LPLRFamide exerts short-term anorexigenic effects that coincide with magnocellular division of the hypothalamic paraventricular nucleus activation

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Abstract:

LPLRFamide is a member of the RFamide peptide family that elicits an anorexigenic effect when centrally injected in chicks although the mechanism mediating this response is poorly understood. Therefore, the purpose of this experiment was to elucidate the hypothalamic mechanism of short-term anorexia after central administration of LPLRFamide in chicks. In Experiment 1 chicks centrally injected with LPLRFamide decreased food intake at 15 min but not 30 min following administration compared to vehicle-injected chicks. For Experiment 2, c-Fos immunoreactivity was quantified in several appetite-associated hypothalamic nuclei and in LPLRF-injected chicks, compared to vehicle-injected chicks, there was an increase in the number of reactive cells in the magnocellular division of the paraventricular nucleus. Lastly in Experiment 3, real time-PCR was performed and hypothalamic proopiomelanocortin (POMC) mRNA abundance was increased in LPLRFamide-injected chicks compared to vehicle-injected chicks. Thus, following central injection of LPLRFamide there is activation of the

paraventricular nucleus of the hypothalamus and increased expression of hypothalamic POMC mRNA in chicks.

Key Words: LPLRFamide, chick, hypothalamus, anorexigenic

1.0 Introduction

The RFamide family of neuropeptides all contain terminal arginine and phenylalanine amino acids on their amino terminus (Dockray, 2004). The first member of this family was identified in mollusks and there have since been many other members identified in a wide variety of species from invertebrates to vertebrates. Thus, neuropeptides of this family apparently arose early in divergent evolution and have been conserved, suggesting fundamental physiological significance. RFamide peptides play roles in appetite-related behaviors, along with other diverse biological functions; their function appears to be dependent on the diversity of the individual N-terminal sequence (Bechtold and Luckman, 2007). LPLRFamide was first isolated from the chicken brain (Ganeshan et al., 1987), and exerts an anorexigenic effect in both chicks and quail (Cline et al., 2010). However, the mechanisms governing the anorexigenic effect of LPLRFamide are poorly understood.

The purpose of this study was to elucidate the central mechanisms by which LPLRFamide exerts its anorexigenic effect. Chicks were administered an intracerebroventricular injection, and food intake was recorded. Following this the number of c-Fos immunoreactive cells was counted in key appetite-associated hypothalamic nuclei followed by quantification of mRNA of some major appetite-associated factors in the hypothalamus.

2. Materials and Methods

2.1 Animals

Hubbard x Cobb-500 chicks were group caged for 1 d, then individually in a room at 30 ± 2 °C and $50 \pm 5\%$ relative humidity where they had ad libitum access to a mash diet 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 08:00 and 13:00 h using 4 d post hatch chicks. 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 (ICV) injection procedure

Chicks were injected using a method adapted (Davis et al., 1979) that does not appear to induce physiological stress (Furuse et al., 1999). The head of the chick was briefly inserted into a restraining device that 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. Chicks were assigned to treatments at random. LPLRFamide (American Peptide, Sunnyvale, CA, USA) was dissolved in artificial cerebrospinal fluid (Anderson and Heisley, 1972) as a vehicle for a total injection volume of 5 μ l with 0.1% Evans Blue dye to facilitate injection site localization and the needle remained at injection depth in the unanesthetized chick for 10 s post injection to reduce backflow. After data collection, the chick was decapitated and its head sectioned along the frontal plane to determine the site of injection. Any chick without dye present in the lateral ventricle system was eliminated from analysis. Sex of chicks was determined visually by dissection.

2.3 Food and water intake

Chicks, fasted for 180 min, were randomly assigned to receive either 0 (vehicle only), 3.0, 7.0 or 15 nmol LPLRFamide by ICV injection. After injection, chicks were returned to their individual home cages and given ad libitum access to both food and water. Food intake was measured by weighing food containers (with accuracy of 0.01 g) every 15 min for 90 min post injection. Water weight (g) was converted to volume (ml; 1 g = 1 ml). Data were analyzed using analysis of variance (ANOVA) within each time point. The initial ANOVA included the main effects of sex and LPLRFamide dose and their interaction. Neither sex nor the interaction was significant, thus a second ANOVA was conducted within each time point with the statistical model including only 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.

2.4 c-Fos immunohistochemistry

Chicks were randomly assigned to receive either vehicle or 15 nmol LPLRFamide by ICV injection. Chicks were allowed ad libitum access to food and water until injection, after which food was withheld to prevent c-Fos immunoreactivity associated with food consumption. Sixty min post injection, as this is the time expected for the most robust c-Fos expression (Muller 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. 60 μ m 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); two parts of the paraventricular nucleus, the magnocellular (PaMC) and parvicellular (PaPC) divisions; lateral hypothalamic area (LHA) and dorsomedial nucleus (DMN) were collected at interaural 2.08 mm, and the arcuate (ARC) at interaural 1.60 mm. Sections, one per chick per respective interaural position, were processed immediately after collection. Procedures for the c-Fos immunohistochemistry assay 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 captured for each section. Overlays containing the respective nuclei boundaries were digitally merged with micrographs and the number of c-Fos immunoreactive cells (those with DAB staining) within each respective nucleus counted by a technician blind to treatment. Data were analyzed by ANOVA including the main effect of LPLRFamide dose.

2.5 mRNA abundance of appetite-associated factors in whole hypothalamus

Chicks were randomly assigned to receive either vehicle or 15 nmol LPLRFamide by ICV injection. Chicks were allowed ad libitum access to food and water until injection, after which food was withheld. Sixty minutes following injection, chicks were deeply anesthetized with sodium pentobarbital via cardiopuncture, 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 sec. 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-RNA Lysis reagent (5-Prime, USA) for 2×2 min at 20 Hz with a Tissue Lyser II (Qiagen). After centrifugation for 10 min for $12,000 \times g$ 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 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 v3.0 (Applied Biosystems). Sequences and Accession IDs are provided in Table 1. Amplification efficiency was validated for all primer pairs before use (95–100% efficiency). Real time PCR reactions were performed in duplicate in 10 μL reactions with Fast SYBR Green Master Mix (Applied Biosystems, USA), 0.5 μL each of forward and reverse primers (0.125 μM each) and 3 μL of 10-fold diluted cDNA using a 7500 Fast instrument (Applied Biosystems). PCR was performed under the following conditions: 95°C for 20 sec and 40 cycles of 90°C for 3 sec plus 60°C for 30 sec. A dissociation step consisting of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec was performed at the end of each PCR reaction to ensure amplicon specificity. Data were analyzed using the $\Delta\Delta\text{CT}$ method, where $\Delta\text{CT} = \text{CT target gene} - \text{CT actin}$, and $\Delta\Delta\text{CT} = \Delta\text{CT target sample} - \Delta\text{CT calibrator}$ (Schmittgen and Livak, 2008). Actin

served as the endogenous control and the average of the vehicle-treated chicks served as the calibrator sample. Relative quantities, calculated as $2^{-\Delta\Delta CT}$, were used for ANOVA with the model including the main effect of treatment.

3.0 Results

3.1 Food intake

In Experiment 1, chicks that received a central injection of LPLRFamide decreased food intake (Figure 1). Treatment effects occurred rapidly, 15 min following injection for 7.0 and 15.0 nmol, but the effect diminished by 30 min post-injection. Compensatory feed intake did not occur during the observation period despite that the LPLRFamide effect was not significant by 30 min and thereafter.

3.2 c-Fos immunohistochemistry

In the second experiment, chicks injected with LPRLFamide had changes in hypothalamic c-Fos immunoreactivity (Figure 2). LPLRFamide injection was associated with an increased number of c-Fos reactive cells in the paraventricular nucleus, in particular the PAMC but not the PAPC. The PAMC of LPLRFamide-injected chicks was nearly 2.5 times more reactive than controls. The other nuclei, including the ARC, DMN, VMH and LHA, were not affected by treatment.

3.3 mRNA abundance of appetite-associated factors in whole hypothalamus

In Experiment 3, hypothalamic appetite-associated factor mRNA was affected by LPLRFamide injection (Figure 3). The expression of POMC was greater in whole hypothalamus

of LPRLFamide than vehicle-injected chicks. AgRP, NPY, OT and CRF mRNAs were not affected by LPLRF injection at 1 hour post-injection.

4.0 Discussion

The result of Experiment 1 confirms that LPLRFamide exerts a very rapid and transient reduction in food intake when centrally administered. Experiment 1 was conducted to confirm this effect because food intake responses can sometimes be altered by environment (Woods and Langhans, 2012). Although the same magnitude of food intake reduction was observed in the present study as in our former (Cline et al., 2010), the duration was shorter in the present study. This may be attributed to the stock of chick used. In our former report we utilized Cobb-500 chicks and in the present a Hubbard by Cobb-500 cross. The Hubbard sire may contribute to differences in growth and metabolism as genetic selection is known to alter these traits in chickens (Buzala et al., 2015). Based on the result of Experiment 1, the dose of 15.0 nmol was chosen for Experiments 2 and 3.

Because the hypothalamus is thought to be a major effector of appetite perception, Experiment 2 was designed to determine if an appetite-associated hypothalamic nucleus mediated the effect of LPLRFamide. Only the paraventricular nucleus, in particular the PAMC, had increased c-Fos immunoreactivity after central LPLRFamide injection. This nucleus receives input from other brain regions for the control of appetite and expresses a variety of both anorexigenic and orexigenic factors (Kalra et al., 1999). Several bodies of evidence (Dube et al., 1992; Kalra et al., 1999) implicate the paraventricular nucleus as a critical site for action of neuropeptide Y (NPY) and show that NPY is one of the most orexigenic factors studied in birds (Kuenzel et al., 1987), thus it may be speculated that LPLRF amide exerts its anorexigenic effect via antagonism of NPY at the PVN.

However, the results from Experiment 3 do not provide evidence that NPY originating from PVN soma is affected by LPLRFamide injection. Although it should be granted that downregulation at the peptide level may not necessarily coincide with an effect at the mRNA level. Of the genes evaluated, only POMC mRNA was affected by LPLRFamide injection. POMC, as a precursor protein, can be cleaved into several active molecules many of which are associated with appetite regulation including melanocyte-stimulating hormones (MSHs), corticotrophin (ACTH) and β -endorphin (Millington, 2007) and these can exert both orexigenic (McCormack and Denbow, 1988) and anorexigenic effects (Kawakami et al., 2000; Smith et al., 2009). The most potent and best studied of these factors are the melanocortins, in particular α -MSH. Thus, the increase in POMC may imply that short-term satiety by LPLRFamide is mediated through α -MSH release. However, the greatest localization of POMC with respect to appetite regulation is within the ARC (Millington, 2007), a nucleus that did not have increased c-Fos immunoreactivity. Therefore, the increased POMC mRNA that we detected in whole hypothalamus samples may not be of ARC origin. This may imply its presence in the PVN or that its origin is some other hypothalamic nucleus not related to appetite. The findings are quite contradictory when placed solely in the context of true appetite regulation. However, it should be noted that only a single time following injection was measured in the present study, and changes in c-Fos or at the transcription level may be occurring prior to or later than the sample collection time. As we previously demonstrated (Cline et al., 2010), LPLRFamide injection affected behaviors unrelated to injection including increased time spent in deep rest, thus the hypothalamic changes we observed in the present study do not necessarily imply that they are appetitive in nature (they may be part of a narcoleptic response). This may be interpreted as the

short-term reduction in food intake is not true satiety, but rather a secondary response from a competitive behavior (one that is incompatible with food intake).

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Figure 1. Cumulative feed intake following central injection of LPLRFamide (Experiment 1). Values are expressed as means $\pm$ standard errors; bars with different superscripts are different from each other within a time point ($P < 0.05$), $n = 8$ to 9 per group.

Figure 2. Effect of central injection of LPRLFamide on the number of c-Fos immunoreactive cells in the nucleus of the magnocellular (PAMC) and parvocellular (PAPC) part of the paraventricular nucleus, arcuate nucleus (ARC), dorsomedial hypothalamus (DMN) ventromedial hypothalamus (VMH) and lateral hypothalamus (LHA) (Experiment 2). * denotes different from vehicle ($P < 0.05$). Values are expressed as means $\pm$ standard errors, $n = 8$ per group.

Figure 3. Hypothalamic expression of agouti-related peptide (AGRP), proopiomelanocortin (POMC), neuropeptide Y (NPY), oxytocin (OT) and corticotrophin releasing factor (CRF) mRNA in whole hypothalamus samples from LPLRFamide-injected chicks (Experiment 3).

Values are means $\pm$ standard error; * denotes different from vehicle, $P < 0.05$. $n = 10$ vehicle and 9 LPLRFamide injected chicks.

Table 1. Primers used for real time PCR

Gene ^a	Accession ID.	Sequence 5' to 3' (forward/reverse)
β-Actin	NM_205518.1	GTCCACCGCAAATGCTTCTAA/ TGCGCATTTATGGGTTTTGTT
AgRP	XM_004950992.1	GGTTCTTCAACGCCTTCTGCTA/ TTCTTGCCACATGGGAAGGT
CRF	NM_001123031.1	TCAGCACCAGAGCCATCACA/ GCTCTATAAAAATAAAGAGGTGACATCAGA
NPY	NM_205473.1	CATGCAGGGCACCATGAG/ CAGCGACAAGGCGAAAGTC
OT	XM_001231491.3	TGGCTCTCTCCTCAGCTTGTTAT/GGCACGGCACGCTT AC
POMC	NM_001031098.1	GCCAGACCCCGCTGATG / CTTGTAGGCGCTTTTGACGAT

^aAgRP: agouti-related peptide; CRF: corticotropin-releasing factor, NPY: neuropeptide Y; OT: oxytocin; POMC: pro-opiomelanocortin

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