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

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A B S T R A C T

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 h 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.

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 factor (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., 1995). 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 (Wang et al., 2011; Zorrilla et al., 2003), including chickens (Cline et al., 2009; Denbow et al., 1999; Furuse et al., 1997; Meade and Denbow, 2003; Tachibana et al., 2006), 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 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.

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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 h). At all times, unless otherwise noted, quail had ad libitum access to a mash diet (energy: 2900 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. 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, 0.75 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 s 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 h 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; 10 males and 38 females). After injection, quail were returned to their individual home cages and given ad libitum access to both food and water. Food and water intake were monitored (0.01 g) every 30 min for 180 min and then 6, 16, 21 and 24 h 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 h 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; 9 males and 15 females). 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 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 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 °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 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 °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.

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 h 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 = 11 for each group; 9 males and 13 females). 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 in a series of

Table 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
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), 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).

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 at 6.6 interaural, and the DMN and ARC were collected at 5.4 interaural based on the Kuenzel and Masson chicken stereotaxic atlas (Kuenzel and Masson, 1988). 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 and a chromogenic staining kit (K-25, Santa Cruz, Santa Cruz, CA, USA) (Newmyer et al., 2013; Zhao and Li, 2010). Anatomies were confirmed using digital images from the chicken stereotaxic atlas (Kuenzel and Masson, 1988), and a digital micrograph was captured of each section with a Nikon Eclipse 80i microscope, 4X objective and DS-Ri1 color camera, and images were analyzed using NIS-Elements Advanced Research Software (Nikon) and the stitching method to assemble a complete image of the section. Overlays containing the respective nuclei boundaries (defined within the stereotaxic atlas) 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 behavior test was conducted in the light cycle. The quail were tested in the same room where birds were kept to avoid the stress from novel environment and the experimenter was not visible to the testing bird during the recording. The quail were kept in the 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 (n = 12 for each group; 11 males and 13 females). Following 6 h 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 min post-injection on DVD and the data were analyzed in 5 min intervals using ANY-maze behavioral analysis software (Stoelting, Wood Dale, IL). At 30 min post-injection, food intake was measured. Locomotion (m traveled), the amount of time spent standing, sitting, preening, or in deep rest, as well as the number of defecations, jumps, steps, feed pecking, exploratory pecking, 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 s (and its timing starting 3 s 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 min 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 h 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. 1A). At 6 h, 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 h post-injection. The quail in the 22 pmol-injected group ate less than vehicle-injected birds for the first hour 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. 1B).

The inhibitory effect of 22 and 222 pmol of CRF on cumulative water intake lasted 2.5 and 24 h post-injection, respectively (Fig. 2A). For non-cumulative water intake, the decrease in water intake induced by 22 pmol of CRF lasted for 1 h post-injection while inhibitory effects were observed in the 222 pmol-injected group for up to 1.5 h post-injection (Fig. 2B). At 24 h post-injection, water intake was reduced in the 2 and 22 pmol-injected groups.

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 h post-injection (Fig. 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).

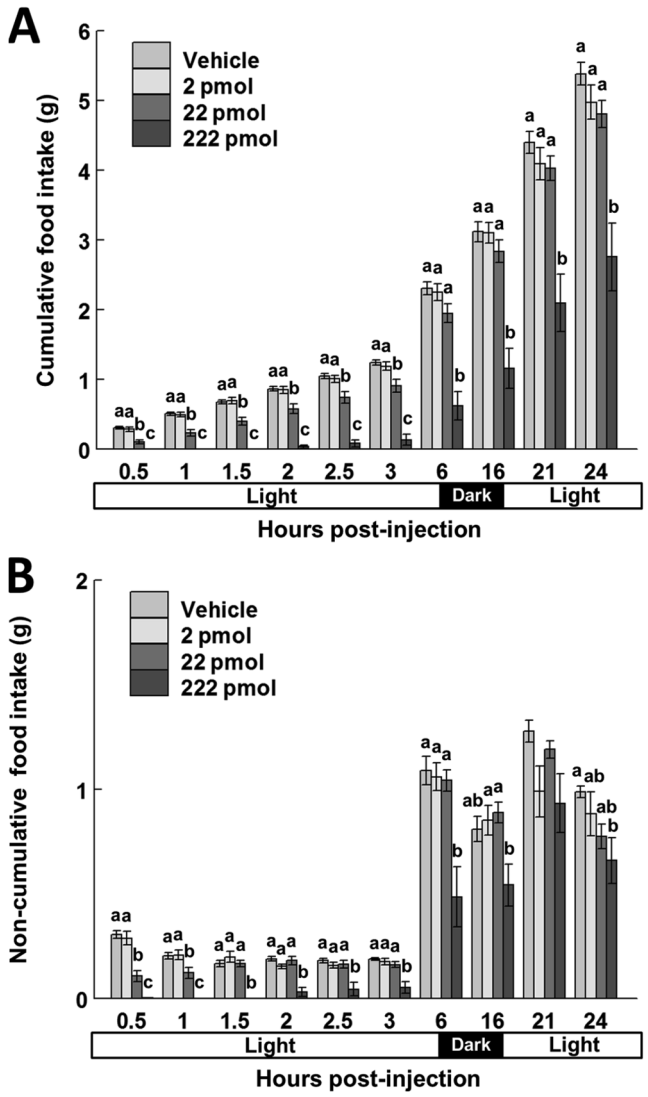

Fig. 1. Cumulative (A) and non-cumulative (B) food intake expressed as a percentage of body weight of Japanese quail at 7 days post-hatch (Experiment 1). Values are means ± S.E.M.; bars with different superscripts are different from each other within a time point ($P < 0.05$). There were 12 quail per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

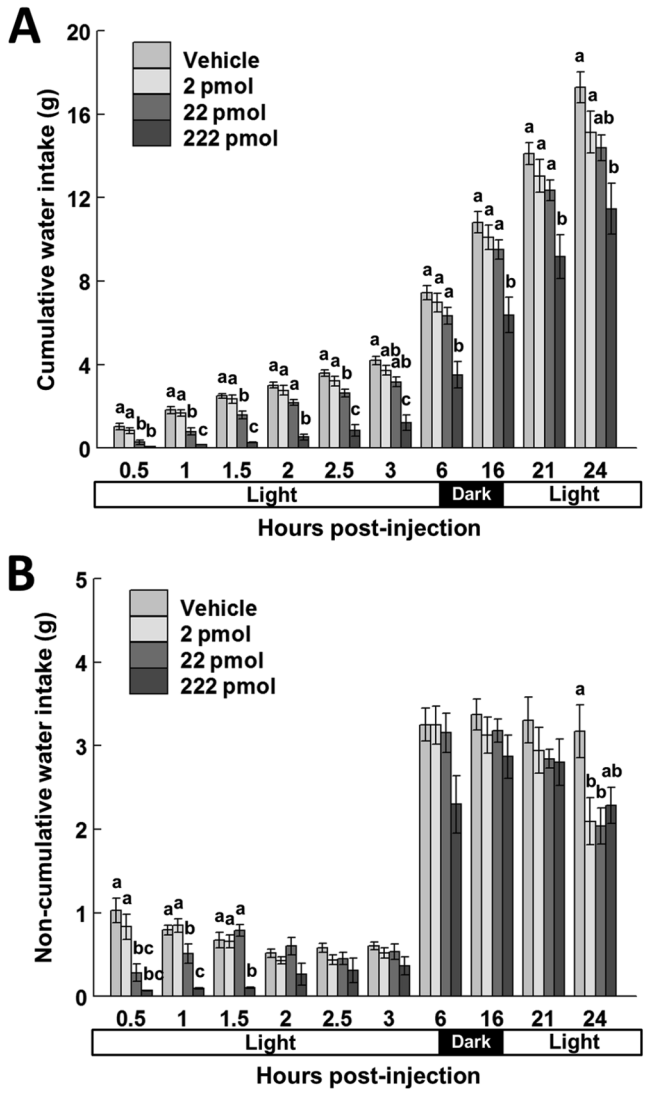

Fig. 2. Cumulative (A) and non-cumulative (B) water intake expressed as a percentage of body weight of Japanese quail at 7 days post-hatch (Experiment 1). Values are means ± S.E.M.; bars with different superscripts are different from each other within a time point ($P < 0.05$). There were 12 quail per group available for analysis.

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 h post-injection (Figs. 4 and 5). 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 min post-injection compared to vehicle-injected birds (Table 2). The CRF-injected quail also displayed fewer jumps at 10 through 30 min post-injection. The numbers of exploratory pecks, defecations, steps, and distance traveled were not affected by CRF injection. From 15 min onward, quail treated with CRF spent more time standing than those from the vehicle-injected group (Table 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 (Denbow et al., 1999; Furuse et al., 1997) 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 min 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 h and food intake returned to baseline at 6 h post-injection. The 222 pmol dose was even more potent, with the anorexic effect persisting for at least 24 h 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

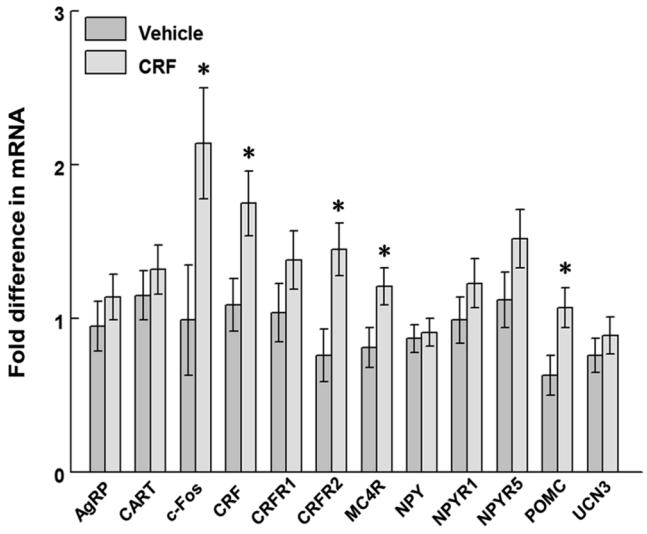

Fig. 3. Effect of ICV administration of CRF (22 pmol) in Japanese quail at 7 days post-hatch on hypothalamic expression of appetite-associated factor mRNA. (*) denotes difference from control ($P < 0.05$). Values are means ± S.E.M. For this experiment, 12 vehicle and 12 CRF-treated Japanese quail were available for the analysis. 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), 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).

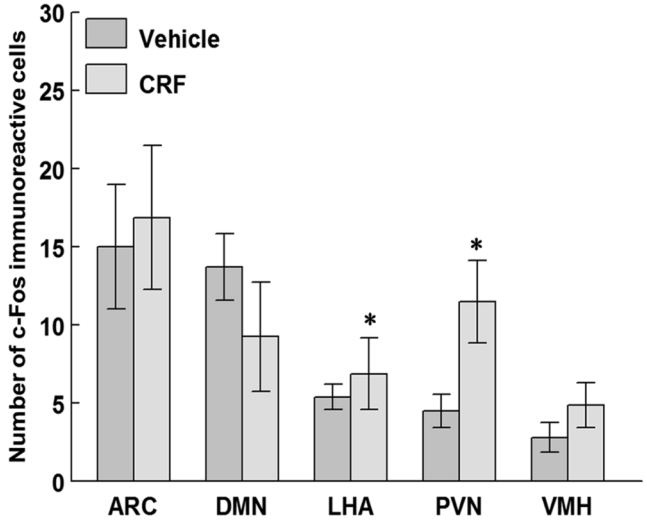

Fig. 4. Effect of ICV administration of CRF (22 pmol) in Japanese quail at 7 days post-hatch on the number of c-Fos immunoreactive cells in the arcuate (ARC), dorsomedial hypothalamic nucleus (DMN), lateral hypothalamic area (LHA), paraventricular nucleus (PVN), and ventromedial nucleus of hypothalamus (VMH). (*) 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.

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). With the PVN being activated after CRF injection, the reduction in water intake could be related to regulation of vasotocin (vasopressin in mammals), which regulates water balance, and is produced in the PVN in response to restraint stress in birds (Nagarajan et al., 2016; Nagarajan et al., 2014).

On a non-cumulative basis, food intake results show that the anorexigenic effects of the 22 pmol dose began to dissipate after 3 h 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 h following ICV injection. Although this method is a gold standard for indirectly assessing neuronal activation (Chung, 2015), a caveat that should be acknowledged is that it does not distinguish between cell-types that express the protein, which could be glial cells in addition to neurons. 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., 1999), 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).

We also measured the hypothalamic mRNA abundance of appetite-associated factors and found that CRF and CRFR2 mRNAs were increased at 1 h 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 (Cullen et al., 2001; Pelleymounter et al., 2000; 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 (Gerets et al., 2000; Takeuchi et al., 1999). 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.,

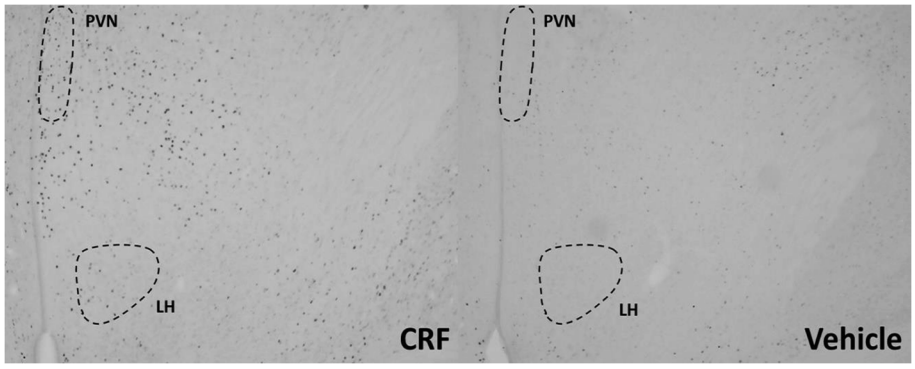

Fig. 5. Photomicrographs of c-Fos immunohistochemistry in the lateral hypothalamus (LH) and paraventricular nucleus (PVN) of the hypothalamus. Treatment is indicated on each photomicrograph.

2000; Strader et al., 2003; Tachibana et al., 2001). The anorexigenic effect of α-MSH is mediated by MC4R, where such an effect could be attenuated by antagonism of the MC4R (Strader et al., 2003; Tachibana et al., 2001), indicating MC4R is an important mediator of the anorexic effect of α-MSH. To date, there is a growing body of evidence that suggests 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 (Kawashima et al., 2008; Lu et al., 2003) 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 (Füzesi et al., 2016; Rho and Swanson, 1987), indicating a possible neural pathway that bridges the signaling between the PVN and LHA, and vasotocin-expressing neurons localize to the LHA in chickens (Nagarajan et al., 2014).

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.02.015.

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