Regulation of the avian central melanocortin system and the role of leptin

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Regulation of the avian central melanocortin system and the role of leptin

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Abstract

The avian central melanocortin system is well conserved between birds and mammals in terms of the component genes, the localisation of their expression in the hypothalamic arcuate nucleus, the effects on feeding behaviour of their encoded peptides and the sensitivity of agouti-related protein (AGRP) and pro-opiomelanocortin (POMC) gene expression to changes in energy status. Our recent research has demonstrated that AGRP gene expression precisely differentiates between broiler breeder hens with different histories of chronic food restriction and refeeding. We have also shown that the sensitivity of AGRP gene expression to loss of energy stores is maintained even when food intake has been voluntarily reduced in chickens during incubation and in response to a stressor. However, the similarity between birds and mammals does not appear to extend to the way AGRP and POMC gene expression are regulated. In particular, the preliminary evidence from the discovery of the first avian leptin genes suggests that leptin is more pleiotrophic in birds and may not even be involved in regulating energy balance. Similarly, ghrelin exerts inhibitory, rather than stimulatory, effects on food intake. The fact that the importance of these important long-term regulators of AGRP and POMC expression in mammals appears diminished in birds suggests that the balance of regulatory inputs in birds may have shifted to more short-term influences such as the tone of cholecystokinin (CCK) signalling. This is likely to be related to the different metabolic fuelling required to support flight.

Keywords: AGRP; POMC; AMPK; leptin; arcuate nucleus; chicken

1. Introduction

The avian melanocortin system genes were all cloned and characterised 14 years ago. They comprise five melanocortin receptors (Takeuchi et al., 1996; Takeuchi et al., 1998; Takeuchi and Takahashi, 1998; Takeuchi and Takahashi, 1999), and the pro-opiomelanocortin (POMC) and agouti-related protein (AGRP) genes encoding their endogenous agonists and antagonists, respectively (Berghman et al., 1998; Takeuchi et al., 1999; Takeuchi et al., 2000). The central melanocortin system shows evolutionarily functional and neuroanatomical conservation in comparison with mammals. Thus, melanocortin signalling is regulated by opposing, agonistic and antagonistic actions of endogenous α-melanocyte-stimulating hormone (α-MSH) and agouti-related protein (AGRP) that, respectively, increase and decrease food intake after central injection (Tachibana et al., 2001; Strader and Buntin, 2003; Strader et al., 2003). Also, the AGRP and POMC genes are expressed in the arcuate nucleus of the hypothalamus where AGRP is co-expressed with neuropeptide Y (NPY) in individual arcuate nucleus neurons (Boswell et al., 2002). In this mini-review we focus on the regulation of the AGRP and POMC genes in birds with emphasis on our recent work on chronic food restriction and natural models of voluntarily reduced food intake. We also consider the implications of the identification and preliminary characterisation in 2014 of the first avian leptin genes.

2. Effects of manipulating energy status

The functional evolutionary conservation of the avian central melanocortin system extends to its response to manipulation of energy status. Most investigations have focused on short-term food deprivation, which increases AGRP mRNA (Phillips-Singh et al.,

70 2003; Higgins et al., 2010; Song et al., 2012). Changes in mRNA appear to reflect
71 parallel changes in peptide because food deprivation increased the number of AGRP-
72 immunoreactive cells in the arcuate nucleus of the ring dove (*Streptopelia risoria*) which
73 were also elevated during the parental phase of the breeding cycle when the birds are in
74 negative energy balance (Strader and Buntin, 2003; Strader et al., 2003). For POMC,
75 Higgins et al. (2010) observed a significant decrease in mRNA after food deprivation and
76 two studies have reported a non-significant decrease (Phillips-Singh et al., 2003; Song et
77 al., 2012). Thus the changes in AGRP and POMC expression during fasting are in the
78 direction predicted from the behavioural effects of their encoded peptides as part of a
79 coordinated counter-regulatory response of hypothalamic appetite control peptides to
80 restore lost energy stores (Phillips-Singh et al., 2003). Higgins et al. (2010) used
81 microarray analysis to explore patterns of gene expression in the hypothalamus of
82 domestic chicks in relation to food deprivation. Pathway analysis software predicted that
83 POMC lies within a network of six interrelated differentially-expressed genes and
84 experimental evidence for the existence of the network was provided from *in vitro*
85 experiments. In addition to POMC, the network contained genes encoding the
86 neuropeptide relaxin-3, the neuropeptide receptors NPY Y5 and somatostatin receptor 5,
87 and the β2 adrenergic and metabotrophic glutamate receptor 8 neurotransmitter receptors
88 (Fig. 1). POMC was the only gene downregulated by fasting while the others in the
89 network were upregulated. This suggests a role for these pathways in the control of
90 appetite within the hypothalamus. There is also suggestive evidence for a role of
91 hypothalamic energy sensing pathways in regulating central melanocortin signalling.
92 Proszkowiec-Weglarz observed strong immunostaining for the energy sensor AMP-

activated protein kinase (AMPK) in the arcuate nucleus and Song et al. (2012) reported changes in AMPK activity indicated by phosphorylation during food deprivation and refeeding that paralleled changes in AGRP gene expression.

In comparison to food deprivation, the effects of chronic food restriction on the central melanocortin system have received less attention. We recently measured AGRP and POMC gene expression in broiler breeder hens subjected to different levels of chronic food restriction and refeeding (Dunn et al., 2013a). Food restriction for six weeks at a level routinely used in the poultry industry to improve reproductive performance strongly stimulated AGRP gene expression. We also made comparisons between hens of the same body mass that had experienced different feeding histories. Thus, we were able to distinguish in terms of significant changes in AGRP mRNA between birds killed at the same body mass that had been either been maintained on an intermediate level of food restriction or more severely restricted and then refed for two weeks. We also observed a difference between restricted birds compared to restricted hens that had been allowed to refeed for two days, with refeeding causing a pronounced decrease in AGRP mRNA. Overall we found that AGRP mRNA provided an integrated and sensitive measure of feeding history and has potential as an objective measure of hunger in animal welfare research. As has been observed in food deprivation studies, there appears to be a tendency for POMC gene expression to be decreased during food restriction. Hen et al. (2006) reported a significant decrease in POMC mRNA after seven days' food restriction in broiler and layer chickens Although we did not observe a significant effect of restriction on POMC expression in our study (Dunn et al. 2013a), we have subsequently observed a significant decrease in chronically restricted broiler chickens in a recent study

with greater sample sizes and statistical power (Dunn et al., unpublished).

3. Seasonal and stress-induced changes in body mass

Seasonal cycles of food intake and body mass have been well studied in birds, particularly in relation to migration, but little is known about the regulatory role of the central melanocortin system (Cornelius et al., 2013). Experiments in white-crowned sparrows (*Zonotrichia leucophrys gambelii*) have suggested that increases in body mass during the migratory period are associated with a regulated change in the level around which body mass is defended, a phenomenon known as a 'sliding set point' or rheostasis (King et al., 1963; Mrosovsky, 1990). One hypothesis is that seasonal changes in appetite and body weight are driven by an altered basal expression of AGRP and POMC in a direction reflecting the behavioural effects of the peptides. For example, to generate a seasonal body mass increase, the expression of AGRP and POMC would be respectively increased and decreased. However preliminary evidence from experiments in the European quail (*Coturnix coturnix*) suggested that AGRP and POMC gene expression were unchanged at a time when food intake and body mass were increasing during the migratory period (Cornelius et al., 2013). These findings are reminiscent of those from studies of seasonal mammals where the rheostatic mechanisms appear to lie in brain areas outside the arcuate nucleus (Ebling, 2014).

Another example of rheostasis that has been well defined in birds is the voluntary loss of appetite and body mass that occurs during incubation, one of several examples of naturally occurring 'animal anorexias' (Savory, 1979; Sherry et al., 1980; Mrosovsky and Sherry, 1980). We have recently examined the expression of AGRP and POMC during

139 incubation in the domestic chicken (Dunn et al., submitted) where hens show reduced
140 feeding behaviour for a three-week period, even though food is freely available. We
141 hypothesised that gene expression would be unchanged because the decreased body mass
142 represents a rheostatic change in the defended level. However, unexpectedly, AGRP was
143 increased in incubating birds compared to laying hens. To control for the possibly
144 confounding fact that the reproductive system is regressed in incubating birds, we
145 imposed food restriction on laying hens to induce a mass loss comparable to that in
146 incubating birds. AGRP expression was similarly high in the incubating and food
147 restricted groups and was significantly reduced in restricted birds allowed to refeed.
148 These findings suggest that AGRP expression remains sensitive to nutritional state during
149 incubation, which may be of adaptive significance in allowing a hen to terminate
150 incubation if her energy stores fall too low. However, the normally anabolic effects
151 associated with increased AGRP mRNA must be overridden by inhibitory inputs. POMC
152 is a candidate because its mRNA was increased in incubating birds on the boundary of
153 significance, although hypothalamic vasoactive intestinal polypeptide (VIP) may also be
154 involved.

155 To consider another example of voluntarily reduced food intake in birds, we
156 examined AGRP and POMC gene expression six days after transferring domestic
157 chickens from individual housing in cages to social housing in pens, a stressor that
158 induced a 70% reduction in food intake (Dunn et al., submitted). The results were
159 comparable to incubation, with AGRP mRNA being significantly elevated in transferred
160 birds together with significantly increased POMC mRNA. Again, the results suggested
161 that AGRP gene expression is sensitive to energy status and this may be important to

allow the birds to recover from the effects of the stressor. Similar to incubation, it is possible that increased POMC expression plays a role in stress-induced inhibition of food intake as has been suggested in some mammalian studies (Liu et al., 2007).

4. Regulation of AGRP and POMC gene expression by leptin

As noted in Section 3, a period of two days' refeeding following prolonged food restriction in broiler chickens results in a rapid decrease in AGRP gene expression. The endocrine signals underlying this response, together with those responsible for increased AGRP expression after energy deprivation or restriction, are currently uncertain. In mammals, leptin is well established as an important regulator of AGRP and POMC gene expression, upregulating POMC and downregulating AGRP (Friedman, 2009). However progress in understanding the role of leptin in regulating the central melanocortin system in birds has been slow because the existence of leptin genes in avian genomes has been controversial for twenty years. Early claims in the literature that bird leptin genes had been cloned were challenged and there was no evidence for their existence from sequenced avian genomes and other genomic resources (Friedman-Einat et al., 1999; Pitel et al., 2010). However, in 2013, a leptin-like sequence was released into public databases during annotation of the zebra finch (*Taeniopygia guttata*) genome that has led to leptin-like genes being identified in, currently, ten bird genomes (Prokop et a., 2014; Friedman-Einat et al., 2014; Huang et al., 2014; Friedman-Einat and Seroussi, 2014). These show a similar syntenic relationship with surrounding genes as other vertebrate leptins. The fact that avian leptin proved difficult to clone by traditional methods is explained by the approximately 30% amino acid identity to mouse and human leptin, together with a high

185 (up to 80%) GC content in the coding region. Recombinant peregrine falcon (*Falco*
186 *peregrinus*), zebra finch, and rock dove (*Columba livia*) leptins activate the leptin
187 receptor (LEPR) in avian LEPR reporter gene cell lines, and also the JAK-STAT pathway
188 through phosphorylation of STAT3 (Prokop et al., 2014; Huang et al., 2014; Friedman-
189 Einat et al., 2014). The tissue distribution of expression of the newly discovered leptin
190 genes has only been studied in two species to date. In the zebra finch real time PCR
191 showed expression of leptin primarily in the brain and the pituitary gland, of which the
192 latter was also the most abundant site of expression of the LEPR (Huang et al., 2014).
193 The pituitary was also the main site of expression of the rock dove LEPR while leptin
194 was largely expressed in the liver and gonads (Friedman-Einat et al., 2014). In both
195 species, leptin expression in adipose tissue was low or undetectable. Thus, although
196 limited, the expression data suggest that leptin cannot be regarded as being predominantly
197 an adipose tissue hormone in birds to the extent it is in mammals and that it shares a more
198 widespread distribution comparable to that observed in other non-mammalian vertebrates
199 (Crespi and Denver, 2006; Rønnestad et al., 2010). This led Millar (2014) in a
200 commentary on the discovery of avian leptin-like genes to suggest that they may
201 primarily be involved in pituitary-gonadal function rather than energy balance regulation,
202 although studies investigating avian leptin expression in response to nutritional
203 manipulation have yet to be performed. Previous investigations in birds had suggested
204 that putative leptin protein and LEPR binding activity are very low or undetectable in the
205 blood in several avian species (Richards et al., 2000; Adachi et al., 2008; Hen et al.,
206 2008; Yosefi et al., 2010). Also, although short isoforms of the avian LEPR have been
207 identified (Liu et al., 2007) the most abundant avian LEPR isoform is orthologous to the

mammalian Rb long isoform and none has so far been shown to be equivalent to the mammalian Ra short isoform thought to be involved in the transport of leptin into the brain. Collectively, these data may indicate a more paracrine role for leptin in birds, although rock dove serum was recently shown to activate a LEPR reporter bioassay (Friedman-Einat et al., 2014).

Much of the investigation into the role of leptin in regulating energy balance in birds has focused on the domestic chicken. However, the existence of functional leptin genes in chickens, Japanese quail and the mallard duck has yet to be proven (Friedman-Einat and Seroussi, 2014). Friedman-Einat and Seroussi (2014) suggest that this may be associated with changes in appetite regulation linked to domestication, and that it is in line with the observation that a pegylated leptin antagonist that inhibited activation of a LEPR bioassay reporter system by rock dove leptin was ineffective in altering food intake and body mass in chickens (Gertler et al., 2014). However, that conclusion is dependent on leptin having a physiological role in energy balance regulation in the chicken, which may not be the case. Also, if chicken leptin is absent from the genome, it might be expected that the LEPR would also be missing or degenerate as appears to be the case with kisspeptin in birds (Pasquier, 2014). It is therefore possible that leptin may yet be identified in chickens, and the recent discoveries in other species have provided a new impetus and scope for comparative analysis. It is certainly the case that mammalian leptin inhibits food intake in chickens and other birds after central or peripheral administration (Denbow et al., 2000; Cerasale et al., 2011). Similar inhibitory results have been obtained after administration of the ‘chicken leptin’ protein that shares 97% amino acid sequence identity to mouse leptin and is evidently artifactual (Raver et al., 1998; Löhmus et al.,

231 2003). Given the new information about the low sequence identity of avian leptins with
232 mammalian leptins, these results need to reevaluated using recombinant avian leptin
233 proteins. This applies to the only study in which the effects of mammalian leptin
234 ('chicken leptin') were investigated in relation to AGRP and POMC expression (Dridi et
235 al., 2005). Here, leptin was administered peripherally to young broiler chickens for six
236 hours after which hypothalamic AGRP and POMC gene expression were measured. No
237 change in expression was detected for either gene, in contrast to what would be expected
238 from mammalian studies. If leptin does regulate AGRP and POMC expression in birds, it
239 would be expected that the LEPR is co-expressed with those genes in arcuate nucleus
240 neurons. However, in our experience, the chicken LEPR long isoform is difficult to detect
241 by *in situ* hybridisation because although detectable by PCR it is not highly expressed in
242 the hypothalamus. Although we were able to increase the sensitivity of radioactive
243 oligonucleotide *in situ* hybridisation sufficiently to detect expression of low-abundance
244 prolactin receptor in the chicken hypothalamus, we were unable to detect expression of
245 the LEPR at the same time using the same methodology (T. Boswell, unpublished).
246
247 **5. Regulation of AGRP and POMC gene expression by other neuroendocrine signals**
248 In the absence of information regarding leptin's possible role in regulating AGRP
249 and POMC gene expression, a small number of investigations have focused on other
250 candidate signals. Several studies have been performed in the domestic chick in relation
251 to insulin, the circulating concentrations of which decrease with fasting in birds as well as
252 in mammals (Christensen et al., 2013). Insulin was established in mammals as a regulator
253 of arcuate nucleus appetite peptide gene expression before the discovery of leptin. It

254 stimulates POMC gene expression and inhibits that of NPY and AGRP, corresponding to
255 an inhibitory effect of centrally-administered insulin on food intake (Porte et al., 2002).
256 Insulin also inhibited food intake after central injections in domestic chicks, an effect
257 blocked by melanocortin receptor antagonists (Honda et al., 2007; Shiraishi et al., 2008).
258 Chick NPY neurons were demonstrated to co-express the insulin receptor and it is likely,
259 but was not determined, that those same individual neurons expressed AGRP given the
260 co-expression of NPY and AGRP previously observed in birds (Boswell et al., 2002;
261 Shiraishi et al., 2011). Co-expression of the insulin receptor with α-MSH was also
262 observed in the arcuate nucleus (Shiraishi et al., 2011). Two independent studies in
263 chicks reported increased gene expression of POMC in the chick brain after central
264 insulin injection but no effect on AGRP mRNA in contrast to an inhibitory effect on NPY
265 mRNA (Honda et al., 2007; Shiraishi et al., 2008). The apparent absence of an inhibitory
266 effect of insulin on AGRP gene expression may point to a regulatory difference between
267 birds and mammals although observations need to be extended to adult chickens and to
268 other species.

269 With regard to other neuroendocrine signals, Byerly et al. (2009) combined *in vivo*
270 and *in vitro* studies on chicken hypothalami from fat and lean chicken lines to investigate
271 the possible regulation of appetite peptide gene expression by brain-derived neurotrophic
272 factor (BDNF), triiodothyronine (T3) and corticosterone. BDNF did not affect AGRP and
273 POMC gene expression but T3 was found both *in vitro* and *in vivo* to upregulate AGRP
274 mRNA and downregulate POMC mRNA. A specific effect of T3 on AGRP gene
275 expression would not be predicted from the fact that circulating T3 concentrations are
276 decreased by fasting in chickens (Harvey and Klandorf, 1983) at the time AGRP mRNA

277 is increased. However it is possible that T3 concentrations within the hypothalamus may
278 differ from those in the blood as a result of local conversion of thyroxine. Fasting
279 increases plasma corticosterone concentrations in birds as in mammals (Harvey and
280 Klandorf, 1983) and it would therefore be predicted that corticosterone would increase
281 AGRP expression and decrease POMC expression in line with mammalian studies
282 (Makimura et al., 2003). However Byerly et al. (2009) reported that while corticosterone
283 administration *in vitro* and *in vivo* decreased POMC mRNA, it did not affect AGRP gene
284 expression. Liu et al. (2012) replicated the suppressive effect of corticosterone on POMC
285 expression after peripheral administration in laying hens, but also reported decreased
286 AGRP mRNA. However, central injection of the glucocorticoid receptor agonist
287 dexamethasone in broiler chicks produced no change in AGRP or POMC gene expression
288 (Liu et al., 2014). The lack of consensus may be because adrenalectomy and
289 glucocorticoid replacement together with consideration of corticosterone and insulin
290 interactions are needed to demonstrate a stimulatory response for AGRP mRNA
291 unambiguously (Makimura et al., 2003). Such studies have not been performed in birds.
292 An additional candidate neuroendocrine feedback signal to the arcuate nucleus that
293 we have investigated recently is tonic signalling by cholecystokinin (CCK). Investigation
294 into the genetic basis of growth rate between lines of fast- and slow-growing chickens
295 revealed the importance of an apparently cis-acting regulatory region downstream of the
296 CCKAR (CCK1 receptor) that explained 19% of the difference in body mass between the
297 lines. Birds with the high-growth haplotype at that locus showed lower expression of
298 CCKAR throughout the body together with decreased sensitivity to the inhibitory effects
299 of exogenous CCK administration (Dunn et al., 2013b). Thus high growth rate was

associated with a lower overall tone of CCK signalling. When we investigated AGRP and POMC gene expression, we found that AGRP mRNA was significantly higher in the high-growth haplotype compared to heterozygotes and the low-growth haplotype but there was no difference in POMC mRNA. These data suggest that AGRP gene expression is sensitive to the tone of CCK signalling. The effect of CCK on the central melanocortin system has not been widely investigated but sensitivity of the feeding response to CCK injections was reduced in MC4R-knockout mice and MC4R neurons were observed to project to hindbrain regions influenced by CCK signalling (Blevins et al., 2009). It is, of course, possible that the increased AGRP gene expression we observed in the high-growth birds was a secondary consequence of altered CCKAR expression, and we are carrying out further studies to test for a direct regulatory influence of CCK.

6. Future perspectives

It is now well established that the avian central melanocortin system shows conservation with its mammalian counterpart in terms of the genes involved and the neuroanatomical localisation of the neurons expressing the genes within the arcuate nucleus. There is functional conservation with respect to the behavioural effects of the peptides and the response of gene expression to changes in energy status. The precision with which we were able to distinguish between hens with different histories of chronic food restriction and refeeding is remarkable (Dunn et al., 2013a) and from our work that we report in Section 3, sensitivity of AGRP expression to energy status appears to be maintained even in situations where food intake is voluntarily decreased, indicating the functional importance of this mechanism. However a similar degree of conservation does

not appear to extend to the way the AGRP and POMC genes are regulated. There are striking differences in the regulatory effects of the two hormones principally associated with the long-term regulation of energy balance in mammals, leptin and ghrelin. As we review in Section 4, a 20-year search for leptin in birds has finally resulted in the identification of leptin-like genes in several avian orders. However, the encoded proteins show low sequence identity to mammalian leptins and the preliminary evidence from the tissue distribution of gene expression suggests that leptin is more pleiotrophic in birds and may not be primarily involved in regulating energy balance. Ghrelin research in birds has been more straightforward in that there has been no lengthy controversy about its existence. However the behavioural effect of ghrelin on feeding is fundamentally different to the situation in mammals in that there is consistent evidence that ghrelin acts in the brain to inhibit food intake rather than stimulate it (Kaiya et al., 2013). The inhibitory effect appears to be mediated by hypothalamic corticotrophin-releasing factor (Saito et al., 2005) rather than through the central melanocortin system. Two other hormones that influence long-term regulation of arcuate nucleus peptide signalling in mammals, insulin and corticosterone, share more similarity with regulatory effects observed in mammals, but with some differences (Fig. 1). Overall, the influence of the neuroendocrine signals involved in long-term energy balance regulation appear diminished in birds compared to mammals, perhaps indicating that regulation of AGRP and POMC synthesis and activity is under more short-term control. Our preliminary observations suggesting a regulatory influence of CCK support this idea. It is also possible that direct energy sensing within the hypothalamus plays a relatively greater role in regulating the arcuate nucleus neural circuitry in birds. The application of genomic

346 methods has provided a foundation for understanding how changes in the hypothalamic
347 environment may influence central melanocortin signalling (Fig. 1). It should not be
348 surprising that the regulation of energy balance is likely to differ between vertebrate taxa.
349 Birds in particular face very different metabolic challenges from most mammals and from
350 ectothermic vertebrates imposed by the requirements of the intense aerobic activity of
351 flight. Fundamental differences in metabolism such as the relative circulating
352 concentrations of insulin and glucagon, present even in domesticated birds like the
353 chicken, are likely to reflect this. It would be beneficial for future investigations to draw
354 more on the established metabolic differences between birds and mammals, particularly
355 on the relatively greater reliance in birds on lipid metabolism as a source of metabolic
356 fuels. We hope that the impetus to the field provided by the identification of avian leptins
357 will encourage a new approach.
358
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Figure legend

Figure 1. Summary of current knowledge of regulatory inputs (positive and negative influences are indicated) on agouti-related protein (AGRP) and pro-opiomelanocortin (POMC) gene expression in the arcuate nucleus of the avian hypothalamus. Signalling within the hypothalamus is shown in the box. Abbreviations: ADRB2 - β2 adrenergic receptor; AMPK – AMP-activated protein kinase; GRM8 – metabotrophic glutamate receptor 8; NPY5R – neuropeptide Y receptor Y5; RLN3 – relaxin-3; SSTR5 – somatostatin receptor 5.