cycles of 90 ℃ for 3 seconds plus 60 ℃ for 30 seconds. A dissociation step consisting of 95 ℃ for 15 seconds, 60 ℃ for 1 minute, 95 ℃ for 15 seconds and 60 ℃ for 15 seconds was performed at the end of each PCR reaction to ensure amplicon specificity.

Real-time PCR data was analyzed using the ΔΔCT method with β-actin as the endogenous control and the average of the quail in the vehicle group as the calibrator sample. Relative quantities calculated as $2^{-\Delta\Delta CT}$ were used for statistical analysis (Livak and Schmittgen, 2001). The statistical model included the main effect of the treatment, sex, and their interactions. The mRNA abundance was not affected by sex for any gene measured, thus we removed sex from the model. The data were analyzed by analysis of variance (ANOVA) using JMP 13 Pro (SAS institute, Cary, NC, USA). Differences were considered significant at $P < 0.05$. Data are presented as means ± SE.

3. Results

3.1 Food and water intake

In experiment 1, quail injected with 3.0 nmol of AM ate less than those in the vehicle-injected group and 0.3 nmol-injected group throughout the entire 180 minutes post-injection (Fig. 6.1A). There was an inhibitory effect of the 1.0 nmol dose of AM on food intake at 90 minutes post-injection. On a non-cumulative basis, 3.0 nmol of AM decreased food intake at 30 minutes post-injection (Fig. 6.1B). At 120 minutes post-injection, quail injected with 3.0 nmol of AM ate less than those in both the 0.3 and 1.0 nmol of AM-treated groups.

Similar to food intake, quail injected with 3.0 nmol of AM drank less than those in the vehicle-treated group during the entire observation period (Fig. 6.2A). Except for at 30 minutes post-injection, quail injected with 3.0 nmol drank less than individuals injected with 0.3 nmol of AM. Quail injected with 3.0 nmol AM also drank less than those treated with 1.0 nmol AM at 90 and 120 minutes post-injection. On a non-cumulative basis, quail injected with 3.0 nmol of AM drank less than those in other three groups at 30, 60, and 90 minutes post-injection (Fig. 6.2B).

3.2 Hypothalamic c-Fos immunoreactivity

The number of immunoreactive cells in quail injected with AM increased by about 300% ($P = 0.0004$) over the numbers in vehicle-injected quail in the ARC (Fig. 6.3). c-Fos immunoreactivity was not affected by AM injection in the other nuclei.

3.3 mRNA abundance in the ARC

The AM-injected quail had greater hypothalamic mRNA expressions of CART ($P = 0.0085$) and POMC ($P = 0.0001$) in the ARC (Fig. 6.5). The ICV injection of AM did not

affect the mRNA abundance of AgRP, CRF, CRF receptor sub-types 1 and 2 (CRFR1 and CRFR2, respectively), melanocortin receptor 4 (MC4R), mesotocin receptor (MTR), NPY, or NPY receptor sub-types 1 and 5 (NPYR1 and NPYR5, respectively).

4. Discussion

In the present study, AM inhibited food intake in 7 day-old Japanese quail, consistent with effects reported in rats (Taylor et al., 1996) and chicks (Wang et al., 2014). In rats, the anorexigenic dose threshold of AM was 5.0 nmol at 30 minutes post-injection (Taylor et al., 1996). In birds, the effective anorexigenic doses of AM are lower than for rats; 1.0 and 3.0 nmol in chicks (Wang et al., 2014) and quail in the present study, respectively. The inhibitory effect of AM began to dissipate at 120 minutes post-injection in chicks (Wang et al., 2014) whereas it lasted the entire 180 minutes in quail, implying that AM's effect is more transient in chicks than in quail. Thus, different thresholds and durations for AM's inhibitory effect on food intake were observed across species. These differences in response may result from genetic background, body weight, age, or experimental design, thus it is unclear how much the difference is due to evolutionary divergence vs. environmental influences.

Unlike food intake, the effect of AM injection on water intake is not consistent across species. In the present study, AM was associated with decreased water intake in quail, which is consistent with rats (Murphy and Samson, 1995) but not chicks (Wang et al., 2014). Also, the inhibitory effect of AM on water intake lasted 60 minutes post-injection compared to the 180-minute duration of effect on food intake, implying that AM-induced reductions in food intake are more sustained.

To investigate the hypothalamic mechanism of AMs' anorexigenic effect in Japanese quail, we first measured hypothalamic c-Fos immunoreactivity at 60 minutes post-injection. c-Fos immunoreactivity was increased in response to ICV injection of AM in the ARC with a much more potent response than in other nuclei. The c-Fos protein, an early intermediate transcription factor, is thought to be an indicator of recent activation of a brain region (Kovács, 1998). Thus, our results indicate that the ARC may be involved in mediating the anorexigenic effect of AM in quail. The ARC, located in the mediobasal hypothalamus, is considered to be the primary hypothalamic nucleus integrating the hormonal and metabolic signals from peripheral and central nervous systems (Funahashi et al., 2000). In contrast, chicks injected with AM displayed increased c-Fos immunoreactivity in the PVN, VMH, and DMN (Wang et al., 2014).

We then measured the hypothalamic mRNA abundance of appetite-associated factors in the ARC and found that POMC and CART mRNAs were increased in response to ICV injection of AM. POMC is robustly expressed in the ARC in chickens and quail, similar to mammals (Gerets et al., 2000) (Phillips-Singh et al., 2003), suggesting conserved roles of POMC between mammals and birds. POMC-derived peptides are involved in a series of satiety-associated mechanisms in the hypothalamus, particularly in the ARC (Millington, 2007) and its expression and cleavage are sensitive to alterations in hormonal or nutritional signals (Phillips-Singh et al., 2003). For example, overfeeding and fasting were associated with increased (Hagan et al., 1999) and decreased (Mizuno et al., 1998) hypothalamic POMC mRNA abundance, respectively. Thus, anorexigenic signaling via POMC-derived pathways may be activated via exogenous AM.

AM injection was also associated with an increase in CART mRNA in the ARC. CART is highly expressed in the hypothalamus, in nuclei such as the PVN, SON, LH, ARC and DMN (Vrang et al., 1999; Elias et al., 2001) and this distribution seems to be conserved among species (Gutierrez‐Ibanez et al., 2016). Central administration of CART reduces food intake in 24-hour fasted and normal rats (Kristensen et al., 1998) and chicks (Tachibana et al., 2003), implying that CART plays an inhibitory role in regulating food intake. Also, ARC-specific CART mRNA is greater in lean than obese rats and mice (Kristensen et al., 1998). It is worthwhile to note that POMC and CART are co-expressed in the ARC (Elias et al., 1998) and satiety-associated neurons in the ARC produce both POMC and CART (Dhillo et al., 2002). Thus, POMC and CART in the ARC may contribute to the anorexigenic effect of AM in quail, although evidence is needed at the protein/peptide level to validate this conclusion.

In conclusion, the results suggest that ICV injection of AM activated the ARC in which there was increased mRNA expression of POMC and CART, and that these events might have contributed to the reduced food intake in quail. The present study provides insights on AM-induced hypothalamic physiology that may contribute to a better understanding of the induction of satiety across species.

Table 6.1 Primers for real-time PCR[1].

Gene	Accession No.	Sequences (forward/reverse)
AgRP	XM_015873899.1	GGTTCTTCAACGCCTTCTGCTA/TTCTTGCCACATGGGAAGGT
CART	XM_003643097.3	GCTGGAGAAGCTGAAGAGCAA/GGCACCTGCCCGAACTT
CRF	XM_015855730.1	TCAGCACCAGAGCCATCACA/GCTCTATAAAAATAAAGAGATGACATCAGA
CRFR1	XM_015885973.1	CTGCTGCCCTTGCTGGGAAT/ATCCTCCCCTGGATTGAC
CRFR2	XM_015852926.1	GGATCAAATACAACACCACAAGAAAT/AGCCCACGTCCCATTGC
MC4R	XM_015854466.1	CATCAGCTTGCTGGAGAACGT/GCGAATGGAGGTTCTTGTTCTT
MTR	XM_015875456.1	TGTGCTGGACGCCCTTCT/TCGTGCGGAGCGTTGGT
NPY	XM_0158538M70.1	CATGCAGGGCACCATGAG/CAGCGACAGGGCGAAAGTC
NPYR1	XM_015861016.1	TAGCCATGTCCACCATGCA/GGGCTTGCCTGCTTTAGAGA
NPYR5	XM_015861003.1	GGCTGGCTTTGTGGGAAA/CTGTCTTCTGCTTGCGTTTTGT
POMC	NM_001323229.1	GCCAGACCCCGCTGATG/CTTGTAGGCGCTTTTGATGAT
β-actin	XM_015876619.1	GTCCACCGCAAATGCTTCTAA/TGCGCATTTATGGGTTTTGTT

[1]Primers were designed with Primer Express 3.0 (Applied Biosystems). Abbreviations: agouti-related peptide (AgRP), cocaine and amphetamine-regulated transcript (CART), corticotropin-releasing factor (CRF), CRF receptor sub-types 1 and 2 (CRFR1 and CRFR2, respectively), melanocortin receptor 4 (MC4R), mesotocin receptor (MTR), neuropeptide Y (NPY), NPY receptor sub-types 1 and 5 (NPYR1 and NPYR5, respectively), and pro-opiomelanocortin (POMC).

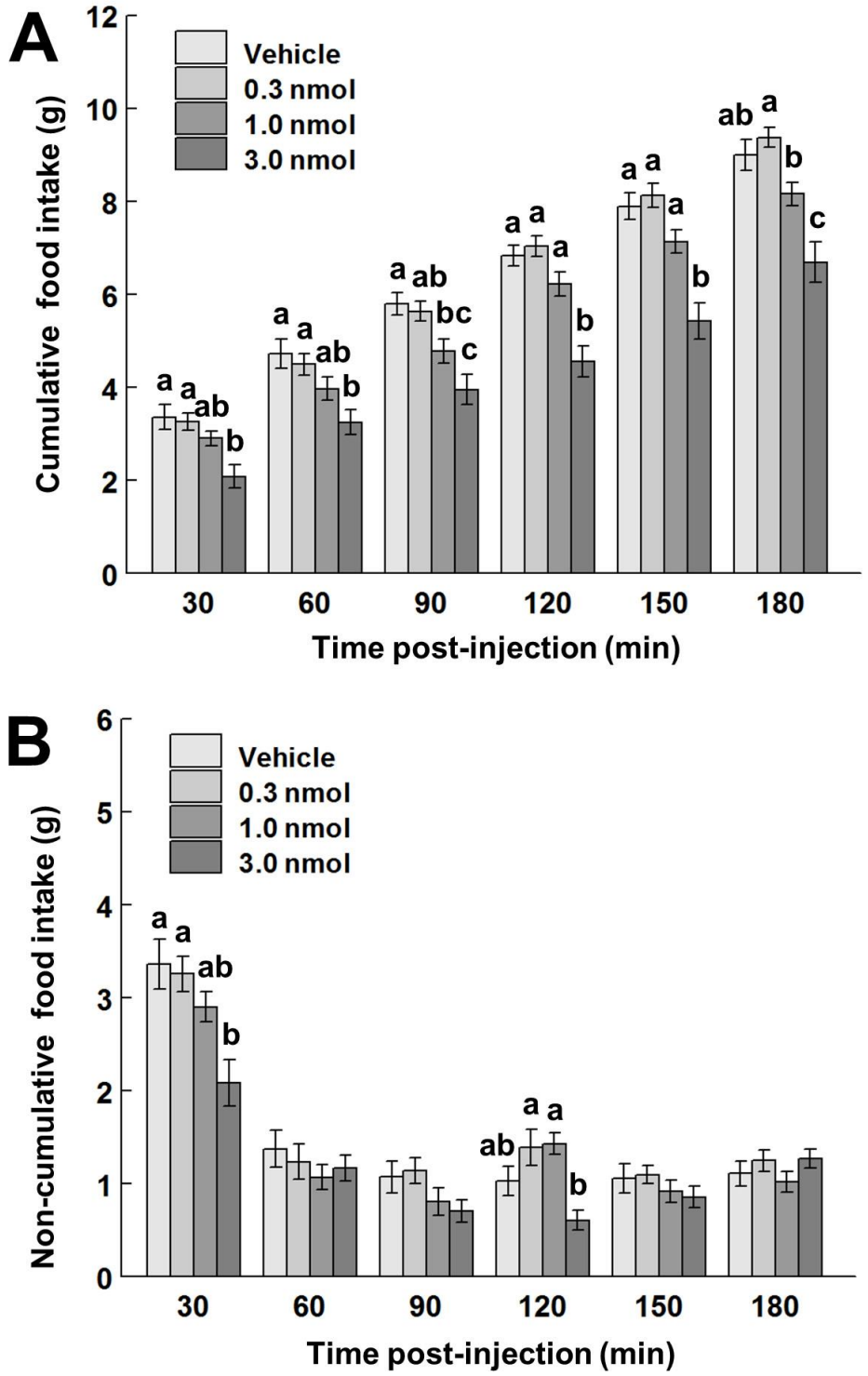

Fig. 6.1 Cumulative (A) and non-cumulative (B) food intake expressed as a percentage of body weight of Japanese quail at 7 days post-hatch (Experiment 1). Values are means ± standard error; bars with different superscripts are different from each other

within a time point ($P < 0.05$). There were 12 quail per group available for analysis.

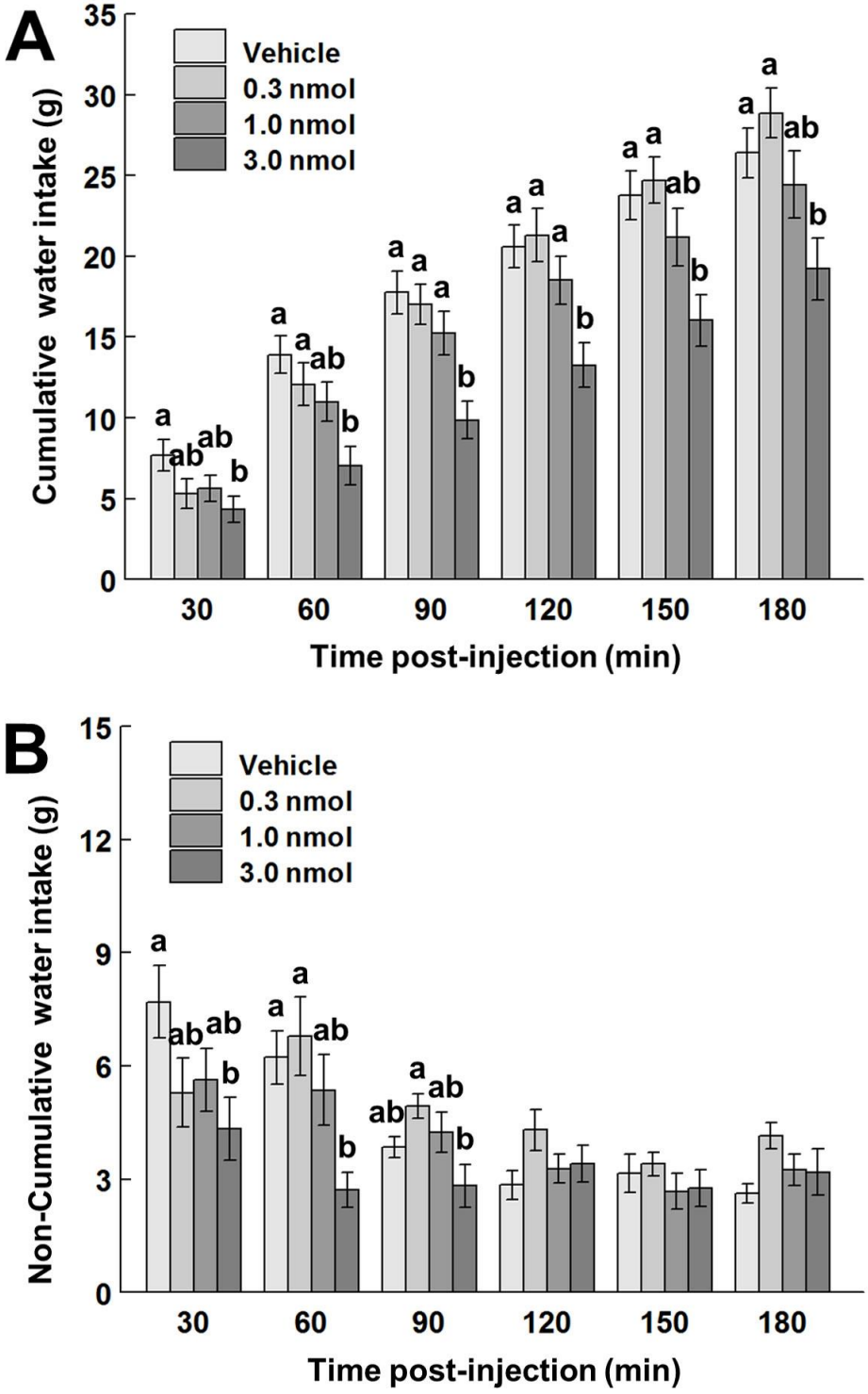

Fig. 6.2 Cumulative (A) and non-cumulative (B) water intake expressed as a percentage

of body weight of Japanese quail at 7 day post-hatch (Experiment 1). Values are means ± standard error; bars with different superscripts are different from each other within a time point ($P < 0.05$). There were 12 quail per group available for analysis.

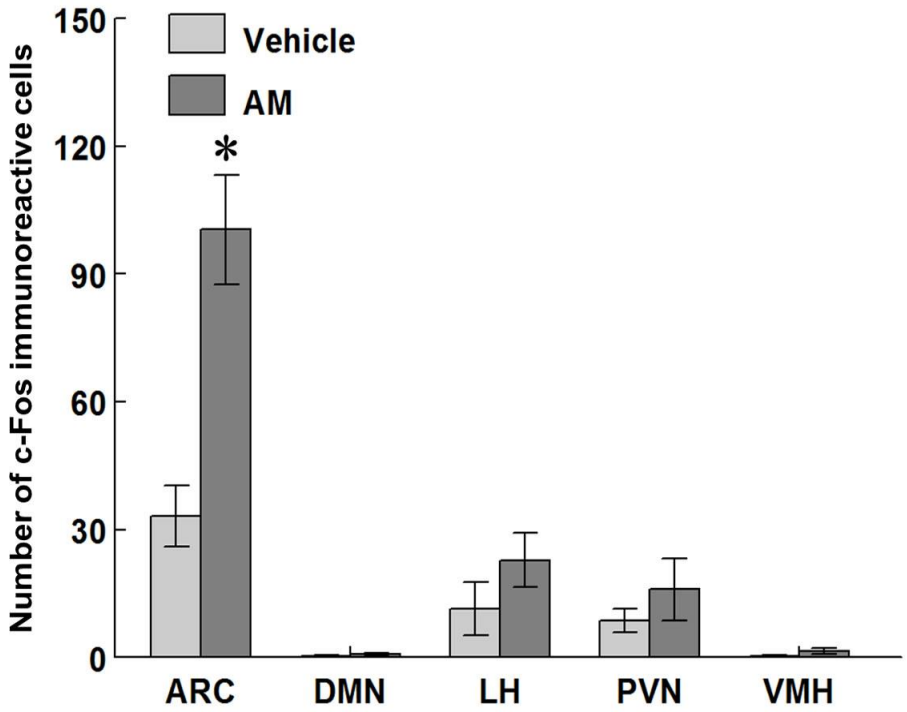

Fig. 6.3 Effect of ICV administration of adrenomedullin (3.0 nmol) in Japanese quail at 7 days post-hatch on the number of c-Fos immunoreactive cells in the arcuate nucleus (ARC), dorsomedial nucleus (DMN), lateral hypothalamus (LH), paraventricular nucleus (PVN), and ventromedial hypothalamus (VMH) (Experiment 2). (*) denotes difference from vehicle ($P < 0.05$). Values are means ± S.E.M. For this experiment, there were 12 quail per group available for analysis.

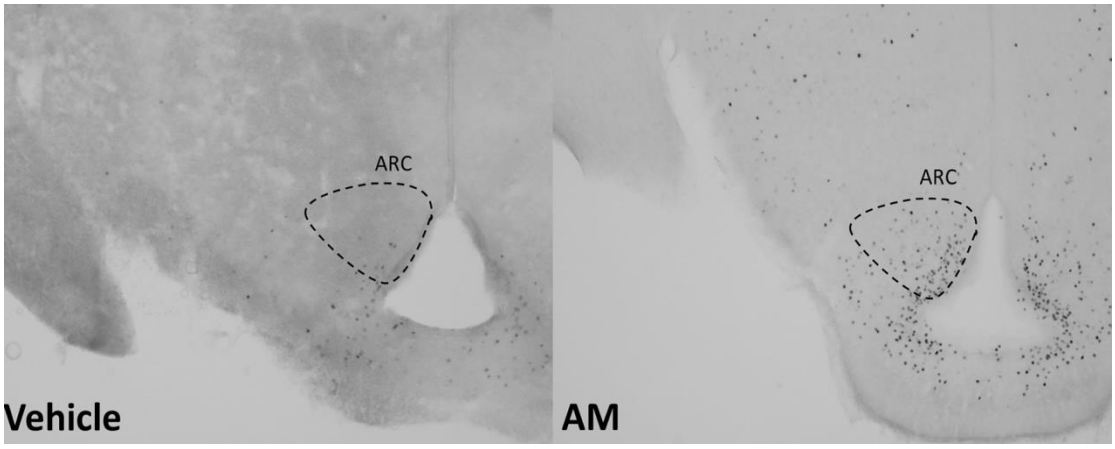

Fig. 6.4 Representative photomicrographs of c-Fos immunohistochemistry in the arcuate nucleus (ARC) of the hypothalamus (Experiment 2). Treatment is indicated on each photomicrograph.

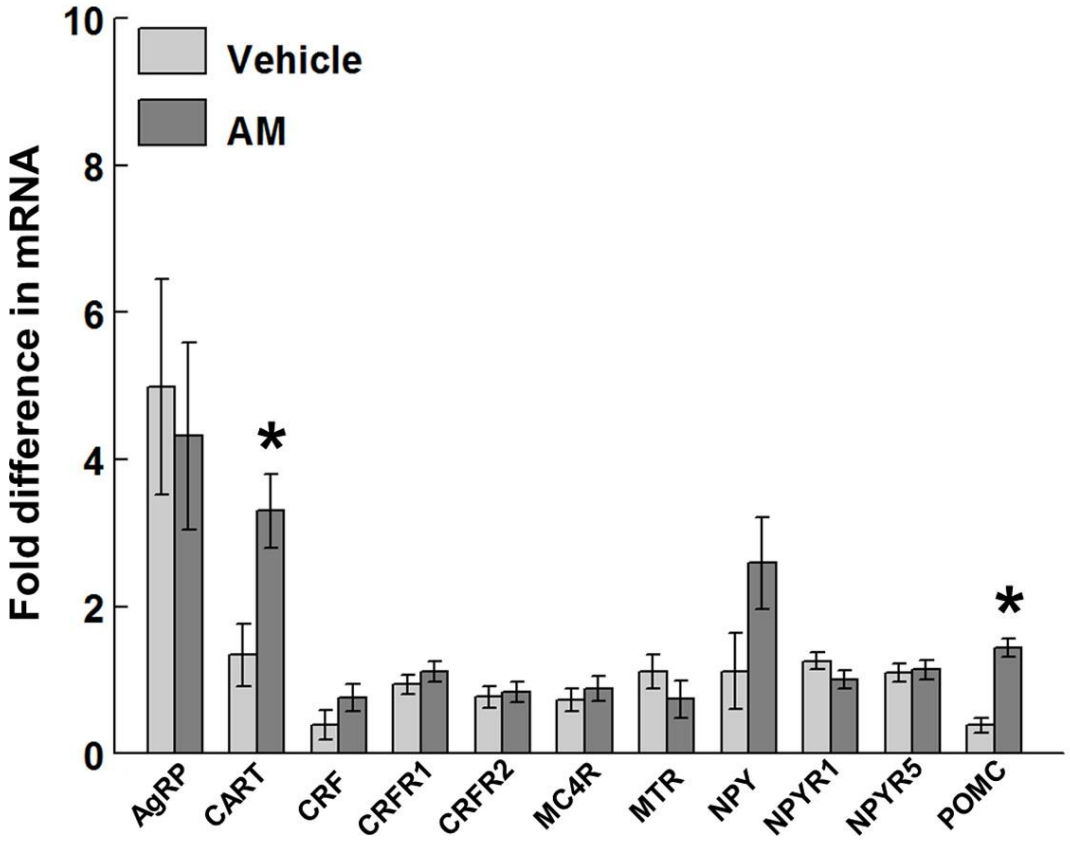

Fig. 6.5 Effect of ICV administration of adrenomedullin (3.0 nmol) in Japanese quail at 7 days post-hatch on appetite-associated factor mRNA in the arcuate nucleus (ARC). (*)

denotes difference from vehicle ($P < 0.05$). Values are means ± S.E.M. For this experiment, there were 12 quail per group available for analysis. Abbreviations: agouti-related peptide (AgRP), cocaine and amphetamine-regulated transcript (CART), corticotropin-releasing factor (CRF), CRF receptor sub-types 1 and 2 (CRFR1 and CRFR2, respectively), melanocortin receptor 4 (MC4R), mesotocin receptor (MTR), neuropeptide Y (NPY), NPY receptor sub-types 1 and 5 (NPYR1 and NPYR5, respectively), and pro-opiomelanocortin (POMC).

Chapter 7: Synthesis

This dissertation consists of a series of experiments designed to determine the hypothalamic mechanisms underlying the regulation of food intake in birds. Since appetite regulation is critical for survival in all species, studying the hypothalamic mechanism of appetite regulation using birds as animal model can provide insights into evolutionary adaptations across various vertebrate species. Also, understanding the differential mechanisms of appetite regulation between birds and mammals may provide novel information about the central regulation of appetie. Thus, findings from these experiments can be applied to explore the hypothalamic signaling pathways that are associated with feeding behavior and energy homeostasis in birds, and may also provide translational value for understanding eating disorders in humans, such as obesity and anorexia.

In Experiment 1, we measured expression of many genes that encode factors associated with the regulation of food intake and stress coping, in chicken lines that have undergone long-term artificial selection for low or high body weight. The primary objective of this experiment was to investigate the mechanism underlying the stress-induced refractoriness to the orexigenic effect of NPY in chickens have been selected for low (LWS). The results showed that the lines responded differently to stress. The most changes were observed in the PVN of LWS chicks, in which several anorexigenic- and stress-associated factors were up-regulated, such as CRF, CRFR1, CRFR2, MC4R, and UCN3. These findings suggested that a potent anorexigenic effect may override the orexigenic effect of NPY, primarily through CRF signaling, eventually resulting in intensified anorexia in the LWS. Furthermore, this hypothesis was supported by results that the

orexigenic effect of NPY was restored in stressed LWS that were treated with astressin (CRF receptor antagonist) before stress exposure. Thus, the results from this experiment demonstrated that there may be a competitive interaction between anorexigenic and orexigenic signaling in the hypothalamus of the LWS in response to stress and that stress-induced activation of the CRF system plays important roles in both appetite regulation and stress coping in chickens.

Since CRF was demonstrated to be an important neuropeptide participating in appetite regulation in the chickens in Experiment 1, we designed Experiments 2 and 3 to further investigate the hypothalamic mechanisms underlying the anorexigenic effects of CRF in chicks and Japanese quail. The chick is a common agricultural species and studying appetite regulation in chicks is beneficial to the poultry industry, while the Japanese quail is more representative of wild birds due to being less artificially-selected. In these two experiments, the bird species showed differential hypothalamic activities in response to central administration of CRF, although similar inhibitory effects on food intake were observed. Firstly, ICV injection of CRF increased c-Fos expression in the PVN, VMH, DMN, and ARC in the chicks whereas it only increased in the PVN and LH in quail. Also, the results from gene expression experiments showed that CRF inhibited hypothalamic gene expression of NPYR1 in chicks whereas it stimulated several anorexigenic factors, such as CRF, CRFR2, MCR4, and POMC in quail. These results indicated that the anorexigenic effect of CRF may be associated with a dampened NPY system in chicks, but with activated CRF and melanocortin systems in quail. At the nucleus level, CRF-induced gene expression changes suggested that the orexigenic NPY system (ARC and DMN) was overridden by anorexigenic signaling through factors including CRF (ARC

and PVN) and MT (VMH), eventually leading to suppression of food intake. Collectively, CRF induced different hypothalamic actions in chicks and quail. This difference may be associated with the different degree of artificial selection or genetic background of these two bird species.

As an anorexigenic neuropeptide, CRF inhibited food intake through the hypothalamus via either enhancing anorexigenic systems or suppressing orexigenic systems. Based on the findings from Experiments 2 and 3, we were interested in determining how other anorexigenic neuropeptides inhibit food intake in birds. Thus, we designed Experiments 4 and 5 to use Japanese quail to determine the hypothalamic mechanisms underlying the anorexigenic effect of NPK and AM. The results from Experiment 4 showed that NPK activated the ARC and PVN, in which expression of several anorexigenic factors increased including CRF, UCN3, CART, and POMC. Meanwhile, expression of several orexigenic factors decreased, such as NPY and AgRP. In Experiment 5, ICV injection of AM activated the ARC which was associated with increased POMC and CART mRNA. Collectively, results of these four experiments suggest a general mechanism where central administration of anorexigenic neuropeptides (CRF, NPK, and AM) inhibited food intake through activating anorexigenic signaling, for example through CRF and melanocortin systems and/or through dampening orexigenic signaling, for example, via NPY and AgRP systems.

Appetite regulation involves an extremely complicated system that includes both the periphery and central nervous system. During the past few decades, it has been demonstrated that the hypothalamus has an essential role in the regulation of food intake via integrating and coordinating metabolic and endocrine signaling from both the

periphery and central nervous system (Arora and Anubhuti, 2006). Also, a hypothalamic regulation model of "first- and second-order neurons" (Hillebrand et al., 2002; Parkinson et al., 2008), was applied to interpret the regulatory mechanism of food intake in mammals. Specifically, satiety or hunger signals from the periphery and other areas of the central nervous system are integrated and processed in the "first-order neurons" in the ARC, and then are transmitted to the "second-order neurons", for example, in the PVN, LH, and VMH, eventually affecting feeding behavior. Although this model originates from findings of human and rodent studies, it is still applicable to interpret our results. According to this model, we first found that the ARC was activated via exogenous neuropeptides (CRF, NPK, and AM) in Experiment 2, 4, and 5, respectively. Within the ARC, a competitive interaction between the anorexigenic and orexigenic factors was observed (for example, CRF in chicks and NPK in quail), eventually generating an inhibitory "net-effect" that may be a key determinant of feeding behavior. Furthermore, once this inhibitory "net-effect" generated in the ARC was transmitted to the "second-order neurons", it correspondingly led to increased inhibitory signaling and/or decreased stimulatory signaling. Among these "second-order neurons", our results suggested that the PVN is a primary target for mediating anorexigenic effects, which is consistent with the finding that the ARC-PVN circuit is a primary signaling pathway participating in central appetite regulation (Tóth and Palkovits, 1998).

Taken together, we investigated the hypothalamic mechanisms underlying appetite regulation in birds, focusing on the anorexia induced by stress or exogenous neuropeptides. The results from these experiments suggest that a complex appetite-associated regulatory network exists in the hypothalamus of birds, including a group of

nuclei and neuropeptides. Furthermore, our studies may provide evidence that the mechanisms of appetite regulation are conserved between mammals and birds, thus demonstrating the high translational value of avian models to study eating disorders. Lastly, it should be noted that many results in our studies are at the transcriptional level. Accordingly, interpretations and conclusions should be made with caution and protein/peptide results should be provided in future studies.

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Appendix A

Immunohistochemistry Protocol for c-Fos Expression

Day "Zero"

Brain fixation, harvest and preparation

Typical brain harvest time is 1 hour following treatment

A. Anesthetize chick via cardiopuncture with sodium pentobarbital

B. Decapitate immediately by cutting as close to the 9th cervical vertebra as possible (long neck)

C. In the hood, perfuse brain with normal saline for 2 minutes using a Fisher peristaltic pump set to speed 1 on the "low" setting

D. Perfuse brain with 4% paraformaldehyde for 6 min with the same peristaltic pump settings

4% Paraformaldehyde	**1000 mL**
NaOH Pellets	5 pellets
Ultrapure Water (Bring to just before boiling (do NOT allow to boil))	500 mL
Add paraformaldehyde while stirring (Stir until solution is clear (long time))	40 g
0.2 M PB	500 mL
Saturated Picric Acid (until dissolved)	0.5 g
Filter Solution	
pH to 7.4 (using HCl or NaOH)	

E. Immediately after paraformaldehyde perfusion flush lines with normal saline

Saline Water	**1000 mL**
NaCl	8.5 g
Ultrapure Water	1000 mL

F. Place head in a cup with identifying number

G. Remove brain from skull as soon as practicable

H. Post fix in 4% paraformaldehyde for at least 30 min

I. Transfer to 20% sucrose in PB until it sinks (about 10 hours at RT)

20 % Sucrose	**100 mL**
Sucrose	20 g
0.1 M PB	fill to 100 mL

J. Transfer to 30% sucrose in PB (about 24 hours at RT)

30 % Sucrose	**100 mL**
Sucrose	30 g
0.1 M PB	fill to 100 mL

(Procedure may be stalled at sucrose steps by placing in 4 C)

K. After it has sunk in 30% sucrose in PB it is ready for cryosectioning

L. Orient brain per Pulles (2007) and block brain frontally immediately behind the arcuate nucleus such that the optic lobe is cut down the middle

M. Collect 60 micron frontal sections at -10 C to -15 C chamber and specimen temperature

N. Place sections in 0.02 M PBS until further processing

(Procedure may be stalled at this point in Storage Buffer at 4℃)

Storage Buffer	100 mL
0.1% Sodium Azide	0.1 g
0.1 M PB	Fill to 100 mL

Day "One"

A. Rinse 3 x in 0.02 M PBS

0.02 M PBS	**1000 mL**
0.2 M PB	100 mL
NaCl	9 g
Ultrapure Water	900 mL

B. Sodium borohydride ($NaBH_4$) treatment (breaks double C=C bonds to improve antibody penetration)

$NaBH_4$	**100 mL**
0.02 M PBS	100 mL
$NaBH_4$	0.1g

a. Incubate for 15 minutes

*Many bubbles may form, stir occasionally to ensure all sections transfer

C. Rinse 2 x in 0.02 M PBS

D. Incubate sections in blocking solution for 1 hour at RT on a shaker

Blocking Solution	**10 mL**
0.02 M PBS	8.7 mL
NGS	1 mL

10% Triton X-100	0.3 mL

E. Rinse sections 2 x 10 min in wash buffer at RT

Wash Buffer	**500 mL**	**1000 mL**
10% Triton X-100	15 mL	30 mL
NGS	250 µl	500 µl
0.02 M PBS	485 mL	970 mL

F. Incubate sections in nonspecific binding solution for 30 min

Non specific binding solution	**1000 mL**
200 proof methanol	485 mL
Ultrapure water	485 mL
50% Hydrogen peroxide	30 mL

G. Rinse sections 3 x in wash buffer at RT

H. Incubate sections with primary antibody at 4 C on shaker
*For assay control, do not add primary antibody

*For chick c-Fos assay use anti c-Fos 1:5000 (1:5000; 5 µl in 25 mL) and incubate 72-96 hours;

*AB429 is in a clear tube with blue cap in the freezer

Primary antibody	**25 mL**
0.02 M PBS	20 mL
Blocking reagent	0.25 g
Heat 35 C, cool (takes a lot of time)	
10% Triton X-100	0.75 mL
NGS	250 µl
0.02 M PBS	Bring to 25 mL

<u>"Day TWO"</u>

A. Rinse sections **3 x** 10 min in wash buffer at RT

Wash Buffer	**500 mL**
10% Triton X-100	15 mL
NGS	250 µl
0.02 M PBS	485 mL

*No shake

B. Incubate sections with biotinylated goat anti-rabbit antibody (Vector) diluted 1:200 (10 drops in 100 mL) in 1% NGS / 0.02 M PBS for **2 hours** at RT on a shaker (one drop of antibody is 50 µl)

Anti-rabbit antibody	**10 mL**
0.02 M PBS	9.9 mL
NGS	0.1 mL
1 drop anti-rabbit antibody	1 drop

C. **Prepare** horseradish peroxidase avidin-biotin complex (Vactastain Elite ABC Kit)

ABC Kit	**10 mL**
0.02 M PBS	10 mL
A	4 drops, vortex
B	4 drops, vortex

*Notice: prepare only!

D. Rinse sections **3 x** 10 min in 0.02 M PBS (not wash buffer)
(Procedure may be stalled at this step by placing in 4 C)

E. Incubate with ABC solution prepared earlier for **1 hour** at RT on a shaker

F. **Prepare** DAB solution (Need to be used within 30- 60 min of preparation time)

DAB	**50 mL**
Tris-HCl buffer	50 mL
Nickel ammonium sulfate	0.075 g
Ammonium chloride	0.02 g
DAB	0.01 g

Tris-HCl buffer	**1000 mL**
Trizma HCl	6.06 g
Trizma Base	1.39 g
ddH2O	Fill to 1000 mL

Glucose Oxidase	**1 mL**
50 mM Sodium acetate buffer	1 mL
Glucose Oxidase	0.2 mg

* Glucose oxidase

* No staining will occur at this point because there is no peroxide present.

10% beta-D glucose solution	**10 mL**
beta-D glucose	1.0 g
ddH_2O	To 10 mL

* Glucose will allow glucose oxidase to generate peroxide and the reaction will begin

a. Staining typically develops for Ni-DAB within 5-10 minutes. You can view the sections under a dissecting microscope as they stain and see the reactions develop in real-time. When the reactions look strong, you can inactivate the DAB by transferring the sections to a well of PBS.

G. Rinse sections 3 x 10 min in 0.05 M Tris-HCl at RT

0.05 M Tris-HCl	**1000 mL**
0.5 M Tris-HCl	100 mL
Ultrapure Water	900 mL

H. Add (~3 mL to the small wells) DAB to well, add 3 µL glucose oxidase, add sections, add 15 µL 10% beta-D glucose

I. Rinse sections 1 x 10 min in 0.05 M Tris-HCl at RT

J. Rinse sections 2 x 10 min in 0.02 M PBS at RT
(Procedure may be stalled at this step by placing in 4 C)

<u>"Day THREE"</u>

A. Mount sections on gelatin coated slides and let them dry

B. Dehydrate slides

Dehydration
5 min 70% EtOH
3 min 70% EtOH
3 min 90% EtOH
3 min 100% EtOH
3 min Histoclear (dangerous!)
3 min Histoclear (dangerous!)

C. Coverslip while still wet with Histoclear using Vectamount

STOCK RECIPES

Tris-HCl

0.5 M Tris-HCl	**1000 mL**
Tris (FW: 121.14)	60.57 g
Ultrapure Water	800 mL
35% HCl (for adjusting pH to 7.6)	30-40 mL
Ultrapure Water	to 1000 mL

[Stock] 0.2 M Dibasic Sodium Phosphate

[Stock] 0.2 M Dibasic Sodium Phosphate	**1000 mL**
Sodium phosphate dibasic anhydrous (Na2HPO4, FW:141.96)	28.39 g
Ultrapure Water	Fill to 1000 mL

[Stock] 0.2 M Monobasic Sodium Phosphate

[Stock] 0.2 M Monobasic Sodium Phosphate	**1000 mL**
Sodium phosphate monobasic nhydrous (NaH2PO4, FW:119.98)	24 g
Ultrapure Water	Fill to 1000 mL

0.2 M Phosphate Buffer (PB)

0.2 M Phosphate Buffer (PB)	**1000 mL**
Stock 0.2 M Sodium Phosphate Dibasic	**1000 mL**
Stock 0.2 M Sodium Phosphate Monobasic	**200 mL**
pH to 7.4 using Dibasic (to increase the pH) or Monobasic (to lower the pH) Stock	

Appendix B

RNA Extraction protocol
(Norgen Biotek, Total RNA Purification Micro Kit (35300))

**Note: Add 10 µL of β-mercaptoethanol to each 1 mL of Lysis Solution FRESH before use under a FUME HOOD!

**Use RNase-free tubes and tips for all steps!

**Prepare 70% ethanol before starting. USE ONLY MOLECULAR BIOLOGY GRADE 100% ETHANOL and RNASE-FREE WATER

**Label tubes and spin-columns before starting experiment

1. Add punch to a 0.65 mL tube containing **300 µL of Lysis Solution**. Shake tube vigorously to submerge punch in liquid. Snap-freeze in liquid nitrogen.
2. Freeze immediately at -80°C or proceed quickly to step 3
3. Thaw to room temperature. Vortex for 30 seconds. Spin down tubes.
4. Incubate at room temperature for 5 minutes
5. Transfer the lysate to a 1.5 mL tube and add **300 µL of 70% ethanol** to the lyate.
6. Vortex for 5 seconds. Spin down tubes!
7. Apply 600 µL of lysate/ethanol mixture to a spin-column and centrifuge for **1 minute at 14,000 x g**
8. If the entire lysate did not pass through the column in the collection tube, centrifuge for an additional minute
9. Discard the flowthrough and reassemble the column and collection tube.
10. For each sample prepare DNase mix (**DO NOT ADD IT TO THE SAMPLE at this point!!!** It does not get added until step 13!!!): **15 µL of DNase I and 100**

µL of Enzyme Incubation Buffer from the Norgen RNase-Free DNase I kit (25710). Mix by GENTLY inverting the tube several times. DO NOT VORTEX (enzymes are sensitive to physical denaturation). Can make master mix for more than 1 sample.

11. Add **400 µL of Wash Solution** to spin column and **centrifuge 2 min at 14,000 x g**
12. Discard the flowthrough and reassemble the spin column and collection tube
13. Apply **100 µL of the RNase-free DNase I solution** that you prepared in step 10 to the column and centrifuge at **14,000 x g for 1 minute**. Ensure that entire solution passes through column. If it did not pass through column, centrifuge for an additional minute.
14. Pipet the flowthrough in the collection tube back on top of the column
15. Incubate at room temperature for 15 minutes
16. Apply **400 µL of Wash Solution** to column and centrifuge **1 min at 14,000 x g**. Again, if entire solution did not pass through column, re-centrifuge
17. Discard flowthrough and reassemble the spin column and tube
18. Apply **400 µL of Wash Solution** to column and centrifuge **1 min at 14,000 x g**.
19. Discard flowthrough and reassemble the spin column and tube
20. Spin column **2 min at 14,000 x g** to dry the resin.
21. DISCARD collection tube
22. Place column into a RNase-free Elution tube (in kit) and apply **20 µL of NUCLEASE-FREE WATER** (NOT PROVIDED IN KIT) to the top of the column.

23. Centrifuge **2 minutes at 200 x g**

24. Centrifuge another **1 min at 14,000 x g**. If the entire volume is not recovered, spin an additional minute at 14,000 x g.

25. STORE eluted RNA at -80°C

Appendix C

ANY-maze behavior analysis

1. Open the AnyMaze program

2. Click New experiment

3. Enter your experiment title

4. Enter name of peptide under ‘Treatment’ and the under Treatments on the right, enter the TOTAL number of animals in your experiment (do not separate by doses)

5. Go to ‘Protocol’

6. Select ‘Video Sources’, click +, ‘New Video Source’, and name the video source anything you want

7. Select ‘Apparatus’, click +, ‘New Apparatus’, and name the apparatus anything you want

8. Click and drag the orange ruler at the bottom of the screen on the right and place it along a distance you have measured on the behavior apparatus. You may need to resize the orange line by clicking and dragging the ends

9. Enter the distance the orange line represents (290 mm for 31 Curie arenas)

10. Use the shapes above the video to outline the ENTIRE apparatus you will be using. Do not only outline the floor of the apparatus

11. Select ‘Keys’, click +, ‘New Key’, enter a behavior you want to analyze by pressing a key and the key you want to use for that behavior

12. Repeat for each behavior you want to analyze by individual key presses (i.e. step, jump peck, etc). Uncheck ‘count this behavior as activity’

13. Leave the way you’d like the key to work as ‘Simple’

14. Repeat for timed behaviors (i.e. sleep, sit, stand, preen, etc) with the exception of how you'd like it to work. For timed behaviors select 'Radio' as the way you'd like it to work

15. Select 'Test Settings and Options', and 'Animal colour'. Choose the color setting that corresponds to how your animals are colored compared to the floor color

16. Select 'Tracking the animal's head' and check 'yes'

17. Select 'Automatic starting of tests' and select 'no – I'll start the tests manually'

18. Select 'What to display while testing'. 'Mark the animal's centre point' and 'shade the entire area of the animal' should be checked. Check 'List any active keys' and select 'show these indicators in the top right corner of the image'

19. Select 'Advanced tracking options' and check 'the apparatus is inside an enclosure'

20. Go to "stages," click "first stage," enter "1800" for test duration (in seconds) if you want to analyze 30 min per bird.

21. Select 'Results, reports, and data'. Select 'Data page' and check the animal information you would like to appear in the data spreadsheet. Click 'Apparatus Measure's and select the ones you would also like to appear in your spreadsheet

22. Go to 'Data' at the top of the screen and click 'Select data to show'. On the right, select the ones you would like to appear in your data table. Be sure 'Segment of Test' is also selected

23. Under 'Actions' on the left of the screen, click 'Set segment length for analysis across time'. Under 'Settings', enter the segment length for analysis, for example if you want your results for every minute, enter 60 and if you want the data broken down by 5 minute intervals, enter 300

24. Go to "Tests" at the top of the screen and press the green arrow when ready to begin

your analysis

25. During the analysis, keys that were set up for timed behavior only have to be pressed once when that behavior starts. When the animal switches to a different timed behavior (i.e. stands and then sits down), press the key for the new timed behavior and it will stop timing the first one and start timing the new behavior