Evidence of a role for neuropeptide Y and monoamines in mediating the appetite-suppressive effect of GH

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Abstract

Among the many responses to GH administration is suppression of voluntary feed intake (FI) in some species, attributed to improvement in the efficiency of nutrient utilization and, therefore, reduced need for ingested substrates. Commercial broiler chickens have been genetically selected for generations for rapid growth, realized largely via the major correlated response of increased voluntary feed consumption. Neuropeptide Y (NPY) and monoamines play very important roles in the central regulation of feeding. Preliminary studies from our laboratory suggest that the appetite-suppressive effect of GH may be independent of its actions as a repartitioning agent, and may involve alterations in NPY expression at the pre-translational level. The purpose of this investigation was to explore the dose–response nature of the appetite-suppressive effect of GH in juvenile broilers, and the possible involvement of NPY and monoamines in this process.

A GH dose–response study was conducted using 8-week-old female broilers infused i.v. with GH in a pulsatile pattern for 7 days at 0, 10, 50, 100 or 200 µg/kg body weight per day. Hypothalamic NPY and epinephrine (EP) concentrations decreased in a dose-related manner with GH. At the highest dosage, voluntary FI decreased 19% ($P<0·05$) and hypothalamic NPY mRNA decreased approximately 50% in the infundibular nuclei and midline region ($P<0·0001$). In contrast, birds pair-fed to the high-GH dosage group did not differ from controls, verifying that changes in NPY and monoamines were not secondary to reduced FI. We conclude that hypothalamic NPY and EP are likely candidates to explore further as mediators of the appetite-suppressive effect of GH.

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Introduction

Growth hormone (GH) is a major anabolic agent with effects on protein, fat and carbohydrate metabolism. Numerous investigators have demonstrated that GH alters voluntary feed intake (FI), but there are marked species differences. A consistent effect on reducing FI is observed in the pig (Klindt *et al.* 1998), and GH attenuates the elevated food consumption exhibited during binge-eating in anorexic/bulemic patients (Vaccarino *et al.* 1994). Alternatively, GH treatment increases FI in rats (Azain *et al.* 1995). The mechanisms underlying the effects of GH on FI have not been explored.

The domestic chicken raised for meat purposes (broiler) has been intensely selected over generations for increased body weight at early ages, derived primarily from the major correlated response of increased voluntary feed consumption (Pym & Nicholls 1979). Contemporary generations of this model display extreme hyperphagia, which results in obesity and attendant health problems similar to the human population (e.g. skeletal, reproduc-tive, cardiovascular), if feed is unrestricted beyond the juvenile period of development. In fact, the ability to regulate energy intake is absent in the broiler (Siegel & Wisman 1966).

GH is secreted in a well-characterized pulsatile pattern in the broiler, with high amplitude peaks occurring at 90-min intervals during early post-hatch development, when growth velocity is maximal (Vasilatos-Younken & Zarkower 1987). In contrast, with increasing age, baseline concentrations decrease and the amplitude of secretory peaks diminishes markedly, so that plasma GH is barely detectable by the late post-hatch period, when birds are still juvenile in development. Research from our laboratory (Vasilatos-Younken 1995) indicates that GH enhancement during the late post-hatch period, either by direct administration of GH or stimulation of endogenous GH secretion via GH-releasing factor administration, depresses voluntary FI, similar to the human. In addition, evidence suggests that birds and mammals have similar feeding control mechanisms (Denbow 1985).

Appetite regulation is very complex, and multiple brain structures and neurotransmitters are known to be involved. The hypothalamus, believed to have an important role in FI regulation, is rich with neurotransmitters and neuropeptides. Among these, neuropeptide Y (NPY) is a potent stimulator of feeding, and is highly conserved among species (Blomqvist *et al*. 1992). The 36 amino acid peptide differs by only a single amino acid (Ser7) in the chicken versus the rat and human, the latter of which are identical. Similar to its action in mammals (Leibowitz 1989), NPY has a robust stimulatory effect on FI in the chicken when administered intracerebroventricularly (Kuenzel *et al*. 1987*a*). Most of the metabolic effects of NPY appear to be centrally mediated in the hypothalamus (Sahu *et al*. 1988, Leibowitz 1989), with the paraventricular nucleus (PVN) principally involved. The PVN receives rich afferents of NPY immunoreactive nerve endings from epinephrine (EP)-containing neurons in the medulla (C1 or C2), norepinephrine (NE)-containing neurons in the medulla (A1) and dorsal pons (A6), and non-catecholaminergic neurons in the infundibular nuclei (IN) (McDonald 1988). The extensive coexistence of NPY and monoamines in the central nervous system (Sawchenko *et al*. 1985) suggests that NPY and monoamines may interact in the hypothalamus to regulate FI.

Monoamines, namely NE, EP, dopamine (DA), and serotonin (5-HT), are believed to have potent and varied effects on eating behavior in the rat, as well as in a variety of other species (Leibowitz 1980, 1989). Hypothalamic stimulation with NE or EP has long been known to potentiate feeding in mammals (Leibowitz 1989) and in the chicken (Denbow *et al*. 1981). This stimulatory effect is anatomically localized, predominantly to the PVN, and it is mediated via $\alpha2$- rather than $\alpha1$-type receptors located on post-synaptic membranes (Leibowitz & Brown 1980). In contrast, intraventricular injection of 5-HT suppresses feeding behavior (Denbow *et al*. 1981, Leibowitz 1985). Bilateral hypothalamic injection of DA induces depression of FI expressed as reduced meal size in the rat (Yang *et al*. 1997), while intracerebral injection of DA has no effect on FI in the chick (Denbow *et al*. 1981).

Recently, GH has been identified as a possible regulator of NPY gene expression. Treatment of rats with GH induces c-fos gene expression in the NPY-containing neurons in the arcuate nucleus (Kamegai *et al*. 1994). Hypophysectomy significantly decreases NPY mRNA levels, and GH treatment restores these levels to those of intact rats (Chan *et al*. 1996). Hypothalamic monoamines are reported to be affected by GH, and may participate in GH autoregulation (Lea & Harvey 1993). The present experiment was designed to investigate the dose–response nature of GH on voluntary FI, and the possible involvement of NPY and monoamines in this process.

Materials and Methods

Animal experiments

All animal procedures were approved by Pennsylvania State University Institutional Animal Care and Use Committee under protocol no. 89R1389 G197.

Over the developmental period prior to sexual maturity, plasma GH concentrations in broilers similar to those used in the present study can range from <10·0 ng/ml (baseline troughs) to over 100 ng/ml (secretory peaks) (Vasilatos-Younken & Zarkower 1987, Johnson *et al*. 1987, R Vasilatos-Younken, unpublished data). A series of dosages (0, 10, 50, 100 and 200 μg/kg body weight (BW) per day) was chosen that was calculated to result in circulating concentrations over this range, and to include the approximate dosage of chicken GH (cGH) that was estimated in a previous study to be maximally effective in terms of metabolic responses (123 μg/kg BW per day; Vasilatos-Younken *et al*. 1988). Birds were selected to be 8 weeks of age at the initiation of infusions, because at this time endogenous GH secretion is low and circulating concentrations are barely detectable (<1·0 ng/ml). This was to avoid superimposing exogenous hormone pulses on pre-existing (endogenous), high-amplitude secretory peaks, as would occur at very young ages, and that would result in a refractory state.

Seven-week-old female broilers were surgically prepared with jugular vein catheters as previously described (Cravener & Vasilatos-Younken 1989). Birds were allowed 3–6 days of postoperative recovery, and normal, pre-surgical behavior, FI and BW gain were confirmed prior to initiation of infusions. When birds were 8 weeks of age (late post-hatch), recombinant cGH (Lucky Biotech, Seoul, Republic of Korea) was infused in a pulsatile pattern for 7 days at one of five dosages (0, 10, 50, 100 or 200 μg/kg BW per day) (ten birds per dosage), and FI was monitored daily. The infusion protocol has been described previously (Vasilatos-Younken *et al*. 1988), and is based on a pulse interval (90 min) and duration (60 min) that replicate the endogenous pattern of GH secretion in young, rapidly growing broilers. Infusions were conducted in two experimental replicates over time, approximately 3 weeks apart, with every treatment included in each experiment.

To control for possible contributions of reduced FI on measured responses, additional, pair-fed groups for GH treatments were established. Pair-feeding of vehicle-infused birds was accomplished by assigning a level of feed restriction to match the amount of feed consumed by the GH treatment groups which exhibited significantly reduced FI in comparison to controls.

FI was measured daily throughout the study. Blood samples were collected on the final day of infusion, immediately following a GH pulse, to determine GH peak amplitudes achieved by infusion. Heparinized blood samples were placed on ice and processed immediately.

Plasma was obtained by centrifugation, aliquoted to multiple vials, and stored at $-70\ °C$ until assayed. Plasma GH was determined by a homologous cGH radioimmunoassay (RIA) as previously described (Vasilatos-Younken 1986). Animals were killed by rapid decapitation. Brains were removed and half of each brain was frozen in isopentene cooled by dry ice to $-30\ °C$ and stored at $-80\ °C$ until NPY hybridization histochemistry was conducted. The other halves were dissected, using the atlas of Kuenzel & Masson (1988). The hypothalamus and the medulla were removed and immediately weighed, frozen in liquid nitrogen, and stored at $-80\ °C$ for NPY RIA assay and monoamine detection.

NPY RIA

Synthetic NPY standard (rat/human) from Peninsula Laboratories (Belmont, CA, USA) was used for standard preparations and for the preparation of labeled NPY. Radioactive ^{125}I was purchased from Amersham International (Arlington Heights, IL, USA). The primary (polyclonal) antibody (Ab$_1$) was rabbit anti-rat NPY(3–8) serum, and was generously donated by Dr Marvin R Brown (Alanex Corporation, San Diego, CA, USA). Anti-rat NPY(3–5) was previously characterized (Allen *et al.* 1991) and did not cross-react with any related peptides, including peptide YY and pancreatic peptide.

NPY was labeled with ^{125}I using the chloramine-T method (Hunter & Greenwood 1962). The iodination procedure was carried out on ice with chilled reagents. Briefly, 5 μg NPY dissolved in 6 μl double-distilled H_2O (ddH_2O) was labeled with 1 mCi Na-^{125}I and 7 μg chloramine-T dissolved in sodium phosphate buffer (50 mM NaPO$_4$, 0·9% NaCl, 25 mM EDTA, 0·1 g/l thimerosal and 0·25% bovine serum albumin (BSA), pH 7·4). The reaction time was 18 s. The reaction was ended by adding 5 μl sodium metabisulfite solution (10 mg/ml in sodium phosphate buffer). Separation of the reaction mixture was achieved by gel filtration on a G-25 PD-10 column with a bed height of 5 cm (Pharmacia, Uppsala, Sweden), and eluted with sodium phosphate buffer. Forty fractions of 2 ml were collected. The peak fraction was diluted and used for immunoassay detection.

Hypothalamic samples were homogenized in 300 μl 0·1 M sodium acetate buffer containing 3–4-dihydroxybenzylamine HBr (DHBA; 48 ng/tube) and ascorbic acid oxidase (325 ng/tube). The samples were then centrifuged at 10 000 g for 10 min at 4 °C and aliquots of the supernatant were removed for monoamine determination and NPY assay. For NPY assay, 120 μl hypothalamic extract were re-extracted with C$_{18}$ Sep-Pak columns (Peninsula Laboratories). The samples were concentrated by lyophilization and brought to 120 μl with assay buffer. Plasma samples (1 ml) were also extracted as for hypothalamic samples. The final volume for plasma samples was 300 μl.

The NPY assay was carried out at pH 7·4 using phosphate buffer (50 mM NaPO$_4$, 0·9% NaCl, 25 mM EDTA, 0·1 g/l thimerosal, 0·25% BSA, pH 7·4). Final dilution of Ab$_1$ (1:45 000 in phosphate buffer) was chosen to achieve a maximal binding of 30–40% [^{125}I]NPY. Standards consisted of synthetic human NPY prepared in phosphate buffer. The initial incubation mixture, containing 10 μl of a 1:10 dilution of hypothalamic extract (50 μl for plasma samples), 490 μl assay buffer (450 μl for plasma) and 100 μl diluted antibody, was incubated for 48 h at 4 °C. On the third day, [^{125}I]NPY (approximately 5000 c.p.m., in 100 μl phosphate buffer per tube) was added, and samples were incubated for 24 h at 4 °C. On the fourth day, 50 μl of a 1:20 dilution of normal rabbit serum and 100 μl of a 1:10 dilution of goat anti-rabbit IgG were added and the mixture incubated for 24 h. The assay was terminated by addition of 2 ml ice-cold sodium phosphate buffer (0·01 M NaPO$_4$ and 0·14 M NaCl). Tubes were centrifuged at 2000 g for 30 min, supernatants decanted, and radioactivity in the pellet measured in a gamma counter (Pharmacia LKB Nuclear Inc, Gaithersburg, MA, USA). The sensitivity of the assay was 250 pg/ml. Inter- and intra-assay coefficients of variation were 20% and 14% respectively, and the concentration at 50% of maximal binding was 1·7 ng/ml.

In situ *hybridization for NPY*

Tissue preparation Using the chicken atlas of Kuenzel & Masson (1988) as an anatomical guide, coronal brain sections (10 μm thick) were obtained with a cryostat (Leica CM1900; Leica Instruments GmbH, Mussloch, Germany). Every fifth section was thaw-mounted onto poly-L-lysine subbed Superfrost/Plus Microscope Slides (Fisher Scientific, Pittsburgh, PA, USA). Slides were stored in air-tight boxes at $-80\ °C$ until hybridization histochemistry was conducted.

Probe preparation A 511 bp pre-proNPY cDNA fragment, containing the full-length rat NPY sequence, was inserted into the EcoRI site of Bluescribe M13(−) vector (Stratagene, La Jolla, CA, USA). The probe was kindly provided by Dr Steven Sabol (Department of Health and Human Services, National Institute of Health, Bethesda, MD, USA) (Higuchi *et al*. 1988). *E. coli* XL-1 blue competent cells (Stratagene) were transformed by electroporation with *E. coli* pulser (Bio-Rad, Hercules, CA, USA). Pulse time was approximately 5 ms, and volts were 1·8 kV. The plasmid, which contained two FspI sites, was linearized overnight at 37 °C with FspI (1·25 unit/μg DNA) (New England Biolabs, Beverly, MA, USA) for antisense RNA, and overnight at 37 °C with HindIII (2·5 unit/μg DNA) (Promega Biotech, Madison, WI, USA) for sense-strand RNA. The linearized plasmid DNA was transcribed with [^{35}S]thio-UTP (New England Nuclear Research Products, Boston, MA, USA) at a

concentration of 50 µM, using T7 and T3 RNA polymerase (Promega Biotec) for sense and antisense probes respectively. Residual DNA was removed by digestion with DNAase (Promega Biotec), and the cRNA probes were purified on a Nensorb column (Stratagene).

The specificity of the rat NPY probe for detection of chicken NPY message in brain tissues has been previously verified by Northern analysis, which demonstrated that a ^{32}P-labeled riboprobe hybridized to complimentary, single-stranded chicken NPY mRNA identically as for rat, and showed the presence of a single 0·8 kb transcript on autoradiography (X Wang, J R Day & R Vasilatos-Younken, unpublished observations).

Single–label *in situ* hybridization histochemistry for NPY mRNA Using the ^{35}S-labeled riboprobe complementary to NPY mRNA, *in situ* hybridization was performed as previously described (Day *et al.* 1990). In brief, after fixation in 4% paraformaldehyde (Sigma, St Louis, MO, USA) in phosphate-buffered saline (137 mM NaCl, 2·7 mM KCl, 4·3 mM $Na_2HPO_4.7H_2O$ and 1·4 mM KH_2PO_4, pH 7·5), tissue sections were rinsed in phosphate buffer and pretreated with 0·25% acetic anhydride (Fisher Scientific) in 0·1 M triethanolamine (pH 8) for 10 min. The slides were dehydrated through a series of ethanol baths (50–100%) and air-dried. The ^{35}S-labeled probe (5 000 000 c.p.m./section) was applied in a hybridization buffer containing 50% formamide (Fisher Scientific), 10% dextran sulfate (Sigma), 5 × Denhardt's (0·5 g Ficoll, 0·5 g polyvinylpyrrolidone, 0·5 g BSA and H_2O to 500 ml), 4 × SSC (0·6 M NaCl and 0·06 M Na_3 citrate.2H_2O, pH 7·0), 25 µg/ml poly A and poly C sulfate (Sigma), 100 mM dithiothreitol (Fisher Scientific) and 0·25 mg/ml yeast transfer RNA (Sigma). Coverslips were applied and the sections were incubated for 3 h in a humidified chamber at 50 °C. After hybridization, the slides were treated with 20 µg RNase-A (Sigma) at 37 °C for 30 min and washed at high stringency in 50% formamide, 0·5 M NaCl, 50 mM sodium phosphate (equal volumes of 1 M NaH_2PO_4 and 1 M Na_2HPO_4) and 1% β–mercaptoethanol (Sigma) at 55 °C for 30 min. Sections were then dehydrated for 3 min each in a series of ethanol baths (50–100%) and air-dried. The slides were dipped in undiluted Kodak NTB-2 emulsion (VWR, Buffalo Grove, IL, USA) that had been heated to 45 °C in a water bath for 45 min, and then air-dried. Sections were exposed for 20 days at 4 °C. Slides were then developed with Kodak D-19, counterstained with cresyl violet (Sigma), dehydrated through a 50–100% ethanol series, and coverslips were applied.

Sections containing the desired region were blindly analyzed by a computer-assisted automated image processing system (Image-1; University Imaging, West Chester, PA, USA) at 1250 × in order to estimate grain density. Grain density was expressed as a percentage of the cell area occupied by grains. Background counts were subtracted from each labeled cell count. A cell was considered labeled only if the density was at least five times greater than the background, and was overlying a counterstained nucleus. Background measurements were taken from emulsion-coated regions of the slides where no tissue was present.

HPLC determination of monoamines

The concentrations of amines (NE, EP, DA, 5-HT) and amine metabolites (dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA)) in supernatants (collected after a 10-min centrifugation at 10 000 *g*, 4 °C) were determined by reverse-phase HPLC (BAS200A; Bioanalytical System, Lafeyette, IN, USA) and electrochemical detection (LC4B detector; Bioanalytical Systems). The samples (spiked with DHBA as an internal standard) were separated on a reverse-phase, 100 × 3·2 mm column of Phase II ODS 3 µm silica particles (MF6213; Bioanalytical Systems), which was eluted with 2·5% (v/v) acetonitrile in ddH_2O in the presence of 0·127 M monochloroacetic acid, 0·03% (w/v) octyl sodium sulfate and 0·65 mM EDTA-Na_2. The flow rate was 0·8 ml/min or 1 ml/min and the amines and metabolites of interest were quantified by peak height. Peak identifications were accomplished by the evaluation of retention times using external standards. A peak in an unknown sample which fell within the range of 95–105% of the external standard retention time for a given monoamine was accepted.

Statistical analysis

Plasma GH data displayed heterogeneity of variance and were transformed to natural log scale prior to statistical evaluation. Dose–response data were analyzed using regression analysis, and signficant effects (linear, quadratic and/or cubic) were retained in the final model for each response. Pair-feeding data were analyzed by analysis of variance according to the model for a completely randomized design. For assessment of treatment effects on FI, each bird served as its own control by calculating the percentage change in average daily FI (g/kg BW per day) for the final 3 days of infusion, relative to the same bird's preinfusion FI (average of the 3 days immediately preceding initiation of treatments). Because FI normally decreases as broilers mature (National Research Council (US) 1994), and this was observed in all birds across treatment groups prior to infusion, FI change was corrected for the average normal decline observed in the controls.

For informational purposes, arithmetic least squares means (LSM) are presented as ± s.e.m. of the LSM in Tables and Figures for all data.

Results

All birds were carefully monitored during the experiments to assess behavior, health status and, upon termination, appearance of internal organs. Birds exhibited normal

Table 1 Plasma GH and NPY concentrations, and hypothalamic NPY concentrations following 7 days of pair-feeding (pairfed-100, pairfed-200). Values are means ± S.E.M. (*n*)

	Control	Pairfed-100[1]	Pairfed-200[2]
Plasma GH (ng/ml)	0·4 ± 0·04 (9)	0·4 ± 0·04 (9)	0·5 ± 0·04 (8)
Plasma NPY (ng/ml)	1·0 ± 0·05 (8)	0·9 ± 0·08 (9)	1·1 ± 0·10 (8)
Hypothalamic NPY (ng/g)	66·3 ± 6·00 (9)	61·8 ± 3·55 (8)	65·5 ± 8·50 (8)

Note: pair-fed birds were not infused with GH. For results of GH infusions, see Results and Fig. 2.
[1]Pair-fed to the 100 µg GH/kg BW per day dosage group; [2]pair-fed to the 200 µg GH/kg BW per day dosage group.
There were no significant differences among groups.

preening behavior and vocalization, water consumption, activity and sleep patterns. Droppings (feces and urine) were normal in consistency, appearance (color) and relative quantity. Internal organs (heart, lungs, liver, kidney and spleen) were normal in appearance/color, texture and lack of abnormal masses.

Effects of pulsatile cGH infusion on circulating GH and FI

Plasma concentrations of GH were elevated in a dose-dependent fashion with GH infusion, and ranged from 0·3 ± 5·7 ng/ml for controls up to 89·0 ± 5·4 ng/ml at the highest dosage (200 µg/kg BW per day) (for detailed profile see Vasilatos-Younken *et al.* 2000). Plasma GH concentrations for pair-fed groups did not differ from controls (Table 1).

Pulsatile infusion of cGH for 7 days had no discernable effect on voluntary FI at the lower dosages administered, but began to depress voluntary FI (7·3 ± 6·3%) at 100 µg/kg BW per day (Fig. 1). This depression was maximal

(19 ± 5·5%) and significant (*P*<0·05) at the highest dosage (200 µg/kg BW per day) administered (Fig. 1).

Effects of pulsatile GH on NPY protein and mRNA levels

The NPY RIA was optimized and validated for determination of plasma and hypothalamic NPY levels in the chicken. Serial dilutions of unknown samples were parallel to the standard curve, and recovery of NPY standard added to Sep-Pak-extracted samples ranged from 95 to 110% (data not shown). Extraction of hypothalamic samples by elution through C-18 (Sep-Pak) columns improved recovery of NPY at concentrations above 10 ng/ml, and this method was therefore used for preparation of both hypothalamic and plasma samples, as previously reported (Stridsberg & Lundqvist 1989).

GH (200 µg/kg BW per day) reduced NPY protein concentration in the hypothalamus compared with controls (*P*<0·065) (Fig. 2). However, plasma NPY
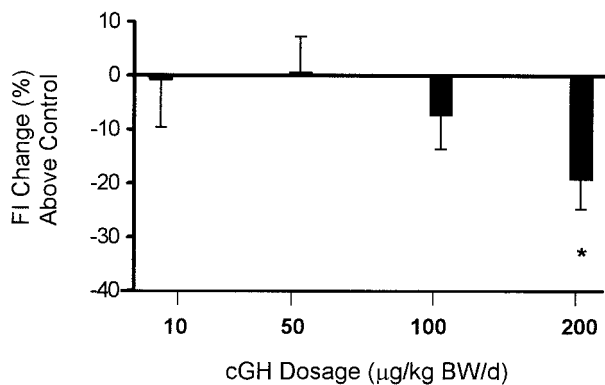

Figure 1 Reduction in voluntary FI following pulsatile i.v. GH infusion for 7 days in 8-week-old, female broiler chickens. Each bird served as its own control by calculating the percentage change in average daily FI (g/kg BW per day) for the final 3 days of infusion, relative to that bird's preinfusion FI (average of the 3 days immediately preceding initiation of treatments). Because FI normally decreases as broilers mature, and this was observed in all birds across treatment groups prior to infusion, FI change for each GH-treated bird was corrected for the average decline observed in the controls. Values are means ± S.E.M. (*n*=9–10 birds per dosage). *P<0·05 compared with pre-infusion FI.
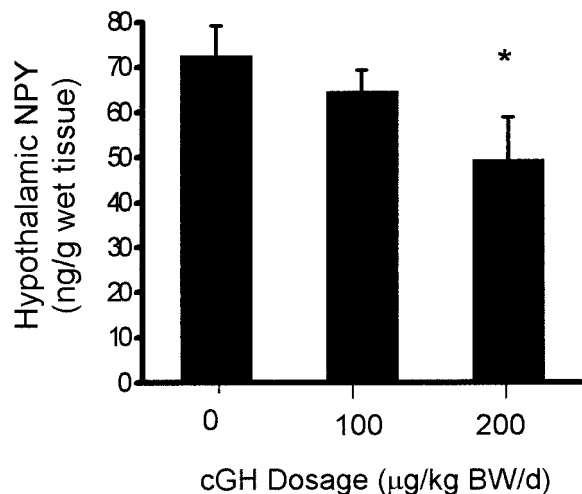

Figure 2 Reduction in hypothalamic NPY following 7 days of pulsatile i.v. cGH infusion in 8-week-old, female broiler chickens. Hypothalamic samples were homogenized in sodium acetate buffer as described in Materials and Methods, and the supernatant was removed for NPY immunoassay. Samples were extracted by elution through C$_{18}$ (Sep-Pak) columns prior to assay. Values are means ± S.E.M. (*n*=9–10 birds per dosage). *P=0·065.
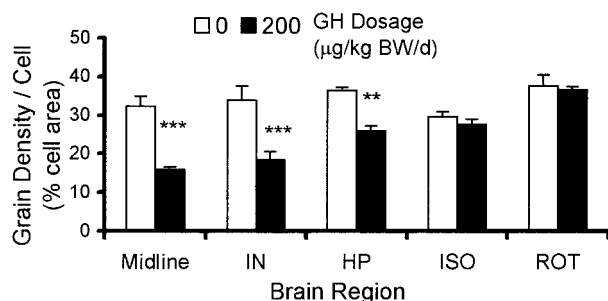
Figure 3 Changes in NPY mRNA in various brain regions (Midline, midline region of the hypothalamus; HP, hippocampus) following pulsatile cGH (200 μg/kg BW per day) infusion for 7 days in 8-week-old, female broiler chickens. Grain density was expressed as a percentage of the cell area occupied by grains. Background counts were subtracted from each labeled cell count. Values are means ± S.E.M. (*n*=3–6 birds/treatment). GH (200 μg/kg BW per day) treatment resulted in a significant reduction in NPY mRNA in the midline region, IN and HP. **P<0·01, ***P<0·001 compared with vehicle-infused controls.
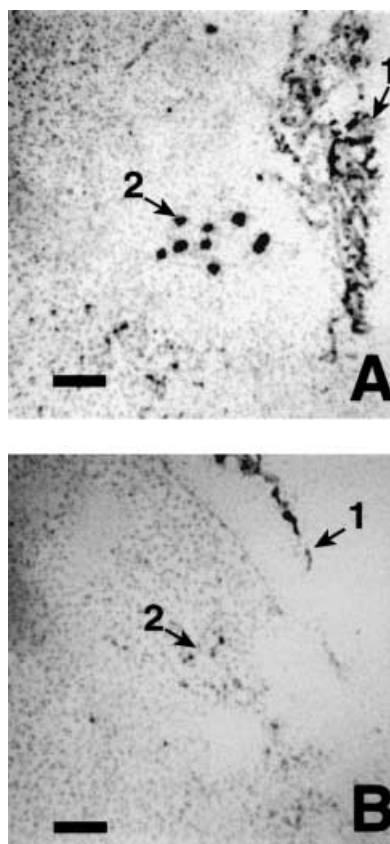

Figure 4 Hypothalamic NPY mRNA expression in the midline region of (A) control and (B) GH-treated (200 μg/kg BW per day), 8-week-old, female broiler chickens. Brightfield photomicrographs of chicken brain sections processed for *in situ* hybridization with ^{35}S-labeled riboprobe complimentary to rat pre-proNPY (anti-sense probe), and dipped in photographic emulsion. Grain density (percentage of cell area occupied by labeled grains; indicated by arrows) is reduced in the midline region (arrow 2) of a GH-treated bird (B), in comparison to control (A). No labeling was observed when sections were hybridized with sense-strand probe (not shown). Arrow 1=third ventricle. Scale bar=40 μm.

concentrations were not affected by GH administration, and averaged 1·0 ± 0·05 ng/ml across all dosages. Both plasma and hypothalamic NPY concentrations in the pair-fed groups were not different from those of the control group (Table 1).

In situ hybridization for NPY message was performed on brain sections from birds of the GH dosage group that exhibited a significant depression in FI (200 μg/kg BW per day), as well as from control and pair-fed groups. At the time this work was conducted, a homologous chicken NPY cDNA was not available. However, a complete map of NPY gene expression in the chicken brain has been generated by our laboratory using the rat pre-proNPY probe used for the present study (X Wang, J R Day & R Vasilatos-Younken, unpublished observations) and found to be identical to data independently published for the chicken brain using a chicken riboprobe (Boswell *et al.* 1998, 1999), and follows the distribution pattern for NPY-containing neurons in the chicken brain based upon previous immunohistochemical studies (Kuenzel & McMurtry 1988). These would confirm that the rat pre-proNPY riboprobe used for *in situ* detection in the present study hybridized to NPY mRNA in the chicken brain and accurately represents expression of the gene.

In emulsion-coated sections, NPY mRNA-containing cells were identified by clusters of silver grains over single neurons. Labeling was specific, and silver grains were not observed over cells when sections were hybridized with sense-strand probe (data not shown). Counted cell area for NPY mRNA in various brain regions did not differ among treatments, within region (data not shown).

At the mRNA level, the NPY signal was markedly reduced with GH treatment (200 μg/kg BW per day) compared with controls, for both the midline region (PVN and periventricularis nucleus (PHN)) (50%; *P*<0·0001)

and IN of the hypothalamus (46%; *P*<0·0001) (Figs 3 and 4). NPY mRNA was also significantly reduced in the hippocampus (Fig. 3). There were no significant differences between control and GH-treated groups in the lateral septal organ (LSO) and neurons around the nucleus rotundus (ROT). No significant differences were found in the midline region and IN between control and pair-fed groups (Table 2).

Effects of pulsatile GH on monoamines and their metabolites

GH decreased hypothalamic EP (y=1253 − 1·83x, *P*=0·009, coefficient of determination (R–Sq)=0·227) (Table 3). The maximal effect was at 200 μg/kg BW per day, at which hypothalamic EP concentrations were reduced 37% versus vehicle-infused. DOPAC was

Table 2 NPY mRNA (grain density; % of total cell area) in the midline and IN region of the brain following 7 days of pair-feeding (pairfed-200) in 8-week-old broiler chickens. Values are means ± S.E.M. (*n*)

	Treatment	
	Control	Pairfed-200[1]
Midline[2]	20·3 ± 1·30 (4)	19·0 ± 1·39 (4)
IN	25·9 ± 1·85 (4)	22·9 ± 1·42 (3)

Note: pair-fed birds were not infused with GH. For results of GH infusion, see Results and Fig. 3.
[1]Pair-fed to the 200 µg GH/kg BW per day dosage group.
[2]PVN and PHN.
Control and pair-fed groups did not differ.

significantly decreased after 7 days of GH treatment ($y = 1189·3 − 2·16x$, $P=0·025$, R–Sq=0·107). The DOPAC/DA ratio, which is an indicator of DA turnover, was significantly decreased as GH dosage increased ($y=2·28 − 0·00374x$, $P=0·007$, R–Sq=0·251), even though hypothalamic DA was not significantly affected by GH treatment. GH had no effect on other hypothalamic monoamines and metabolites (5-HT, HVA, NE and DA/HVA). No significant differences were observed in pair–fed groups for all monoamines measured in the hypothalamus (data not shown).

A GH dose-related increase in the DOPAC/DA ratio in the medulla was observed ($y=3·26+0·05x$, $P=0·015$, R–Sq=0·287) (Table 4). Significant increases in 5-HIAA and DA were not dose–related, but were observed for the 10 µg cGH/kg BW per day treatment dosage compared with the control group ($P<0·05$). No differences were observed for other monoamines (Table 4). Feed restriction had no significant effect on any of the monoamines or their metabolites measured in the medulla (data not shown).

Discussion

The present study was conducted to assess the influence of a physiologically relevant pattern of GH enhancement (pulsatile i.v. infusion) on FI, and possible mechanisms underlying this effect, using an animal model that exhibits relatively high levels of feed consumption. The appetite-depressive effect of GH over a range of dosages was assessed, beginning at an age when endogenous GH secretion is low relative to earlier ages. Because of the precise control possible with microprocessor-controlled, intravenous delivery, circulating concentrations of GH achieved over the full dose–response curve were extremely uniform within dosage groups, and non-overlapping among groups. With this model, a significant depression in voluntary FI was detected only at the highest peak plasma concentration achieved (i.e. at 200 µg GH/ BW per day).

In conjunction with decreased FI, GH administration resulted in a dramatic reduction in hypothalamic NPY protein and gene expression, supporting the idea that regulation is at a pre-translational level. Further, as intra–cerebral injection of NPY induces a robust increase in FI in the chicken (Kuenzel *et al.* 1987*b*), decreased NPY may mediate the anorexia caused by GH, possibly by increasing sympathetic nervous system activity (Stanley 1993). A large body of evidence suggests that NPY of hypothalamic origin, produced primarily in the IN (equivalent to the mammalian arcuate nucleus) of the hypothalamus, may be involved in the control of ingestive behavior in the chicken. In this study, NPY mRNA was depressed in both the IN and midline regions (PVN and PHN) of the hypothalamus, as well as in the hippocampus. Although the hippocampus may participate in the regulation of FI (Davidson & Jarrard 1993), the exact roles of hippocampal NPY on FI are unclear.

In contrast to the response observed in chickens in the present study, GH up–regulates NPY mRNA in the hypothalamus of the rat (Kamegai *et al.* 1994, Chan *et al.* 1996). This species-specific difference may explain the difference in FI response to GH in the rat versus chicken (and other species that respond similarly to the latter, such as the human).

The values presented here for concentrations of NE and DA in the hypothalamus and in the medulla agree well in general with available data for the chick (Kuenzel *et al.* 1987*b*, Siuciak *et al.* 1992). 5-HT and 5-HIAA levels in this study are in agreement with previous studies in the chicken brain using HPLC analysis (Cassone *et al.* 1986, Siuciak *et al.* 1992). Our data for EP, however, were ten times higher than those found by Siuciak *et al.* (1992), and much higher than those reported by Kuenzel *et al.* (1987*b*). It is unlikely that these differences are methodo-logic, as concentrations of other monoamines were similar across studies. Rather, these differences may be due to neurodevelopmental stage, as juvenile (8-week-old) birds were used in the present study, versus infant (2-week-old) chicks previously (Kuenzel *et al.* 1987*b*, Siuciak *et al.* 1992).

GH significantly depressed EP in a dose-dependent manner in the hypothalamus. This is consistent with reports that synthesis and release of catecholamines are reduced after bolus GH administration both in the rat (Stern *et al.* 1975) and in the chicken (Lea & Harvey 1993). EP has been shown to increase FI in the chicken (Denbow *et al.* 1981). The reduction in EP may therefore contribute to the FI-depressive effect of GH as well. Given that a close anatomical association between the adrenergic and NPY neural systems exists (Sawchenko *et al.* 1985), and stimulation of feeding induced by central administration of NPY can be attenuated by prior block–ade of $\alpha2$ adrenoceptors (Clark *et al.* 1988), it is possible that EP may, at least partially, mediate the effect of NPY on feeding behavior.

Table 3 Effect of pulsatile i.v. infusion of GH for 7 days on monoamines and their metabolites in the chicken hypothalamus (pg/mg tissue). Values are means ± S.E.M. (*n*)

	cGH dosage (µg/kg BW per day)				
	0	10	50	100	200
Measurement					
NE	1987 ± 344 (9)	1719 ± 222 (9)	1849 ± 299 (9)	1589 ± 196 (9)	1745 ± 278 (6)
EP**	1464 ± 100 (5)	1177 ± 196 (4)	992 ± 95 (7)	1095 ± 39 (5)	919 ± 91 (8)
5-HT	923 ± 107 (9)	874 ± 123 (9)	872 ± 101 (9)	873 ± 107 (10)	1061 ± 119 (10)
5-HIAA	820 ± 54 (9)	912 ± 94 (9)	885 ± 48 (9)	847 ± 52 (9)	897 ± 76 (10)
5-HIAA/5-HT	0·98 ± 0·13 (9)	1·16 ± 0·16 (9)	1·12 ± 0·14 (9)	0·97 ± 0·10 (8)	0·82 ± 0·10 (9)
DA	447 ± 41 (7)	478 ± 65 (7)	507 ± 62 (5)	438 ± 93 (6)	510 ± 38 (7)
HVA	2052 ± 153 (9)	2189 ± 144 (9)	2260 ± 98 (9)	2184 ± 104 (10)	2068 ± 151 (10)
HVA/DA	4·60 ± 0·22 (7)	4·65 ± 0·52 (7)	4·41 ± 0·53 (5)	6·10 ± 1·54 (6)	4·18 ± 0·19 (7)
DOPAC*	1050 ± 113 (6)	983 ± 90 (9)	908 ± 79 (9)	927 ± 70 (10)	829 ± 83 (10)
DOPAC/DA**	2·72 ± 0·15 (6)	1·95 ± 0·23 (5)	1·71 ± 0·24 (5)	1·95 ± 0·23 (5)	1·59 ± 0·14 (7)

*$P < 0.05$, **$P < 0.01$; significant linear regression.

Although hypothalamic DA was unaffected by GH, the DOPAC/DA ratio was significantly decreased in the hypothalamus. This result is consistent with reduced DA turnover in the hypothalamus in GH transgenic mice (Steger *et al.* 1991). In the medulla, GH affected DA turnover in the opposite direction. Given that injection of DA into the lateral ventricle has no effect on FI in the chick (Denbow *et al.* 1981), the change in DA metabolism observed in this study may not be relevant to GH-induced FI depression.

Feed restriction *per se* was reported to increase levels of NPY mRNA and protein (Sahu *et al.* 1988) and 5-HT content (Krieger 1974) in the hypothalamus of the rat, and 48 h of feed deprivation has been reported to decrease hypothalamic NE and DA levels (Stachowiak *et al.* 1979). In the present study, feed restriction imposed on birds pair-fed to the reduced level of the GH treatment group had no effect on hypothalamic NPY protein and mRNA concentrations and monoamine levels, possibly because

the level of restriction was not comparably severe to the previous studies cited above. However, more importantly, these strongly support that the changes associated with GH treatment are not secondary to the reduced FI that accompanies higher dosages of the hormone.

It is not clear whether the reduction in NPY and EP in response to GH administration is a direct effect on the hypothalamus or via a secondary mediator. Most of the NPY-containing neurons in the arcuate nucleus have been shown to express GH receptor mRNA (Chan *et al.* 1996). Since there is evidence that GH is able to cross the blood–brain barrier (Tannebaum & Ling 1980), it is possible that GH may regulate the activity of NPY neurons in the hypothalamus directly.

Alternatively, companion data from this study demonstrate that GH increases hepatic leptin mRNA in a dose-dependent manner, whereas leptin gene expression is depressed with feed restriction *per se*, as was observed in the pair-fed group (Ashwell *et al.* 1999). Leptin is a

Table 4 Effect of pulsatile i.v. infusion of GH for 7 days on monoamines and their metabolites in the chicken medulla (pg/mg tissue). Values are means ± S.E.M. (*n*)

	cGH dosage (µg/kg BW per day)				
	0	10	50	100	200
Measurement					
NE	1419 ± 194 (9)	1303 ± 129 (8)	1354 ± 128 (7)	1432 ± 333 (10)	1628 ± 362 (10)
EP	1122 ± 368 (4)	507 ± 94 (4)	917 ± 199 (4)	602 ± 118 (5)	1207 ± 388 (6)
5-HT	450 ± 97 (8)	456 ± 95 (8)	398 ± 109 (9)	434 ± 68 (9)	674 ± 144 (9)
5-HIAA	468 ± 56 (9)	678 ± 44 (8)	616 ± 83 (10)	650 ± 71 (10)	523 ± 53 (9)
5-HIAA/5-HT	1·14 ± 0·19 (8)	1·59 ± 0·28 (7)	2·74 ± 0·74 (9)	2·37 ± 0·85 (9)	2·62 ± 1·43 (9)
DA	293 ± 38 (5)	400 ± 15 (5)	317 ± 28 (6)	400 ± 46 (3)	264 ± 29 (5)
HVA	1834 ± 96 (8)	2071 ± 121 (8)	1779 ± 97 (9)	1026 ± 142 (10)	1619 ± 161 (10)
HVA/DA	6·34 ± 0·75 (4)	5·57 ± 0·37 (5)	6·10 ± 0·57 (6)	5·87 ± 0·23 (3)	6·26 ± 0·28 (5)
DOPAC	867 ± 135 (7)	3072 ± 953 (7)	1487 ± 490 (7)	1327 ± 464 (10)	2763 ± 1005 (8)
DOPAC/DA*	2·54 ± 0·56 (3)	6·21 ± 2·44 (4)	5·39 ± 2·99 (5)	5·23 ± 1·72 (3)	14·97 ± 4·95 (5)

*$P < 0.05$; significant linear regression.

peripheral signal informing the brain of starvation (Ahima *et al.* 1996). Among several possible mediators of leptin action within the hypothalamus, NPY is the most important (Erickson *et al.* 1996). Since mice that lack NPY still respond normally to the satiety effects of leptin, other mediators of leptin must exist within the brain. The depressive effect of GH on FI may be due to changes in peripheral leptin production. Leptin in turn may induce changes in hypothalamic NPY (and possibly EP), which result in anorexia.

Our results verify that pulsatile infusion of GH for 7 days, at a dosage that achieved a high physiological peak amplitude concentration of the hormone, significantly suppressed FI, and suggest that NPY and EP are involved in this effect. The reduction in both NPY and EP could account for the sustained depressive effects of GH on feeding, as EP has very brief latencies and short-acting effects (1–30 min), while NPY has long-term effects (up to 4 h) (Stanley *et al.* 1986). Future research is required to confirm whether the effects of GH on hypothalamic NPY and EP are direct, or if secondary mediators are involved.

Acknowledgements

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