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

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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 elucidate the hypothalamic mechanisms that mediate CRF-induced anorexia in 4 day-old Cobb-500 chicks. After intracerebroventricular (ICV) injection of 0.02 nmol of CRF, CRF-injected chicks ate less than vehicle chicks while no effect on water intake was observed at 30 min post-injection. In subsequent experiments, the hypothalamus samples were processed at 60 min 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). In the ARC, CRF-injected chicks expressed more CRF and CRF receptor sub-type 2 (CRFR2) mRNA but less 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.

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 (Gillies and Grossman 1985; Vale et al. 1981). CRF belongs to the CRF family which also includes several structurally and functionally related peptides, such as urocortin (UCN)1, UCN2, and UCN3 (Stengel and Taché 2014). Cellular effects of CRF family peptides are mediated via CRF receptor sub-types 1 (CRFR1) and 2 (CRFR2) (Stengel and Taché 2014). CRF is associated with multiple, diverse physiological functions, such as immunity (Margioris et al. 2017), reproduction (Chand and Lovejoy 2011), stress responses (Kormos and Gaszner 2013), anxiety behavior (Chen 2014) and arousal and locomotor activity (Lowry and Moore 2006; Zitnik 2016).

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 (Cline et al. 2009; Denbow et al. 1999; Furuse et al. 1997; Ohgushi et al. 2001; Zhang et al. 2001). The paraventricular nucleus (PVN) is the major site of synthesis and release of CRF in the hypothalamus (Heinrichs et al. 1993; Mönnikes et al. 1992; Wang et al. 2011). The CRF antagonist, α-helical CRF, attenuated the anorectic effects of CRF injection into the PVN but not other hypothalamic nuclei such as the ventromedial hypothalamus (VMH) (Kalra et al. 1999). Additionally, central injection of α-helical CRF augments NPY-induced hunger (Heinrichs et al. 1992), and blunts the reduction in food intake caused in response to restraint (Krahn et al. 1986) and tail pinch (Heinrichs et al. 1992) stress and exercise (Rivest and Richard 1990), which suggests that endogenous CRF plays a physiological role in appetite regulation. This is consistent with observations that endogenous

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CRF production is affected by a variety of factors that affect feeding behavior, including drugs such as fenfluramine (Appel et al. 1991), obesity (Richard et al. 1996), and tumor-induced anorexia (McCarthy et al. 1993). Similarly, we demonstrated that anorexic chickens subjected to stressors at hatch were later refractory to the food intake-stimulating effects of NPY (Wang et al. 2017), and that this intensification of anorexia was associated with up-regulated CRF and CRFR expression in the PVN and could be blocked by pretreatment with astressin, a non-selective CRFR antagonist (Wang et al. 2017).

The CRFR2 is regarded as the primary receptor through which the anorexigenic effects of CRF are mediated, as demonstrated with pharmacological inhibitors (Cullen et al. 2001; Pelleymounter et al. 2000) 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 (Cha et al. 2008; Levin 2006).

In chickens, the avian species for which the most information exists, CRF inhibits food intake (Cline et al. 2009; Denbow et al. 1999; Furuse et al. 1997; Ohgushi et al. 2001; Zhang et al. 2001) 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) (Kamisoyama et al. 2009; Tachibana et al. 2007), leptin (Gardner et al. 1998), cholecystokinin (CCK) (Tachibana et al. 2012) and UCN3 (Yeh et al. 2016).

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 nuclei play important roles in the regulation of feeding behavior, such as the arcuate nucleus (ARC), PVN, VMH, dorsomedial hypothalamic nucleus (DMN), and lateral hypothalamic area (LHA) (Arora and Anubhuti, 2006). The ARC is thought to be a feeding control center (Funahashi et al. 2000), which contains two functionally discrete neuronal populations carrying out opposite effects on appetite regulation including the orexigenic, neuropeptide Y (NPY)/agouti-related peptide (AgRP) and anorexigenic, pro-opiomelanocortin (POMC)/ cocaine and amphetamine-regulated transcript (CART) (Hillebrand et al. 2002; Parkinson et al. 2008) neurons.

Other neuropeptides and receptors exerting either stimulatory or inhibitory effects on feeding behavior have been identified, such as melanin-concentrating hormone (MCH) (Ando et al. 2000), mesotocin (avian ortholog of oxytocin; MT) (Masunari et al. 2013), melanocortin receptors 3 and 4 (MC3R and MC4R, respectively) (Sobrino Crespo et al. 2014) and neuropeptide S (NPS) (Cline et al. 2007), although their specific role in hypothalamic regulation of appetite in birds has not been elucidated. In the present study, we measured the gene expression of these and several other appetite-related factors in the hypothalamus after CRF injection in order to determine whether they might be involved in CRF-induced reduction of food intake. 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 molecular mechanisms 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 °C and 50 ± 5% relative humidity and 24 h of light. Chicks were handled daily to adapt to handling and minimize stress, with ad libitum access to food (21.5% crude protein and 3000 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. 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 was based on the the Kuenzel and Masson chicken stereotaxic atlas (Kuenzel and Masson 1988). Anatomical landmarks were visually determined by using a restraining device and plastic tubing sheath. In particular, the restraining device is a piece of clay with scales that was molded with the use of a chick cadaver. The restraining device leaves the cranium exposed and coordinates the injection point at 2 mm anterior and 0.75 mm lateral from the sagittal suture. The plastic tubing sheath over the needle was used to control injection depth (1.5 mm). The needle remained at injection depth in the un-anesthetized chick for 5 s 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 visually the presence of dye in the lateral ventricle. Any chick without dye present in the lateral ventricle system was eliminated from analysis. Sex of chicks was determined visually by dissection and gonadal inspection at the time of decapitation.

2.3. Experiment 1: study design and measurement of food and water intake

Chicks, fasted for 180 min, were randomly assigned to receive either vehicle or 0.02 nmol CRF, the dose of which was based on (Zhang et al. 2001; 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 min post-injection.

2.4. Experiment 2: 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 min prior to injection, after which food was withheld to prevent c-Fos immunoreactivity associated with food consumption. 60 min 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 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. Several 60 µm coronal sections that contained appetite-related nuclei based on the anatomical description of Kuenzel and Masson (Kuenzel and Masson 1988) were collected in 0.02 M phosphate-buffered saline (PBS) containing 0.1% sodium azide using a cryostat at −15 °C. Sections were processed immediately after collection. Procedures for c-Fos immunohistochemistry were performed as described (Newmyer et al. 2013) using rabbit polyclonal anti-c-Fos at a dilution of 1:20,000 (K-25, Santa Cruz, CA, USA). For assay controls, normal rabbit serum was substituted for the primary antibody. 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. Although the discussion of results alludes to neuronal mechanisms, this method of protein detection and counting does not distinguish between neurons and glial cells.

2.5. Experiment 3: total RNA isolation from the whole hypothalamus, reverse transcription, and real-time PCR

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 min prior to injection, at which time food was withheld to prevent molecular changes associated with differences in food consumption. At 60 min 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 s. 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 °C for 10 min, 37 °C for 120 min and 85 °C for 5 min. 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 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 °C for 20 s and 40 cycles of 90 °C for 3 s plus 60 °C for 30 s. A dissociation step consisting of 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 s was performed at the end of each PCR reaction to ensure amplicon specificity.

2.6. Experiment 4: total RNA isolation from hypothalamic nuclei, reverse transcription, and real-time PCR

The design was the same as for Experiment 3 except for that at 60 min 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 min of perfusion, brains were sectioned in a cryostat at −10 °C into 500 µm thick coronal sections in the direction from rostral to caudal: the LHA, PVN and VMH were collected at plate 8.0, 7.4, and 6.8 respectively, and the ARC and 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 °C. 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 s and incubated at room temperature for 5 min before adding 70% molecular biology-grade ethanol, and total RNA was isolated following the manufacturer's instructions for the Total RNA Purification Micro Kit and RNase-Free DNase I (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.

2.7. Statistical analyses

All data were analyzed using the Fit Model platform of JMP Pro 13 (SAS Inst., NC). Homogeneity of variance was evaluated for all variables using the Fit Y by X platform and the Unequal Variances test and Levene's test. Food intake data were analyzed using analysis of variance (ANOVA), with the statistical model including the main effect of treatment. Immunohistochemistry (immunoreactive cell counts) data were analyzed by ANOVA and the model included the main effect of CRF treatment within each nucleus. Real-time PCR data for whole hypothalamus samples (Experiment 3) 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. Real-time PCR data for nucleus samples were analyzed the same as for Experiment 3 except that the statistical model included the main effect of CRF treatment within nucleus. Statistical significance was set at $P < .05$ for all experiments.

3. Results

3.1. Food and water intake

At 30 min post-injection, CRF-injected chicks ate about 75% less than vehicle-injected chicks ($P = .0019$) while water intake was not affected by CRF injection ($P = .1852$) (Fig. 1).

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

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

[a] 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).

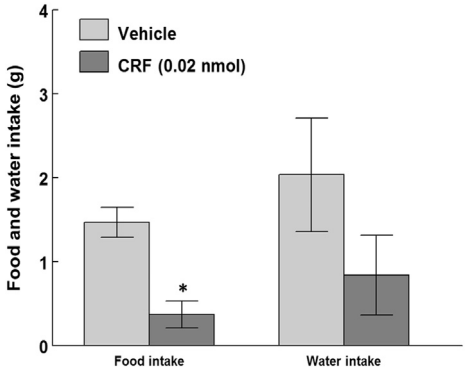

Fig. 1. Effect of intracerebroventricular injection of corticotropin-releasing factor (CRF; 0.02 nmol) in 4 day-old Cobb-500 chicks on food and water intake at 30 min post-injection. (*) denotes difference from vehicle-injected group ($P < .05$). Values are means ± SEM. For this experiment, 10 vehicle- and 10 CRF-treated chicks were available for the analysis.

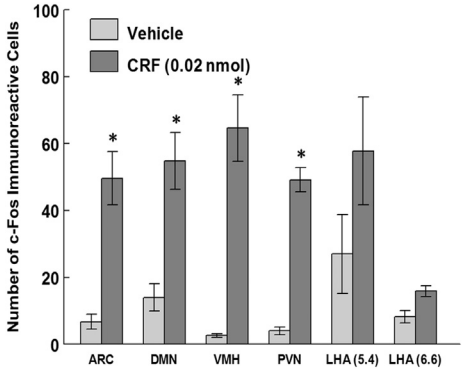

Fig. 2. Effect of intracerebroventricular injection of corticotropin-releasing factor (CRF; 0.02 nmol) in 4 day-old Cobb-500 chicks on the number of c-Fos immunoreactive cells in the arcuate nucleus (ARC), dorsomedial hypothalamic nucleus (DMN), ventromedial hypothalamus (VMH), paraventricular nucleus (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 ($P < .05$). Values are means ± SEM. For this experiment, 6 vehicle- and 8 CRF-treated chicks were available for the analysis.

3.2. c-Fos immunohistochemistry

The number of c-Fos immunoreactive cells in chicks treated with CRF increased by about 740% ($P = .0044$) and 390% ($P = .0057$) over the vehicle-injected chicks in the ARC and DMN, respectively (Fig. 2). In the VMH and PVN, immunoreactive cell numbers increased 2500% ($P = .0004$) and 1200% ($P < .0001$), 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. 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-treated chicks at 60 min post-injection ($P = .0492$) (Fig. 3B). Melanin-concentrating hormone (MCH), agouti-related peptide (AgRP), neuropeptide Y (NPY), and neuropeptide Y receptor sub-types 2, 5 and 6 (NPYR2, 5 and 6, respectively) mRNAs were not affected by CRF injection.

3.4. mRNA abundance of appetite-associated factors in hypothalamic nuclei

In the ARC, CRF-injected chicks expressed greater CRF ($P = .0352$) and CRFR2 ($P = .0463$) mRNAs (more than four-fold up-regulation) but less AgRP ($P = .0014$), NPY ($P = .0079$), and NPYR1 ($P = .0146$) mRNA than vehicle chicks (Fig. 4A). The ARC expression of leptin receptor (LEPR), mesotocin (MT), neuropeptide S (NPS), Pro-opiomelanocortin (POMC), NPYR2, NPYR5 and CRFR1 mRNA was not different between CRF-injected and vehicle chicks. In the PVN, CRF-injected chicks expressed more CRFR2 mRNA than vehicle-treated chicks ($P = .0022$) (Fig. 4B). The mRNA abundance of NPY, CRF, CRFR1, MT, NPS, NPYR5, POMC and UCN3 in the PVN was not affected by ICV injection of CRF. In the DMN, NPYR1 mRNA was down-regulated in CRF-injected chicks ($P = .0105$) (Fig. 5A). The CRF treatment did not affect the mRNA abundance of NPY, CRFR1, CRFR2, MT, NPS, CRF, and POMC in the DMN. In the VMH, CRF-injected chicks had almost four times greater quantities of MT mRNA than chicks from the vehicle

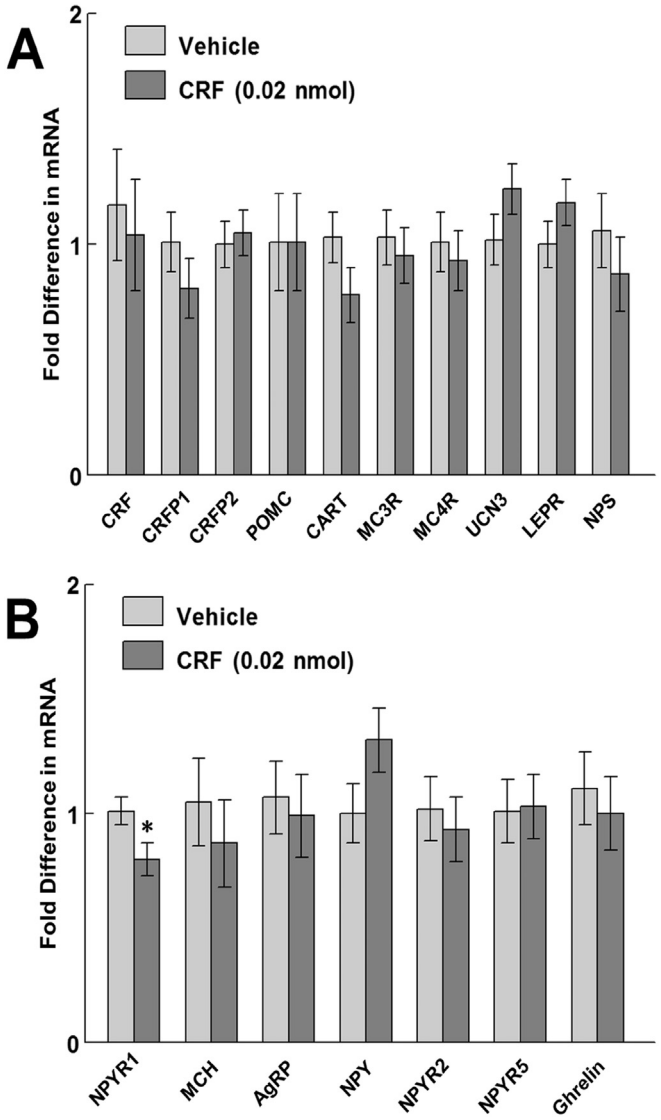

Fig. 3. Effect of intracerebroventricular administration of corticotropin-releasing factor (CRF; 0.02 nmol) in 4 day-old Cobb-500 chicks on expression of anorexigenic (A) and orexigenic (B) – associated factors in whole hypothalamus at 60 min post-injection. (*) denotes difference from vehicle-injected group ($P < .05$). 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).

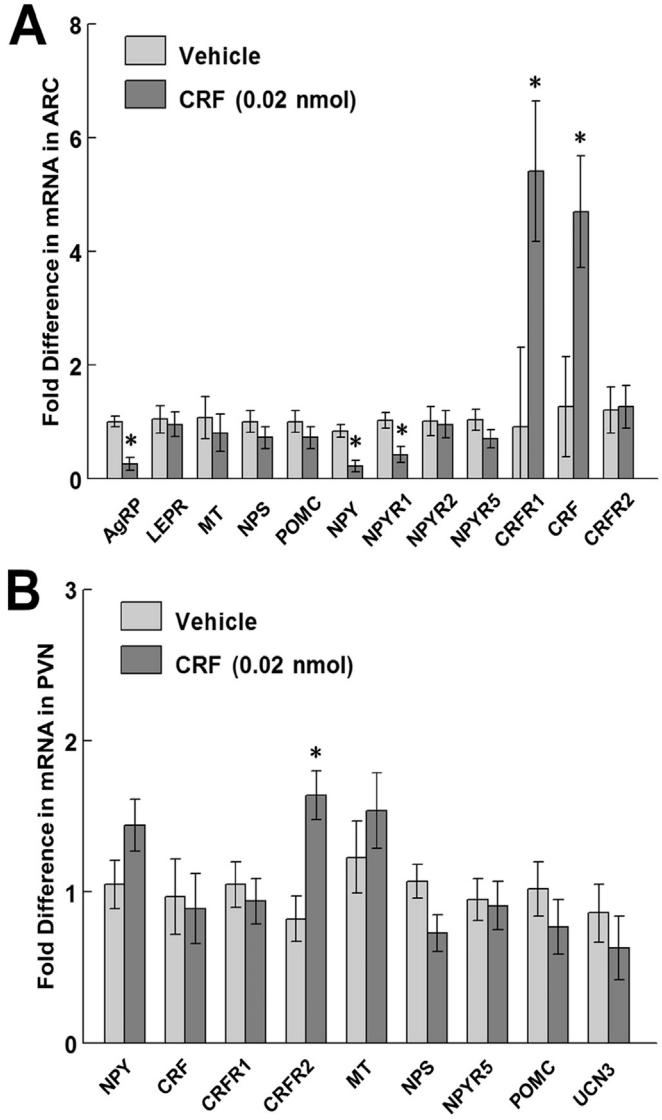

Fig. 4. Effect of intracerebroventricular administration of corticotropin-releasing factor (CRF; 0.02 nmol) in 4 day-old Cobb-500 chicks on mRNA in the arcuate nucleus (ARC) (A) and paraventricular nucleus (PVN) (B) at 60 min post-injection. (*) denotes difference from vehicle-injected group ($P < .05$). Values are means ± SEM. For this experiment, 12 vehicle and 12 CRF-treated chicks were available for the analysis. Abbreviations: agouti-related peptide (AgRP), CRF receptor sub-types 1 and 2 (CRFR1 and CRFR2, respectively), leptin receptor (LEPR), 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).

group ($P = .0094$) (Fig. 5B). The expression of NPY, CRFR2, LEPR, NPYR1, CRFR1, and NPS was not different between CRF- and vehicle-treated chicks.

4. Discussion

The CRF is a potent anorexigenic factor in chickens (Cline et al. 2009; Denbow et al. 1999; Furuse et al. 1997; Tachibana et al. 2006). Central administration of 2–200 pmol of CRF suppressed food intake dose-dependently in 2 day-old broiler chicks that were fasted for 3 h 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 that were 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 min 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

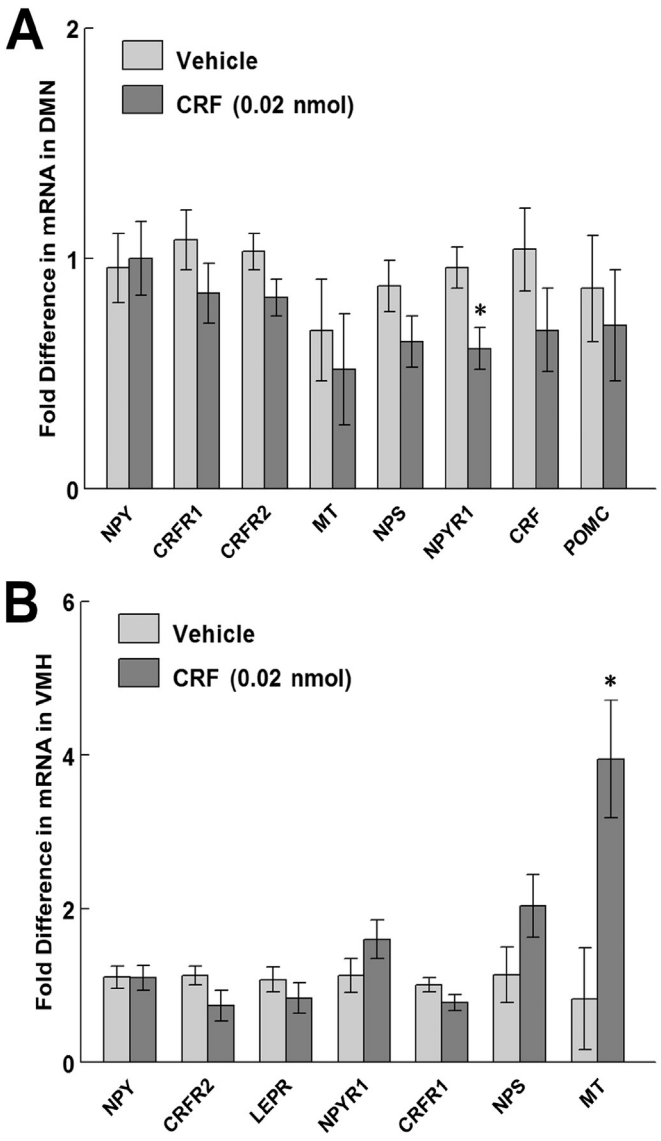

Fig. 5. Effect of intracerebroventricular administration of corticotropin-releasing factor (CRF; 0.02 nmol) in 4 day-old Cobb-500 chicks on mRNA in the dorsomedial hypothalamic nucleus (DMN) (A) and ventromedial hypothalamus (VMH) (B) at 60 min post-injection. (*) denotes difference from vehicle-injected group ($P < .05$). Values are means ± SEM. For this experiment, 12 vehicle and 12 CRF-treated chicks were available for the analysis. Abbreviations: CRF receptor sub-types 1 and 2 (CRFR1 and CRFR2, respectively), leptin receptor (LEPR), mesotocin (MT), neuropeptide S (NPS), neuropeptide Y (NPY), NPY receptor sub-types 1, 2 and 5 (NPYR1, NPYR2 and NPYR5, respectively), and Pro-opiomelanocortin (POMC).

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 that 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 those in 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 (Danielsa et al. 2001; Kanatani et al. 1996) 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 (Chance et al. 2007; Kishi et al. 2005), 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 NPY 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.

MT, the non-mammalian equivalent of oxytocin in birds, increased in the VMH in response to CRF treatment. ICV injection of MT 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 MT,

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

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