Neuropeptides 53 (2015) 29–35

Contents lists available at ScienceDirect

Neuropeptides

journal homepage: www.elsevier.com/locate/ynpep

The central anorexigenic mechanism of adrenocorticotropic hormone involves the caudal hypothalamus in chicks

Steven L. Shipp [a], Jiaqing Yi [a], Sami Dridi [b], Elizabeth R. Gilbert [a], Mark A. Cline [a,*]

[a] Department of Animal and Poultry Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA
[b] Center of Excellence for Poultry Science, University of Arkansas, Fayetteville, AR, USA

ARTICLE INFO

Article history:
Received 2 February 2015
Received in revised form 3 July 2015
Accepted 31 July 2015
Available online 8 August 2015

Keywords:
Adrenocorticotropic hormone
Hypothalamus
Food intake
Chick

ABSTRACT

Adrenocorticotropic hormone (ACTH), consisting of 39 amino acids, is most well-known for its involvement in an organism's response to stress. It also participates in satiety, as exogenous ACTH causes decreased food intake in rats. However, its anorexigenic mechanism is not well understood in any species and its effect on appetite is not reported in the avian class. Thus, the present study was designed to evaluate central ACTH's effect on food intake and to elucidate the mechanism mediating this response using broiler chicks. Chicks that received intracerebroventricular (ICV) injection of 1, 2, or 4 nmol of ACTH reduced food intake, under both *ad libitum* and 180 min fasted conditions. Water intake was also reduced in ACTH-injected chicks under both feeding conditions, but when measured without access to feed it was not affected. Blood glucose was not affected in either feeding condition. Following ACTH injection, c-Fos immunoreactivity was quantified in key appetite-associated hypothalamic nuclei including the ventromedial hypothalamus (VMH), dorsomedial hypothalamus, lateral hypothalamus (LH), arcuate nucleus (ARC) and the parvo- and magno-cellular portions of the paraventricular nucleus. ACTH-injected chicks had increased c-Fos immunoreactivity in the VMH, LH, and ARC. Hypothalamus was collected at 1 h post-injection, and real-time PCR performed to measure mRNA abundance of some appetite-associated factors. Neuropeptide Y, pro-opiomelanocortin, glutamate decarboxylase 1, melanocortin receptors 2–5, and urocortin 3 mRNA abundance was not affected by ACTH treatment. However, expression of corticotropin releasing factor (CRF), urotensin 2 (UT), agouti-related peptide (AgRP), and orexin (ORX), and melanocortin receptor 1 (MC1R) mRNA decreased in the hypothalamus of ACTH-injected chicks. In conclusion, ICV ACTH causes decreased food intake in chicks, and is associated with VMH, LH, and ARC activation, and a decrease in hypothalamic mRNA abundance of CRF, UT, AgRP, ORX and MC1R.

1. Introduction

Adrenocorticotropic hormone (ACTH) is a 39 amino acid peptide derived from pro-opiomelanocortin (POMC); and has primarily been studied for its involvement in the hypothalamic–pituitary–adrenal (HPA) axis. When a stressor is perceived, corticotropin releasing factor (CRF) is released from the hypothalamus, which serves as a secretagogue of adenohypophysis derived-ACTH into circulation (Vale et al., 1981). One effect of circulating ACTH is the synthesis and release of corticosterone from the adrenal cortex, which helps the animal cope with the stressor. Although this was the first and best understood physiological significance of ACTH, it also participates in many other aspects of physiology including decreased growth rates in birds (Davison et al., 1979), lowered immunity in both birds and mammals (Kang et al., 2012; Thaxton et al., 1968), decreased hippocampal cell proliferation (Onoue et al., 2014), and depressive-like behavior (Tokita et al., 2012).

In chickens, ACTH is cleaved from POMC in the pars distalis and cephalic lobe of the pituitary gland (Gerets et al., 2000). While mostly found in the pituitary, there are ACTH immunoreactive fibers found in the arcuate nucleus of the rat hypothalamus (Csiffary et al., 1990), and because the arcuate nucleus is involved in food intake, it is not surprising that ACTH affects satiety. ACTH can also be found in the amygdala, cerebral cortex, cerebellum, and brain stem (Civelli et al., 1982; Palkovits et al., 1987; Pilcher and Joseph, 1984).

ACTH decreases feeding in rats and zebra fish, and centrally is believed to bind to the melanocortin 4 receptor (MC4R) in rats (Al-Barazanji et al., 2001), and the melanocortin 2 receptor (MC2R) under fed conditions, or MC4R under fasted conditions in fish (Cerda-Reverter et al., 2011; Josep Agulleiro et al., 2013). Although ACTH's effect on food intake has been documented in rats and fish, the central mechanism is still poorly understood, and not one of the effects has been documented in the avian class.

To evaluate the effects of ACTH on food intake in chicks, we administered ACTH through intracerebroventricular injection (ICV), and food and water intake were measured for 180 min post injection. To study the effects on the hypothalamus, c-Fos immunoreactive cells were

* Corresponding author.
E-mail address: macline2@vt.edu (M.A. Cline).

http://dx.doi.org/10.1016/j.npep.2015.07.005
0143-4179/

quantified as a measure of neuronal activity following ICV ACTH and gene expression of feeding-related hypothalamic neuropeptides were also examined. Finally, a comprehensive behavioral analysis was also conducted.

2. Materials and methods

2.1. Animals

Unsexed Hubbard × Cobb-500 cross chicks (*Gallus gallus*) were obtained from a commercial Hatchery on the morning of hatch. Chicks were caged individually at 30 ± 1 °C and 50 ± 5% relative humidity; with free access to a mash diet (21.5% crude protein 3000 kcal ME/kg) and tap water. All experiments were conducted between 08:00 and 11:00 using 4 day post hatch chicks and each experiment used chicks from separate hatches. All experimental procedures were performed according to the National Research Council publication, Guide for Care and Use of Laboratory Animals and were approved by the Virginia Polytechnic Institute and State University Institutional Animal Care and Use committee.

2.2. Intracerebroventricular (ICV) injection

Chicks received ICV injections from the methods adapted from Davis et al. (1979). The head of the chick was momentarily inserted into a restraining device that left the cranium exposed and allowed for free-hand injection. Injection coordinates were 3 mm anterior to the coronal suture, 1 mm lateral from the sagittal suture, and 2 mm deep targeting the left lateral ventricle. Anatomical landmarks were determined visually and by palpation. Injection depth was controlled by placing a plastic tubing sheath over the needle. The needle remained *in vivo* in the unanesthetized chick for 5 s to reduce backflow. Human ACTH (American Peptide Company, Sunnyvale, CA, Molecular weight: 2933.5) was dissolved in artificial cerebrospinal fluid to act as a vehicle, with an injection volume of 5 μL, with 0.06% Evan's Blue dye to determine injection site location. After data collection, each bird was decapitated and the brain sectioned to determine site of injection. Any chick with no dye present was eliminated from analysis. The sex of each chick was determined visually by dissection.

2.3. Experiment 1: food and water intake in chicks fed ad libitum

Chicks were randomly assigned to receive an ICV injection of either 0 (vehicle), 1, 2, or 4 nmol ACTH. Immediately following injection, chicks were returned to their cages, where they were given access to food and water *ad libitum*. Food and water were measured every 30 min for 180 min post injection. Water weight was converted to volume (mL; 1 g = 1 mL). Blood glucose levels were also collected 180 min post injection *via* a OneTouch glucose monitoring system. Data were analyzed using analysis of variance (ANOVA) at each time point using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC). The model included ACTH dose, sex and the interaction of sex with ACTH dose. Sex and the interaction of sex and ACTH dose were not significant and were eliminated from the model (and the effect of sex was not tested in subsequent experiments). If significant treatment effects were found, Tukey's multiple comparison test was used to separate the means at each time period. The number of chicks used in each experiment is given in figure legends. For this and all proceeding experiments, statistical significance was set at $P < 0.05$.

2.4. Experiment 2: food and water intake in 180 min fasted chicks

Procedures were the same as 2.3 except each chick was fasted for 180 min prior to the experiment.

2.5. Experiment 3: water intake

Procedures were the same as 2.3 except each chick was fasted for 180 min prior to the experiment and water intake was measured without access to feed.

2.6. Experiment 4: c-Fos immunohistochemistry

Each chick was randomly assigned to receive either vehicle or 2 nmol ACTH by ICV injection. Chicks were fasted for 180 min prior to the injection, after which food was not provided 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 (Muller et al., 1984), chicks were decapitated, 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 anatomies described by Puelles (2007) were collected in 0.02 M phosphate buffered saline (PBS) containing 0.1% sodium azide using a cryostat at −15 °C. The ventromedial hypothalamus (VMH), paraventricular nucleus (PVN), lateral hypothalamic nucleus (LH), and dorsomedial nucleus (DMN) were collected at interaural 2.08 mm, and VMH, LH, and the arcuate nucleus (ARC) at interaural 1.60 mm. Sections were processed immediately after collection. Procedures for c-Fos immunohistochemistry were based on those of (Zhao and Li, 2010) and were performed as described by 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). Anatomy was 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 by ANOVA including the main effect of ACTH dose.

2.7. Experiment 5: whole hypothalamus total RNA extraction and food intake related gene expression

Each chick was randomly assigned to receive either vehicle or 2 nmol ACTH by ICV injection. Chicks were fasted for 180 min prior to the injection, after which food was not provided. Sixty minutes following ACTH injection, chicks were decapitated and brains removed. The inverted brain was submerged in liquid nitrogen such that the most ventral aspect of the optic lobe was level with the surface of the liquid nitrogen for 11 s. Perpendicular to the midline suture, a cut was made at the septopallio-mesencephalic tract and at the third cranial nerves. At 2.0 mm parallel to the midline, two cuts were made. The final cut was made from the anterior commissure to 1.0 mm ventral to the posterior commissure. This block comprising the hypothalamus was stored in RNAlater (Qiagen) and homogenized using 5 mm stainless steel beads (Qiagen) and 1 ml Isol Lysis reagent (5-Prime, USA) for 2 × 2 min at 20 Hz with a Tissue Lyser II (Qiagen). After incubation and centrifugation for 10 min for 12,000 × g at 4 °C, the supernatant was removed and total RNA separated, following the manufacturer's instructions (5-Prime). Following the step of addition to 70% ethanol, mixtures were transferred to spin columns and total RNA purified using the RNeasy Mini Kit (Qiagen, USA), including the optional on-column RNase-free DNase I step (Qiagen, USA). The eluted total RNA samples were evaluated for integrity by agarose-formaldehyde gel electrophoresis and concentration and purity assessed by spectrophotometry at 260/280/230 nm.

Single-strand cDNA was synthesized from 200 ng total RNA in 20 μl reactions with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), following the manufacturer's instructions. Reactions