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Research report

Neuropeptide Y is associated with changes in appetite-associated hypothalamic nuclei but not food intake in a hypophagic avian model

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HIGHLIGHTS

- Low body weight selected chicks to not respond to neuropeptide Y with increased food intake.
- High body weight selected chicks do respond to neuropeptide Y with increased food intake.
- Both lines have similar hypothalamic c-Fos immuoreactivity.

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ABSTRACT

While neuropeptide Y (NPY) has been studied extensively per its pronounced role in food intake stimulation as well as its role in central pathways governing eating disorders, it has to our knowledge not been studied in polygenic models of hypo- and hyperphagia. Thus, the present study was designed to measure central NPY-associated food intake in lines of chickens that have undergone long-term genetic selection for low (LWS) or high (HWS) body weight and exhibit hypo- and hyperphagia, respectively. LWS chicks did not respond with any magnitude of altered food intake to any dose of NPY tested, while HWS chicks responded to all doses of NPY at similar magnitudes throughout the duration of observation. Both lines responded with similar increases in c-Fos immunoreactivity in the lateral hypothalamus and both divisions of the paraventricular nucleus; there were no significant line or line by treatment interactions. These data support the hypothesis that differences exist in the central NPY system of chicks from LWS and HWS lines and may provide novel insight for understanding NPY control of appetite.

1. Introduction

Demonstrated as one of the most potent endogenous orexigenic and one of the most abundant of all neurotransmitters, it is thus not surprising that neuropeptide Y (NPY) has received considerable attention in appetite research. Potent food intake increase associated with centrally administered NPY was demonstrated in the rat [1] two years after its isolation from the porcine brain [2]. The NPY gene and NPY's stimulatory effect on food intake are highly conserved across classes from fish and amphibians to higher mammals including humans (reviewed in 3). While pronounced orexigenic effects associated with NPY implies its validity as an attractive target in the development of pharmacological strategies for eating disorders, NPY influences on food intake regulation and in the development of appetite-related disorders are difficult to study by using only singular genetic modification models [3]. Thus, we examined central mechanisms of NPY-induced food intake in polygenic models of hypo-and hyperphagia. These unique avian models, having undergone over 50 years of divergent selection for body weight at 8 weeks of age, currently differ in body weight by more than ten-fold at selection age. Members of the low (LWS) body weight line are hypophagic and some exhibit anorexia [4], while the high (HWS) body weight line is comprised of compulsive eaters that all become obese [5]. That these lines have been long-term selected for body weight suggests that they may accurately represent polygenic obesity and anorexia. Because these disorders in humans are often associated with genetic polymorphisms [6], studying these populations may provide a more thorough understanding of the central mechanisms contributing to these conditions and more closely model some human counterparts than do single-gene animal models.

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2. Materials and methods

2.1. Animals

The lines of White Plymouth Rock chickens used in this study are from a long-term divergent selection experiment for low or high body weight at 8 weeks of age [7,8]. The founder population consisted of crosses of 7 inbred lines with the LWS and HWS selected lines maintained as closed populations. Review of the selection program may be found in Dunnington and Siegel [9], Siegel and Wolford [10], LeRauzic et al. [11], and Marquez et al. [8]. Eggs obtained from age contemporary parents from S_{51} generation parental stocks were incubated in the same machine. After hatch, chicks were group caged for 2 d, then individually in a room at 30 ± 2 °C and 50 ± 5% relative humidity where they had ad libitum access to a mash diet (20% crude protein, 2,685 kcal ME/kg) and tap water. The individual cages allowed visual and auditory contact with other chicks. Chicks were handled twice daily to adapt to handling. All trials were conducted between 11:00 and 16:00 h using 5 d post hatch chicks. Data in each experiment were recorded from both lines concurrently and injections were performed sequentially (LWS, HWS, LWS, HWS and so forth). Experimental procedures were performed according to the National Research Council publication, Guide for Care and Use of Laboratory Animals and were approved by the Radford University Institutional Animal Care and Use Committee.

2.2. Intracerebroventricular (i.c.v.) injection procedure

S_{52} generation free-feeding chicks from both lines were injected using a method adapted from Davis et al. [12]. The head of the chick was briefly inserted into a restraining device that left the cranium exposed and allowed for free-hand injection. Injection coordinates were 3 mm anterior to the coronal suture, 1 mm lateral from the sagittal suture, and 2 mm deep targeting the left lateral ventricle. Anatomical landmarks were determined visually and by palpation. Injection depth was controlled by placing a plastic tubing sheath over the needle. The needle remained at injection depth for 5 s post-injection to reduce backflow. Chicks were assigned to treatments at random. For preliminary trials, porcine NPY (American Peptide) was used. Afterwards and for all results described in figures and tables, chicken NPY was used. Chicken and porcine NPY peptide sequences differ by two amino acids (5%). Chicken NPY (YPSKPDSPGEDAPAEDMARYYSALRHYINLITRQRY, AnaSpec, San Jose, CA, USA) was dissolved in avian artificial cerebrospinal fluid as a vehicle for a total injection volume of 5 μL with 0.06% Evans Blue dye. After data collection, each chick was decapitated and its head sectioned coronally to determine site of injection. Any chick without dye present in the lateral ventricle system was eliminated from analysis. Sex was determined visually by dissection.

2.3. Experiment 1: food intake

Chicks were randomly assigned to receive 0 (vehicle only), 0.3, 1, or 3 nmol chicken NPY by i.c.v. injection. After injection, chicks were returned to their individual cages and given ad libitum access to both food and water, with individual food containers weighed (0.01 g) every 30 min for 180 min post injection. Data were analyzed using analysis of variance (ANOVA) at each time point. The preliminary analysis included sex, NPY dose, line, and interactions among them. Because neither sex nor interactions involving sex were significant, they were removed from the model. Because the line by NPY dose interaction was significant, we conducted a secondary one-way ANOVA within line with the main effect of NPY dose and Tukey's method of multiple comparisons was used to separate means. Because LWS and HWS chicks consume different amounts of food as a result of differences in body size, food and water intake data were adjusted for body weight at each time point by dividing the amount each chick consumed by its body weight (0.01 g) immediately prior to its i.c.v. injection and multiplying by 100.

2.4. Experiment 2: c-Fos immunohistochemistry

Chicks were randomly assigned to receive either vehicle or 3 nmol chicken NPY by i.c.v. injection (protocol detailed above). Chicks were allowed ad libitum access to food and water until injection, at which time food was withheld to prevent c-Fos immunoreactivity associated with food consumption. One hour post injection (as this is the time expected for the most robust c-Fos expression [13], 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 1 h 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 40 μm coronal sections that contained appetite-related nuclei based on anatomies described by Puelles et al. [14] 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 (DM) were collected at interaural 2.08 mm, the arcuate (ARC) at interaural 1.12 mm, and the nucleus of the solitary tract (NTS) at interaural −7.04 mm. Sections were processed immediately after collection. Procedures for c-Fos immunohistochemistry were based on those of Zhao and Li [15]. Free-floating sections were pre-blocked for 1 h with 10% normal goat serum (NGS) and 0.3% Triton X-100 in 0.02 M PBS. To inhibit endogenous peroxidase activity, sections were incubated in 1.5% hydrogen peroxide and 50% methanol in deionized water for 30 min. Following a 3×10 min wash in wash buffer (0.05% NGS and 0.3% Triton X-100 in 0.02 M PBS), sections were incubated with rabbit polyclonal anti-c-Fos at a dilution of 1:20,000 (K-25, Santa Cruz, Santa Cruz, CA, USA) in PBS containing 0.3% Triton X-100, 1% NGS, and 1% blocking reagent (11096176001, Roche Diagnostics, MA, DE) for 48 h under slow oscillation at 4 °C. For assay controls, the primary antibody was substituted with normal rabbit serum. Sections were then rinsed 3×10 min in wash buffer and incubated with biotinylated goat anti-rabbit secondary antibody at a dilution of 1:200 (Vector Laboratories, CA, USA) in PBS containing 1% NGS for 2 h at room temperature. Following a rinse with PBS, sections were processed with avidin–biotin–horseradish peroxidase complex at a dilution of 1:200 (Vectastain Elite ABC Kit, Vector Laboratories). Reactions were visualized with the DAB Substrate Kit for Peroxidase (Vector Laboratories) for 45 s, mounted on gelatin-coated slides and cover-slipped with VectaMount (Vector Laboratories). 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 by ANOVA using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC).

3. Results

3.1. Experiment 1: food intake

We found that i.c.v. administration of chicken NPY did not affect food intake at any time in LWS chicks (up to 3 h post-injection, Fig. 1). However, in HWS chicks, all three NPY doses tested were associated with a similar magnitude of increased food intake (approximately 200%) at all observation times.

3.2. Experiment 2: c-Fos immunohistochemistry

NPY was associated with increased c-Fos immunoreactivity in both LWS and HWS chicks, but in no case was there a significant line or line by treatment interaction. Ergo, comparisons are presented for treatments (Table 1). NPY-treated chicks had increased c-Fos immunoreactive cells in both the magnocellular (PaMC) and

Table 1
Effect of i.c.v. NPY on mean number of c-Fos immunoreactive cells in the magnocellular division of the paraventricular nucleus (PaMC), parvicellular division of the paraventricular nucleus (PaPC) dorsomedial nucleus (DM), ventromedial nucleus (VMH), lateral hypothalamus (LH), arcuate (ARC), and nucleus of the solitary tract (NTS) in chicks from lines LWS and HWS. There were no line or line by treatment interactions, ergo means are expressed across treatment. Number of chicks = 9–11 and 10–14 per LWS and HWS/NPY dose, respectively.

Nucleus	Treatment	Number of immunoreactive cells
PAMC	Vehicle	20.37
		$p = 0.0005$
	NPY	55.44
PAPC	Vehicle	32.54
		$p = 0.0023$
	NPY	56.43
DM	Vehicle	38.88
		$p = 0.0841$
	NPY	51.76
VMH	Vehicle	28.65
		$p = 0.9960$
	NPY	28.63
LH	Vehicle	90.61
		$p = 0.0002$
	NPY	207.69
ARC	Vehicle	61.08
		$p = 0.0507$
	NPY	87.54
NTS	Vehicle	40.72
		$p = 0.0587$
	NPY	55.93