Hypothalamic Agouti-Related Protein Expression Is Affected by Both Acute and Chronic Experience of Food Restriction and Re-Feeding in Chickens

Hypothalamic agouti-related protein expression is affected by both acute and chronic experience of food restriction and re-feeding in chickens

I.C. Dunn[1], P.W. Wilson[1], T.V. Smulders[2], V.Sandilands[3], R.B. D'Eath[3], and T. Boswell[4]

Abbreviated title: AGRP and food restriction in chickens

[1]University of Edinburgh, Roslin Institute, and Royal (Dick) School of Veterinary Studies, Easter Bush, Scotland, UK;

[2]Institute of Neuroscience, and Centre for Behaviour and Evolution, Newcastle University, England. UK;

[3]SRUC, Auchincruive and Easter Bush, Scotland, UK.

[4]School of Biology, Institute of Neuroscience, and Centre for Behaviour and Evolution, Newcastle University, England. UK;

Key words: AGRP, chronic food restriction, broiler breeder chicken, re-feeding

Correspondence to: Timothy Boswell, School of Biology, Ridley Building, Newcastle University, Newcastle upon Tyne, NE1 7RU, United Kingdom; timothy.boswell@ncl.ac.uk

Abstract

The central melanocortin system is conserved across vertebrates. However, in birds, little is known about how energy balance influences orexigenic agouti-related protein (AGRP) and anorexigenic pro-opiomelanocortin (POMC) expression, despite the fact that commercial food restriction is critical to the efficient production of poultry meat. To enable contrasts to be made between levels of food restriction, between hens with the same body weight but different feeding experience, and between hens moved from restricted feeding to *ad libitum* feeding for different periods, five groups of broiler-breeder hens were established between six and 12 weeks of age with different combinations of food restriction and release from restriction. AGRP and neuropeptide Y (NPY) expression in the basal hypothalamus was significantly increased by chronic restriction but only AGRP mRNA levels reflected recent feeding experience: hens at the same body weight which had recently been on *ad libitum* feeding showed lower expression than restricted birds. AGRP expression also distinguished between hens released from restriction to *ad libitum* feeding for different periods. In contrast, POMC and cocaine- and amphetamine-regulated transcript (CART) mRNA levels were not different. These results demonstrate for the first time in birds that although AGRP mRNA reflected differences between a bird's weight and its potential weight or set point, it also discriminated strongly between differing acute experiences of food intake at the same body weight. Therefore AGRP expression potentially provides an integrated measure of food intake experience and an objective tool to assess a bird's perception of satiety in feeding regimes for improved poultry welfare.

Introduction

The existence of a neuronal network that regulates food intake and energy balance in the hypothalamic arcuate nucleus of vertebrates is well established and involves the central melanocortin system (1). Melanocortin signalling within the hypothalamus is mediated by melanocortin 3 and 4 receptors (MC3R and MC4R) in the paraventicular nucleus that exert inhibitory effects on food intake and body fat content (2, 3). Two main melanocortin receptor (MCR) ligands have been implicated in the action of the receptors on energy balance in the hypothalamus. Agouti-related protein (AGRP) exerts an antagonistic action on the MC3R and MC4R (4) and therefore induces obesity when it is genetically over-expressed, and stimulates food intake after injection into the brain (5, 6). In contrast, α-melanocyte-stimulating hormone (α-MSH), encoded by the pro-opiomelanocortin (POMC) gene, acts as an agonist on MCRs and inhibits food intake after central administration (7, 8). The neurons expressing AGRP and POMC are present in two distinct populations within the arcuate nucleus and co-express neuropeptide Y (NPY) and cocaine-and-amphetamine-regulated transcript (CART), respectively (1). The appetite-stimulatory AGRP/NPY and inhibitory POMC/CART cells are sensitive to energy status because they express receptors for hormones such as leptin and insulin and have access to blood-borne nutrients such as glucose, fatty acids and amino acids (9). AGRP/NPY and POMC/CART gene expression are, respectively, up- and down-regulated by food deprivation (10, 11) and the frequency of action potentials was increased in AGRP/NPY neurons of food-deprived mice recorded *in vitro* (12). The regulatory effect of leptin in coordinating this response in mammals has received particular attention (12, 13).

Although best characterised in mammals, the melanocortin system is evolutionarily ancient, with components being detectable in elephant sharks (*Callorhinchus milii*) and sea lampreys

(*Petromyzon marinus*) as well as in several teleost fish species (14, 15). The involvement of the melanocortin system in regulating energy balance has also been conserved: transgenic overexpression of AGRP in zebrafish resulted in obesity (16). Among non-mammalian amniotes, the melanocortin system of birds has been best studied (17, 18). Five melanocortin receptors have been cloned in the chicken, including the MC3R and MC4R (19, 20). AGRP and POMC have been localised in the arcuate nucleus of the domestic chicken (*Gallus gallus*), Japanese quail (*Coturnix japonica*), and ring dove (*Streptopelia risoria*) (21-25). The avian arcuate nucleus was formerly known as the infundibular nucleus (26) but current nomenclature favours the use of the mammalian name for this structure (27). The high degree of evolutionary conservation of the neuroendocrine network is emphasised by co-expression in the Japanese quail arcuate nucleus of AGRP and NPY mRNAs in individual neurons, corresponding to the situation in mammals (22). Melanocortin system function also appears to be conserved between birds and mammals because AGRP, α-MSH, and the α-MSH agonist melanotan-II (MT-II) exert opposing stimulatory (AGRP) and inhibitory (α-MSH, MT-II) effects on food intake after exogenous administration in several avian species (28-31). Also, biosynthesis of AGRP and NPY mRNAs and peptides are increased after food deprivation in the domestic chicken, Japanese quail and ring dove (23, 30, 32). Increased food intake after fasting in birds appears to be mediated primarily by upregulation of AGRP and NPY expression because no significant changes in POMC or CART expression were observed in Japanese quail and domestic chickens after a fast (33). Although AGRP and NPY expression are nutritionally sensitive in birds, the regulation of their expression, together with that of other arcuate nucleus neuropeptides, is uncertain because birds appear to lack a leptin ortholog (34-36).

In contrast to mammals, there has been relatively little work on melanocortin system and other arcuate nucleus neuropeptides in birds, particularly in relation to the effects of chronic food restriction (as distinct from acute food deprivation/ fasting). We used broiler breeder chickens as a model for investigation, because they are routinely food restricted in the poultry industry, leading to concerns that hunger impairs their welfare (37). Broiler breeders are the parents of broiler chickens, which are reared for meat, accounting for over one third of the world's agricultural animal protein production (38). The genetic loci under selection for high food intake and growth rate in broiler chickens also influence reproduction (39) and the reproductive performance of fully-fed broiler breeder chickens is impaired, in part from an over-activity of the reproductive axis (40). Thus, routine food restriction of breeders during the growth phase improves reproductive performance and the birds' overall health but is seen in itself as impairing welfare, the so called 'broiler breeder paradox' (41). During the rapid growth phase (assessed in the present study) this represents around 25% of *ad libitum* intake, but is much milder during the egg laying period from around 20 weeks of age. To explore methods to optimise the level of food availability in relation to reproductive performance but balanced against animal welfare concerns, it would be useful to define internal markers of feeding motivation in chickens. Specific experimental activation of AGRP neurons in mice has recently been shown to increase motivation for feeding and promote food-seeking behaviour (42, 43). We reasoned that because AGRP expression is increased after food deprivation in mammals and birds as part of a counter-regulatory response to energy deficit, its expression may also be increased in chickens that are under-weight during the growth phase as a result of chronic food restriction, and would vary in proportion to the amount of restriction imposed and also in response to re-feeding. To take this further we also set out to assess the sensitivity of AGRP expression as an indicator of recent feeding history in groups of chickens that had experienced either food restriction or a period of re-feeding following

restriction, but which had attained the same body weight. While the dynamics of changes in AGRP and POMC gene expression following food deprivation or restriction and re-feeding have been studied in several mammalian species (44-47), little information is available in non-mammalian vertebrates, and we are not aware of investigations in any vertebrate that have examined the effect on the melanocortin system of recent feeding history in animals at the same body weight.

Using this approach, we demonstrate in the present study that AGRP expression in broiler breeder chickens is closely associated with the level of food restriction and responds rapidly to re-feeding. The level of AGRP gene expression also allows discrimination between hens at the same body weight but with different recent experience of food restriction or re-feeding.

Materials and methods

Animals

Female broiler breeders (Ross 308 line) were housed three to a pen with four pens per treatment (n=12/treatment). Lighting, nutritional composition of the food, and dietary restriction from day-old to six weeks of age was implemented according to the 2007 management manual (http://en.aviagen.com/ross-308/). Briefly, a starter (19% crude protein) and a grower diet (15% crude protein) with an energy density of 11.7 MJ/kg was fed from 0-4 and 4-12 weeks of age, respectively. In the standard commercial protocol, these diets are available *ad-libitum* from 0-1 week and thereafter step-wise to 44 g/bird/day at 6 weeks of age and 58 g/bird/day by 12 weeks of age. Management procedures from six weeks are provided below for each experiment. The experiment was performed under a United

Kingdom Home Office Project Licence and birds were humanely killed as specified in Schedule 1 of the United Kingdom Animals (Scientific Procedures) Act 1986.

Experiment 1

This experiment was designed to contrast commercial restriction (R) with *ad libitum* feeding (AL) and to highlight those genes which had the greatest potential to act as markers.

From six weeks of age, hens were subjected to two treatments. Half the pens were maintained on the recommended restricted diet (R), temperature and lighting regime (http://en.aviagen.com/ross-308/) and half were maintained on the recommended diet and temperature but were given *ad libitum* access to food (AL). All hens were killed over two days (see description for Experiment 2, below) at 12 weeks of age.

Experiment 2

This experiment was designed to contrast the effects on marker gene expression of feeding history during the rapid growth phase. It included a comparison between hens at the same body weight that had experienced different feeding histories of either *ad libitum* feeding, or restricted feeding in the two weeks prior to killing.

From six weeks of age hens were subjected to five treatments:

1) *Ad libitum* (AL)

2) Maintenance on a commercial quantitative restriction programme (R).

3) Maintenance on a commercial quantitative restriction programme before being fed *ad libitum* for two days prior to death (R+2d). The beginning of *ad libitum* feeding was staggered to match the dates the hens were killed (see below).

4) Maintenance on two times the commercial quantitative restriction programme (2R) to achieve a body weight intermediate between the AL and R groups.

5) Feeding to a body weight intermediate between commercially restricted and *ad libitum*-fed hens based on growth trajectories in previous experiments. This was achieved by maintenance on a commercial quantitative restriction programme (R) to approximately 10 weeks of age followed by *ad libitum* feeding for the final 14 days (R+14d).

All birds were humanely killed at 12 weeks of age. Because a limited number of dissections could be performed in a day, birds were killed over two days in Experiment 1 and four days in Experiment 2. Dissections took place throughout the day from 1.5 hours after lights-on until the early dark phase, 8 hours after lights on. This was after the daily food ration had been provided to the restricted birds. Individual hens were dissected from each treatment in turn to minimise the effects of sampling time. For the groups released onto *ad libitum* feeding, the day of release was adjusted in relation to the day an individual was killed.

Food intake was averaged over the week prior to the end of the experiment adjusted for date of death. The exception was for the R+2d group, which was averaged over the two days prior to the end of the experiment.

RNA isolation and reverse transcription

The basal hypothalamic dissection procedure used has been previously described (48). The anterior pituitary gland and neural lobe were removed and the basal hypothalamus was isolated by making incisions at the caudal margin of the optic chiasma and rostral to the roots of the oculomotor nerves. The dissection extended laterally 1 mm either side of the third

ventricle and dorsally 2 mm from the surface of the median eminence. The tissue was dissected immediately after death, frozen in liquid nitrogen, and stored at -80°C. RNA was extracted from up to 100 mg of tissue using Ultraspec II reagent (AMS Biotechnology, Abingdon, UK) and Lysing Matrix D tubes in a FastPrep Instrument (MP Biomedicals, Cambridge, UK). Total RNA was reverse transcribed using NotI-(dT)18 primer and a First-Strand cDNA synthesis kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's protocol. cDNA samples were diluted x15 for use in real-time PCR

Real-time PCR (RT-PCR) assays

Gene specific primers were designed using Primer3 (49) to amplify products of approximately 200 bp across intron/exon boundaries. AGRP was amplified using forward primer 5'- AGGCCAGACTTGGATCAGATG (positions 220-240 of NM_001031457) and reverse primer 5'- ACTCCAGGAGGCGGACAC (positions 362-379); for POMC the forward primer was 5'- AACAGCAAGTGCCAGGACC (positions 88-106 of NM_001031098) and the reverse primer 5'- ATCACGTACTTGCGGATGCT (positions 214-233); for NPY the forward primer was 5'-TGGAAAGAGATCAAGCCCA (positions 230-248 of NM_205473) and the reverse primer 5'- CAATGGCTGCATGCACTG (positions 416-433); and for CART the forward primer was 5'-GCCGCACTACGAAAAGAAGT (positions 192-211 of XM_003643097) and the reverse primer 5'-GAAAGGAGTTGCACGAGGTGC (positions 299-319). The chicken lamin B receptor gene was used as a control gene for normalisation, amplified by forward primer 5' – GGTGTGGGTTCCATTTGTCTACA (positions 1464-1486 of NM_205342) and reverse

primer 5'- CTGCAACCGGCCAAGAAA (positions 1526-1543). Standards were produced by gel purification of PCR products using a QIAquick gel extraction kit (Qiagen Ltd., Crawley, West Sussex, UK) and their concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Loughborough, Leicestershire, UK). Serial dilutions of standards were made to create standard curves for real-time PCR quantification. Real-time PCR reactions were run on an MX3000p real-time PCR machine (Agilent Technologies, Cheshire, UK) using the following conditions: 95^{o}C for 2 min, 40 cycles of 95 ^{o}C for 15 s, 60^{o}C for 30 s. Real time PCR reactions (25 µl) were run using 10 µl cDNA template together with SYBR green master mix (VHBio Ltd, Gateshead, UK) and gene specific primers (100 nM). No-template controls were also included. Samples and standard curves were run in duplicate on the same 96-well plate. Standards were diluted to produce top standards detectable after approximately 15 PCR cycles. Assays were analysed using MxPro software (Agilent Technologies) and neuropeptide expression was expressed as a ratio in relation to lamin B receptor expression (50) measured in the same samples.

Statistical analysis

ANOVA was used for statistical analysis of data using Genstat 12th edition (VSN International Ltd, Oxon, UK). Log transformation of the corrected values was used to give approximate normality and consistency of variances of the residual values after model fitting. In Experiment 1, hens were killed over two days and in Experiment 2, over four days. Hens in each treatment were housed in four pens: in Experiment 2 this level of variance was confounded with day of kill. Additionally two assay runs were used in the RT-PCR assay of mRNA in Experiment 2. These sources of variance were accounted for in the ANOVA.

Where appropriate, *post hoc* comparisons were made using least significant differences. The level of significance was $P<0.05$.

Results

Experiment 1

As expected, the body weight of restricted (R) hens was significantly lower than that of the hens fed *ad libitum* (AL) (1467 vs. 3458 g; $P<0.001$) and, at 12 weeks of age, food intake of the R hens was approximately 27% of that eaten by the AL hens (232 g/day vs. 58 g/day) (Fig. 1). Neuropeptide mRNA measurements in this experiment focused on AGRP and POMC expression in the basal hypothalamus. Food restriction resulted in an approximately 58-fold increase in the expression of AGRP ($P<0.001$) but no difference was observed in POMC expression between treatments.

Experiment 2

The aim of producing groups of hens with the same body weight but different feeding histories was achieved (Table 1 and Fig. 2). As in Experiment 1, the mean body weights of AL and R hens (3563±62 g and 1410±32 g, respectively) were strongly significantly different ($P<0.001$) but, as intended, they were the same in the R+14d and 2R groups (2255±42 g and 2272±44 g. respectively; Table 1 and Fig. 2). In general individual hens from food restricted groups showed fat stores that were greatly reduced in comparison to the *ad libitum*-fed group and were too small for routine dissection. There were some differences in specific organ

weights between the R+14d and 2R groups (Table 1). The 2R group had lower mean pituitary and oviduct weights and a longer mean tarsus length than the R+14d group. Mean body weight was different between the R and R+2d groups (1410±32 g and 1732±29 g, respectively) but the difference was accounted for by the weight of food (approximately 300 g) removed from the food storage organ, the crop, of the hens at *post mortem*. Food intake averaged over the days immediately prior to the end of the experiment was R, 58 g/bird/day; 2R, 116 g/bird/day; R+14d, 203 g/bird/day; R+2d, 234 g/bird/day; AL, 227 g/bird/day.

As in Experiment 1, there was a large difference (167-fold) in the expression of AGRP in the basal hypothalamus between AL and R hens (Fig. 3A). The expression level of AGRP in 2R hens was between that in AL and R hens (although it was closer to AL). *Post hoc* tests demonstrated significant differences in AGRP expression between all of the feeding treatments (minimum $P<0.01$). Specifically, the level of expression in the 2R group was higher than in the R+14d group, and the expression level in the R group was higher than in the R+2d group. However both these pairs of groups had the same body weight (Fig. 2). There was a gradation in the level of expression dependent on the duration of the release from food restriction (i.e. the duration of *ad libitum* feeding prior to death), with the AL group being lower than in the R+14d group, and the R+2d group showing the highest expression.

Neuropeptide mRNA measurements in Experiment 2 were extended to include NPY and CART. For NPY (Fig. 3B), the general pattern of expression was similar to what we observed for AGRP. However the differences in NPY expression were of lesser magnitude, with an 8-fold difference between the AL and R groups compared with a 167-fold difference for AGRP. Also, no significant differences were observed between the 2R and R+14d groups, which had similar mean body weights but different feeding histories. In contrast to AGRP and NPY, we observed no significant treatment effect for POMC and CART mRNAs (Fig.

3C and D). The birds that were on the highest level of restriction (R), and those just released (2R), had high variability in POMC expression. However, when we performed correlation analysis between gene expression and body weight within the AL and R groups, we found no significant correlations for POMC or any of the other neuropeptides we measured.

Discussion

For the first time in birds we have demonstrated that AGRP gene expression in the basal hypothalamus is elevated after chronic quantitative food restriction. Moreover, AGRP appears to be an integrated measure of the chronic and acute feeding state, being affected by both the difference between actual and potential body weight, and the acute feeding history over a number of days. The level of AGRP expression in hens of the same body weight was lower in hens with recent access to *ad libitum* food. Conversely the expression of POMC and CART were unaffected by differences in acute or chronic quantitative food restriction. The expression of NPY showed a similar pattern of expression to AGRP but the changes were smaller in magnitude and the ability to discriminate between the different treatments was reduced.

Our findings extend observations of changes in AGRP, NPY and POMC and CART biosynthesis in relation to acute food deprivation (24-48 h) in birds to the situation of prolonged food restriction. AGRP and/or NPY mRNA and immunoreactivity were increased following food deprivation (24-48 h) in Japanese quail, ring doves, and chickens (23, 24, 33) while POMC and CART mRNA was decreased or unchanged (23, 33, 51). Also, the number of observable AGRP-immunoreactive cell bodies was increased in the ring dove arcuate nucleus in the post-hatch phase of the breeding cycle when the parent birds are in negative

energy balance (24). Our findings from the present study suggest commonality between the acute and chronic regulation of arcuate nucleus neuropeptide genes in birds, with energy deficit consistently inducing increased expression of AGRP and NPY. Moreover, the level of AGRP and NPY expression corresponded to the level of food restriction and returned towards baseline after two days' re-feeding. More variable results have been obtained in mammalian studies. For example, AGRP and NPY expression was increased after prolonged food restriction in sheep, Siberian hamsters (*Phodopus sungorus*), rats, and golden spiny mice (*Acomys russatus*) (44, 46, 52, 53). However, in other rat studies, no effect of chronic restriction on AGRP and NPY expression was reported (45, 47). Also, whilst AGRP and NPY expression were increased after chronic food restriction in rats, only NPY mRNA levels were decreased after two days' re-feeding (54). In comparison, the broiler breeder chicken appears to be a particularly responsive model for investigating the dynamics of AGRP and NPY expression in relation to body weight and re-feeding. Our observations of unchanged POMC and CART gene expression after food restriction suggest that orexigenic drive to the MCR is provided primarily by increased biosynthesis of AGRP and NPY during food restriction rather than through decreased anorexigenic drive from reduced POMC and CART synthesis. With a relatively small sample size Hen et al. (31) observed a significant decrease in POMC expression after 50% food restriction of one-month-old broiler chickens for one week suggesting that decreased anorexic drive from POMC neurons may be relevant under some experimental conditions.

Our measurements in the present study were focused on neuropeptide gene expression so that the extent to which changes in mRNA are reflected in altered neuropeptide protein synthesis is uncertain. However, the available evidence from birds indicates that AGRP-like and NPY-like immunoreactivity is detectable and that changes in immunoreactive cell numbers reflect

nutritional state (24, 55). As in mammals, NPY has been demonstrated to be co-expressed with AGRP in individual arcuate nucleus neurons in Japanese quail (22). As we had observed previously in older hens (56) a difference in NPY expression between *ad libitum*-fed and food-restricted hens was clearly apparent, with the restricted-fed hens having 8-fold higher expression. However NPY mRNA measurement was unable to discriminate significant differences between the2R and R+14d groups. However, unlike AGRP, which is expressed only in the arcuate nucleus, NPY is expressed in other hypothalamic nuclei within our basal hypothalamic dissection, and it may be this dilution that has reduced the sensitivity of measurement in comparison with AGRP. This could be confirmed with in situ hybridisation studies.

Our observations indicate that AGRP and NPY expression is closely associated with nutritional status, both in relation to food restriction and re-feeding. In the hypothalamus in mammals, signalling of AGRP/NPY neurons is closely linked to local energy sensing pathways such as AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR), the activities of which, in turn, are influenced by circulating metabolic hormones such as ghrelin and leptin, and by nutrients such as glucose and amino acids (57-59). There is evidence that the AMPK pathway has been conserved in chickens (60), and in broiler chicks the amount of phosphorylated AMPKα was significantly increased after food deprivation and subsequently reduced by re-feeding in parallel with changes in AGRP and NPY (but not POMC and CART) gene expression (33). While leptin and ghrelin are important nutritional signals to hypothalamic neurons in mammals their status in birds is much less clear. The leptin gene appears to be absent from avian genomes (34, 36) and central infusion of mammalian leptin did not influence AGRP gene expression in broiler chickens (61). Also, although ghrelin is expressed in birds, its effect on food intake is

inhibitory rather than stimulatory as in mammals (62) and this extends to an inhibitory, rather than stimulatory, action of AMPK activity after icv injection of ghrelin in chickens (63). The role of insulin in regulating AGRP and NPY expression is worthy of further investigation because central administration of insulin decreases food intake in chicks, and co-localisation of insulin receptors and NPY has been observed in arcuate nucleus neurons in the chick brain (64, 65). Also, Song et al. (33) observed that circulating insulin concentrations were decreased during food deprivation, when AGRP and NPY expression was increased, and AGRP and NPY mRNA levels and were normalised along with plasma insulin concentrations after re-feeding. A pronounced effect on AGRP gene expression was observed after re-feeding in the present study. AGRP mRNA differed significantly between hens of the same body weight that had been food-restricted compared to previously restricted hens that had fed freely for two weeks before sampling. Also, AGRP expression was markedly and significantly decreased following two days' *ad-libitum* re-feeding of restricted hens. These effects may have been mediated by circulating hormones and/or nutrients, but the possibility that gut-brain signalling may also play a role is raised by the observation that hypothalamic AGRP gene expression was increased in fast-growing chicken genotypes with alleles for reduced expression of the cholecystokinin A receptor (CCKAR) (66).

Our observations of increased AGRP and NPY gene expression in restricted hens, together with the known anabolic effects of the respective peptides in birds (28, 29), are consistent with increased AGRP and NPY signalling being part of a compensatory drive to restore body weight to a defended set point (or range, (67)). Because we performed the experiment during the growth phase, the differences in body weight we observed were due not only to reduced fat stores in restricted birds (which were minimal) but also to increased growth in *ad libitum*-fed birds of all bone and body organs measured, together with early development of the

reproductive system (increased oviduct weight). However, the fact that AGRP and NPY expression were significantly decreased and approaching baseline levels in the re-fed R+2d group, which had an equivalent body composition and growth stage to the fully restricted R group, indicates that changes in AGRP and NPY expression are driven primarily by changes in nutritional state rather than simply being associated with a particular stage of growth or reproductive development. The comparison between the 2R and R+14d groups revealed that recent feeding history could be discriminated by measurement of AGRP, but not NPY, gene expression because AGRP mRNA was significantly lower in the R+14d group.

In conclusion, both AGRP and NPY gene expression provide an indication of how far a bird has deviated from its defended body weight and, uniquely, AGRP expression potentially provides an integrated measure of food intake experience. If AGRP expression was only a short-term indicator of satiety, then we would not have observed differences between *ad libitum*-fed hens with different histories of restriction. Similarly, if AGRP mRNA level was solely a long-term indicator of drive towards a defended body weight, then we would not have observed differences between hens at the same body weight but with different access to food. Thus AGRP is a potential marker of an integrated level of satiety that mirrors behaviours such as increased foraging in hens given similar levels of food restriction. As such, it would allow us to evaluate the effect of interventions aimed at improving the welfare of food-restricted chickens in the context of the 'broiler-breeder paradox', where food restriction is necessary to improve the birds' health and reproductive performance, but causes hunger (68, 69). For example, a reduction in AGRP expression in birds fed diets containing fibre (70), and therefore experiencing more gut-fill, would suggest that they are more satiated (less hungry). It may also be possible to understand how the degree of satiety indicated by AGRP expression interacts with the varying level of restriction used in the poultry industry, which is around 25% at 12 weeks of age and is reduced as the hens reach sexual maturity. Before we

can be sure of these points though, we need to understand better the relationship between AGRP expression and behaviour and, in particular with feeding motivation (71, 72) which is an important measure in relation to animal welfare (73).

Acknowledgements

The work described in this paper was funded by DEFRA grant AW1141 'Quantifying the subjective state of feed restricted broiler chickens using behavioural and neurochemical measures'. The Roslin Institute is funded by the BBSRC through Institute Strategic Grant funding and SRUC receives funding from the Scottish Government. The authors would like to thank the Kim Bernard and her staff in the poultry unit at Roslin for the excellent animal care.

References

1. Cone RD. Anatomy and regulation of the central melanocortin system. *Nat Neurosci* 2005; **8**: 571-578.

2. Huszar D, Lynch CA, FairchildHuntress V, Dunmore JH, Fang Q, Berkemeier LR, Gu W, Kesterson RA, Boston BA, Cone RD, Smith FJ, Campfield LA, Burn P, Lee F. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* 1997; **88**: 131-141.

3. Butler AA, Kesterson RA, Khong K, Cullen MJ, Pelleymounter MA, Dekoning J, Baetscher M, Cone RD. A unique metabolic syndrome causes obesity in the melanocortin-3 receptor-deficient mouse. *Endocrinology* 2000; **141**: 3518-3521.

4. Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen YR, Gantz I, Barsh GS. Antagonism of central melanocortin receptors in vitro and in vivo by Agouti-related protein. *Science* 1997; **278**: 135-138.

5. Graham M, Shutter JR, Sarmiento U, Sarosi I, Stark KL. Overexpression of Agrt leads to obesity in transgenic mice. *Nat Genet* 1997; **17**: 273-274.

6. Rossi M, Kim MS, Morgan DGA, Small CJ, Edwards CMB, Sunter D, Abusnana S, Goldstone AP, Russell SH, Stanley SA, Smith DM, Yagaloff K, Ghatei MA, Bloom SR. A C-terminal fragment of Agouti-related protein increases feeding and antagonizes the effect of alpha-melanocyte stimulating hormone in vivo. *Endocrinology* 1998; **139**: 4428-4431.

7. Poggioli R, Vergoni AV, Bertolini A. ACTH-(1-24) and alpha-MSH antagonize feeding-behavior stimulated by kappa opiate agonists
Peptides 1986; **7**: 843-848.

8. Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature* 1997; **385**: 165-168.

9. Belgardt BF, Okamura T, Bruening JC. Hormone and glucose signalling in POMC and AgRP neurons. *J Physiol (Lond)* 2009; **587**: 5305-5314.

10. Brady LS, Smith MA, Gold PW, Herkenham M. Altered expression of hypothalamic neuropeptide messenger-RNAs in food-restricted and food-deprived rats. *Neuroendocrinology* 1990; **52**: 441-447.

11. Mizuno TM, Mobbs CV. Hypothalamic Agouti-related protein messenger ribonucleic acid is inhibited by leptin and stimulated by fasting. *Endocrinology* 1999; **140**: 814-817.

12. Takahashi KA, Cone RD. Fasting induces a large, leptin-dependent increase in the intrinsic action potential frequency of orexigenic arcuate nucleus neuropeptide Y/Agouti-related protein neurons. *Endocrinology* 2005; **146**: 1043-1047.

13. Cowley MA, Smart JL, Rubinstein M, Cordan MG, Diano S, Horvath TL, Cone RD, Low MJ. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* 2001; **411**: 480-484.

14. Cerda-Reverter JM, Agulleiro MJ, Guillot RR, Sanchez E, Ceinos R, Rotllant J. Fish melanocortin system. *Eur J Pharmacol* 2011; **660**: 53-60.

15. Vastermark A, Schioth HB. The early origin of melanocortin receptors, agouti-related peptide, agouti signalling peptide, and melanocortin receptor-accessory proteins, with emphasis on pufferfishes, elephant shark, lampreys, and amphioxus. *Eur J Pharmacol* 2011; **660**: 61-69.

16. Song Y, Cone RD. Creation of a genetic model of obesity in a teleost. *FASEB J* 2007; **21**: 2042-2049.

17. Boswell T, Takeuchi S. Recent developments in our understanding of the avian melanocortin system: Its involvement in the regulation of pigmentation and energy homeostasis. *Peptides* 2005; **26**: 1733-1743.

18. Bungo T, Shiraishi J-i, Kawakami S-I. Hypothalamic melanocortin system on feeding regulation in birds: A review. *J Poult Sci* 2011; **48**: 1-13.

19. Takeuchi S, Takahashi S. A possible involvement of melanocortin 3 receptor in the regulation of adrenal gland function in the chicken. *Biochim Biophys Acta: Mol Cell Res* 1999; **1448**: 512-518.

20. Takeuchi S, Takahashi S. Melanocortin receptor genes in the chicken - Tissue distributions. *Gen Comp Endocrinol* 1998; **112**: 220-231.

21. Gerets HHJ, Peeters K, Arckens L, Vandesande F, Berghman LR. Sequence and distribution of pro-opiomelanocortin in the pituitary and the brain of the chicken (Gallus gallus). *J Comp Neurol* 2000; **417**: 250-262.

22. Boswell T, Li QS, Takeuchi S. Neurons expressing neuropeptide Y mRNA in the infundibular hypothalamus of Japanese quail are activated by fasting and co-express agouti-related protein mRNA. *Mol Brain Res* 2002; **100**: 31-42.

23. Phillips-Singh D, Li Q, Takeuchi S, Ohkubo T, Sharp PJ, Boswell T. Fasting differentially regulates expression of agouti-related peptide, pro-opiomelanocortin, prepro-orexin, and vasoactive intestinal polypeptide mRNAs in the hypothalamus of Japanese quail. *Cell Tissue Res* 2003; **313**: 217-225.

24. Strader AD, Buntin JD. Changes in agouti-related peptide during the ring dove breeding cycle in relation to prolactin and parental hyperphagia. *J Neuroendocrinol* 2003; **15**: 1046-1053.

25. Yuan LX, Ni YD, Barth S, Wang YF, Grossmann R, Zhao RQ. Layer and broiler chicks exhibit similar hypothalamic expression of orexigenic neuropeptides but distinct expression of genes related to energy homeostasis and obesity. *Brain Res* 2009; **1273**: 18-28.

26. Kuenzel WJ. Central neuroanatomical systems involved in the regulation of food-intake in birds and mammals. *J Nutr* 1994; **124**: S1355-S1370.

27. Puelles L, Martinez-de-la-Torre M, Paxinos G, Watson C, Martinez S. The chick brain in stereotaxic coordinates: An atlas featuring neuromeric subdivisions and mammalian homologies. Oxford: Academic Press, 2007.

28. Kawakami S, Bungo T, Ando R, Ohgushi A, Shimojo M, Masuda Y, Furuse M. Central administration of alpha-melanocyte stimulating hormone inhibits fasting- and neuropeptide Y-induced feeding in neonatal chicks. *Eur J Pharmacol* 2000; **398**: 361-364.

29. Tachibana T, Sugahara K, Ohgushi A, Ando R, Kawakami SI, Yoshimatsu T, Furuse M. Intracerebroventricular injection of agouti-related protein attenuates the anorexigenic effect of alpha-melanocyte stimulating hormone in neonatal chicks. *Neurosci Lett* 2001; **305**: 131-134.

30. Strader AD, Schioth HB, Buntin JD. The role of the melanocortin system and the melanocortin-4 receptor in ring dove (Streptopelia risoria) feeding behavior. *Brain Res* 2003; **960**: 112-121.

31. Hen G, Yosefi S, Simchaev V, Shinder D, Hruby VJ, Friedman-Einat M. The melanocortin circuit in obese and lean strains of chicks. *J Endocrinol* 2006; **190**: 527-535.

32. Zhou WD, Murakami M, Hasegawa S, Yoshizawa F, Sugahara K. Neuropeptide Y content in the hypothalamic paraventricular nucleus responds to fasting and refeeding in broiler chickens. *Comp Biochem Physiol, A: Mol Integr Physiol* 2005; **141**: 146-152.

33. Song ZG, Liu L, Yue YS, Jiao HC, Lin H, Sheikhahmadi A, Everaert N, Decuypere E, Buyse J. Fasting alters protein expression of AMP-activated protein kinase in the hypothalamus of broiler chicks (Gallus gallus domesticus). *Gen Comp Endocrinol* 2012; **178**: 546-555.

34. Friedman-Einat M, Boswell T, Horev G, Girishvarma G, Dunn IC, Talbot RT, Sharp PJ. The chicken leptin gene: Has it been cloned? *Gen Comp Endocrinol* 1999; **115**: 354-363.

35. Dunn IC, Girishvarma G, Talbot RT, Waddington D, Boswell T, Sharp PJ. Evidence for low homology between the chicken and mammalian leptin genes In: Dawson A, Chaturverdi CM, eds. *Avian Endocrinology*. New Delhi: Narosa Publishing House 2002: 327-336.

36. Pitel F, Faraut T, Bruneau G, Monget P. Is there a leptin gene in the chicken genome? Lessons from phylogenetics, bioinformatics and genomics. *Gen Comp Endocrinol* 2010; **167**: 1-5.

37. Mench JA. Broiler breeders: feed restriction and welfare. *Worlds Poult Sci J* 2002; **58**: 23-29.

38. FAO. FAO World Food Outlook 2008. *http://wwwfaoorg/ag/againfo/themes/en/meat/backgroundhtml* 2008.

39. Podisi BK, Knott SA, Dunn IC, Law AS, Burt DW, Hocking PM. Overlap of quantitative trait loci for early growth rate, and for body weight and age at onset of sexual maturity in chickens. *Reproduction* 2011; **141**: 381-389.

40. Ciccone NA, Dunn IC, Sharp PJ. Increased food intake stimulates GnRH-I, glycoprotein hormone alpha-subunit and follistatin mRNAs, and ovarian follicular numbers in laying broiler breeder hens. *Domest Anim Endocrinol* 2007; **33**: 62-76.

41. Decuypere E, Hocking PM, Tona K, Onagbesan O, Bruggeman V, Jones EKM, Cassy S, Rideau N, Metayer S, Jego Y, Putterflam J, Tesseraud S, Collin A, Duclos M, Trevidy JJ, Williams J. Broiler breeder paradox: a project report. *Worlds Poult Sci J* 2006; **62**: 443-453.

42. Aponte Y, Atasoy D, Sternson SM. AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. *Nat Neurosci* 2011; **14**: 351-355.

43. Krashes MJ, Koda S, Ye C, Rogan SC, Adarns AC, Cusher DS, Maratos-Flier E, Roth BL, Lowell BB. Rapid, reversible activation of AgRP neurons drives feeding behavior in mice. *J Clin Invest* 2011; **121**: 1424-1428.

44. Henry BA, Rao A, Ikenasio BA, Mountjoy KG, Tilbrook AJ, Clarke IJ. Differential expression of cocaine- and amphetamine-regulated transcript and agouti related-protein in chronically food-restricted sheep. *Brain Res* 2001; **918**: 40-50.

45. Bi S, Robinson BM, Moran TH. Acute food deprivation and chronic food restriction differentially affect hypothalamic NPY mRNA expression. *Am J Physiol-Regul Integr Comp Physiol* 2003; **285**: R1030-R1036.

46. Gutman R, Hacmon-Keren R, Choshniak I, Kronfeld-Schor N. Effect of food availability and leptin on the physiology and hypothalamic gene expression of the golden spiny mouse: a desert rodent that does not hoard food. *Am J Physiol-Regul Integr Comp Physiol* 2008; **295**: R2015-R2023.

47. Kinzig KP, Hargrave SL, Tao EE. Central and peripheral effects of chronic food restriction and weight restoration in the rat. *Am J Physiol Endocrinol Metabol* 2009; **296**: E282-E290.

48. Lal P, Sharp PJ, Dunn IC, Talbot RT. Absence of an effect of naloxone, an opioid antagonist, on luteinizing-hormone release in vivo and luteinizing-hormone- releasing hormone-I release in vitro in intact, castrated, and food restricted cockerels. *Gen Comp Endocrinol* 1990; **77**: 239-245.

49. Rozen S, Skaletsky HJ. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, eds. *Bioinformatics Methods and Protocols: Methods in Molecular Biology.* Totowa, NJ, : Humana Press 2000: 365-386.

50. McDerment NA, Wilson PW, Waddington D, Dunn IC, Hocking PM. Identification of novel candidate genes for follicle selection in the broiler breeder ovary. *BMC Genomics* 2012; **13**: 494.

51. Higgins SE, Ellestad LE, Trakooljul N, McCarthy F, Saliba J, Cogburn LA, Porter TE. Transcriptional and pathway analysis in the hypothalamus of newly hatched chicks during fasting and delayed feeding. *BMC Genomics* 2010; **11**.

52. Mercer JG, Moar KM, Logie TJ, Findlay PA, Adam CL, Morgan PJ. Seasonally inappropriate body weight induced by food restriction: Effect on hypothalamic gene expression in male Siberian hamsters. *Endocrinology* 2001; **142**: 4173-4181.

53. Bertile F, Oudart H, Criscuolo F, Le Maho Y, Raclot T. Hypothalamic gene expression in long-term fasted rats: relationship with body fat. *Biochem Biophys Res Commun* 2003; **303**: 1106-1113.

54. Sucajtys-Szulc E, Turyn J, Goyke E, Korczynska J, Stelmanska E, Slominska E, Smolenski RT, Rutkowski B, Swierczynski J. Differential effect of prolonged food restriction

and fasting on hypothalamic malonyl-CoA concentration and expression of orexigenic and anorexigenic neuropeptides genes in rats. *Neuropeptides* 2010; **44**: 17-23.

55. Mirabella N, Esposito V, Squillacioti C, De Luca A, Paino G. Expression of agouti-related protein (AgRP) in the hypothalamus and adrenal gland of the duck (Anas platyrhynchos). *Anat Embryol* 2004; **209**: 137-141.

56. Boswell T, Dunn IC, Corr SA. Hypothalamic neuropeptide Y mRNA is increased after feed restriction in growing broilers. *Poult Sci* 1999; **78**: 1203-1207.

57. Morrison CD, Xi X, White CL, Ye J, Martin RJ. Amino acids inhibit Agrp gene expression via an mTOR-dependent mechanism. *Am J Physiol Endocrinol Metabol* 2007; **293**: E165-E171.

58. Yang Y, Atasoy D, Su HH, Sternson SM. Hunger States Switch a Flip-Flop Memory Circuit via a Synaptic AMPK-Dependent Positive Feedback Loop. *Cell* 2011; **146**: 991-1002.

59. Watterson KR, Bestow D, Gallagher J, Hamilton DL, Ashford FB, Meakin PJ, Ashford ML. Anorexigenic and orexigenic hormone modulation of mammalian target of rapamycin complex 1 activity and the regulation of hypothalamic agouti-related protein mRNA expression. *Neurosignals* 2012; **28**: 28.

60. Proszkowiec-Weglarz M, Richards MP, Ramachandran R, McMurtry JP. Characterization of the AMP-activated protein kinase pathway in chickens. *Comp Biochem Physiol B: Biochem Mol Biol* 2006; **143**: 92-106.

61. Dridi S, Swennen Q, Decuypere E, Buyse J. Mode of leptin action in chicken hypothalamus. *Brain Res* 2005; **1047**: 214-223.

62. Kaiya H, Furuse M, Miyazato M, Kangawa K. Current knowledge of the roles of ghrelin in regulating food intake and energy balance in birds. *Gen Comp Endocrinol* 2009; **163**: 33-38.

63. Xu P, Siegel PB, Denbow DM. Genetic selection for body weight in chickens has altered responses of the brain's AMPK system to food intake regulation effect of ghrelin, but not obestatin. *Behav Brain Res* 2011; **221**: 216-226.

64. Honda K, Karnisoyama H, Saneyasu T, Sugahara K, Hasegawa S. Central administration of insulin suppresses food intake in chicks. *Neurosci Lett* 2007; **423**: 153-157.

65. Shiraishi J-I, Tanizawa H, Fujita M, Kawakami S-I, Bungo T. Localization of hypothalamic insulin receptor in neonatal chicks: Evidence for insulinergic system control of feeding behavior. *Neurosci Lett* 2011; **491**: 177-180.

66. Dunn IC, Meddle SL, Wilson PW, Wardle C, Law AS, Bishop V, Hindar C, Robertson GW, Burt DW, Ellison SJL, Morrice DM, Hocking PM. Decreased expression of the satiety signal receptor CCKAR is responsible for increased growth and body weight during the domestication of chickens. *Am J Physiol Endocrinol Metabol* 2013 **304** E909-E921.

67. Speakman JR, Levitsky DA, Allison DB, Bray MS, de Castro JM, Clegg DJ, Clapham JC, Dulloo AG, Gruer L, Haw S, Hebebrand J, Hetherington MM, Higgs S, Jebb SA, Loos RJF, Luckman S, Luke A, Mohammed-Ali V, O'Rahilly S, Pereira M, Perusse L, Robinson TN, Rolls B, Symonds ME, Westerterp-Plantenga MS. Set points, settling points and some alternative models: theoretical options to understand how genes and environments combine to regulate body adiposity. *Dis Models Mech* 2011; **4**: 733-745.

68. Decuypere E, Bruggeman V, Everaert N, Li Y, Boonen R, De Tavernier J, Janssens S, Buys N. The Broiler Breeder Paradox: ethical, genetic and physiological perspectives, and suggestions for solutions. *Br Poult Sci* 2010; **51**: 569-579.

69. D'Eath RB, Tolkamp BJ, Kyriazakis I, Lawrence AB. 'Freedom from hunger' and preventing obesity: the animal welfare implications of reducing food quantity or quality. *Anim Behav* 2009; **77**: 275-288.

70. Sandilands V, Tolkamp BJ, Kyriazakis I. Behaviour of food restricted broilers during rearing and lay - effects of an alternative feeding method. *Physiol Behav* 2005; **85**: 115-123.

71. Savory CJ, Lariviere JM. Effects of qualitative and quantitative food restriction treatments on feeding motivational state and general activity level of growing broiler breeders. *Appl Anim Behav Sci* 2000; **69**: 135-147.

72. Nielsen BL, Thodberg K, Malmkvist J, Steenfeldt S. Proportion of insoluble fibre in the diet affects behaviour and hunger in broiler breeders growing at similar rates. *Animal* 2011; **5**: 1247-1258.

73. Dawkins MS. The science of animal suffering. *Ethology* 2008; **114**: 937-945.

Legends

Fig. 1 *Comparison of a) body weight, b) food intake, and basal hypothalamic expression of c) AGRP and d) POMC mRNA in food restricted and ad libitum-fed broiler breeder hens at 12 weeks of age (n=12). Bars represent standard error of the mean. Statistical differences between the treatments are represented where appropriate on the graphs. AGRP and POMC gene expression are expressed as a ratio of expression of LBR in the same samples.*

Fig. 2 *Body weight trajectories of hens fed ad libitum (AL ●); maintained on a commercial quantitative restriction programme (R ○); maintained on a commercial quantitative restriction programme before being fed ad libitum for two days prior to death (R+2d ▼); restricted to a body weight intermediate between R and AL birds by being fed ad libitum 2 weeks prior to death (R+14d Δ); or maintained on two times the commercial quantitative restriction programme to achieve a body weight intermediate between the AL and R group (2R ■). All treatments were applied from 6 weeks of age; prior to that all hens were maintained on a restricted diet. Note that the difference between body weight at R and R+2d is due to the weight of food in the crop.*

Fig. 3 *Expression in the basal hypothalamus of hens subject to the treatments in Fig. 2 of* ***A)*** *AGRP. Individual comparisons were all significant at P<0.001 except R vs. R+2d and R+2d vs. 2R which are significant at P<0.01.* ***B)*** *NPY . Individual comparisons were all significant at P<0.01 except AL vs. R+14d which was significant at P<0.05. The difference between 2R or R+2d and R+14d was not significant.* ***C)*** *POMC. ANOVA showed no significant effect of treatment. D) CART . ANOVA showed no significant effect of treatment. The dark-filled columns indicate groups fed ad libitum at the time of sampling and the grey columns the groups on different levels of restriction. The braces indicate comparisons where the hens had the same body weight but differed in their access to food at the time of sampling, i.e. ad libitum or restricted.*