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Review

Melanocortins and body weight regulation: Glucocorticoids, Agouti-related protein and beyond

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ABSTRACT

In the intervening three decades since Panksepp observed for the first time that centrally administered α-melanocyte stimulating hormone decreased food intake (Panksepp and Meeker, 1976), a wealth of data have accrued to firmly establish melanocortin signaling as a central regulator of food intake and fat mass. Advances in molecular biology have not only allowed detailed studies of spontaneously occurring obese mice with altered melanocortin signaling to be undertaken but also permitted the generation of a plethora of mouse models with precise perturbations at critical steps in the melanocortin system to finesse further the cellular and molecular architecture of relevant pathways. In this article we focus in upon a number of these mouse models which continue to help us tease apart the complexities of this critical system. Further, we review data on the important interaction between pro-opiomelanocortin derived peptides and the adrenal system and the relationship between agonist and antagonist peptides acting at central melanocortin receptors.

Contents

1. Introduction

1.1. Pro-opiomelanocortin (POMC)

Pro-opiomelanocortin (POMC) is the archetypal polypeptide precursor. *In toto* it is functionally inert but requires extensive, tissue-specific post-translation modification to generate a range of smaller, biologically active peptides. These include adrenocorticotrophic hormone (ACTH) and α-, β- and γ-melanocyte stimulating hormone (MSH), collectively known as the melanocortins. The action

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of the melanocortin peptides is mediated by a family of five G protein-coupled seven transmembrane domain receptors, known as melanocortin receptors type 1 to type 5 (melanocortin MC_1 receptor to melanocortin MC_5 receptor). Melanocortin MC_1 receptor is expressed on melanocytes, melanocortin MC_2 receptor is expressed in the adrenal cortex and is the target for pituitary-derived ACTH whilst melanocortin MC_5 receptor is found in sebaceous glands. However, the melanocortin MC_3 and MC_4 receptors are both highly expressed in regions of the brain known to be involved in the control of food intake and energy balance (Cone, 1999).

1.2. The central melanocortin system

The hypothalamus acts as a primary sensor of alterations in energy stores by receiving and integrating neural, metabolic and humoral signals from the periphery. Within the arcuate nucleus of the hypothalamus are two separate populations of neurons which express either POMC or both neuropeptide Y and Agouti-related protein (AgRP) (Schwartz et al., 2000). A unique feature of the central melanocortin system is that in addition to the range of POMC-derived ligands, there exists a second peptide produced in the hypothalamus that has biological activity at melanocortin receptors. Thus, whereas POMC-derived melanocortins α- and β-MSH are agonists, AgRP is a potent melanocortin antagonist at the melanocortin MC_3 and MC_4 receptors (see below for further exploration of the role of AgRP). From the arcuate nucleus, these two populations of neurons project to other brain areas that are involved in energy homeostasis. These include other hypothalamic regions such as the paraventricular nucleus, lateral hypothalamus and the dorsomedial nucleus. In addition to the hypothalamus, POMC neurons are also found in the brainstem, in particular within the nucleus of the solitary tract. The nucleus of the solitary tract is the primary site for innervation by vagal afferents from the gut (Schwartz, 2000) and it may be that the melanocortin system within the nucleus of the solitary tract may also be important in integrating short-term gut-derived satiety signals (Fan et al., 2004). Indeed, there do appear to be some intriguing and important differences between POMC neurons in the nucleus of the solitary tract and the arcuate nucleus. A study from Bjorbaek's group demonstrated that although fasting induced a fall in POMC mRNA in both regions, in contrast to the arcuate nucleus, this reduction was not reversed by leptin administration (Huo et al., 2006). Furthermore, again in sharp contrast to the arcuate nucleus, leptin did not cause STAT-3 phosphorylation or c-fos activation within nucleus of the solitary tract POMC neurons, suggesting therefore that leptin signaling via POMC-derived peptides in the central nervous system (CNS) occurs entirely via hypothalamic POMC neurons.

2. POMC deficiency

The first report of the phenotype of two children congenitally lacking POMC gene products appeared in 1998. As a result of ACTH deficiency, both subjects presented in early childhood with the metabolic consequences of hypocortisolemia and went on to develop severe, early-onset obesity associated with hyperphagia, due to reduced hypothalamic melanocortinergic signaling. Both probands also had pale skin and red hair because of reduced signaling through melanocortin MC_1 receptor on melanocytes in skin and hair follicles. Three additional children with an identical phenotype have subsequently been reported by the same group. More recently, however, colleagues in Cambridge have reported a child of Turkish origin with severe obesity and hypoadrenalism due to POMC deficiency who does not have red hair (Farooqi et al., 2006). The retention of dark, eumelanin-rich hair in this child indicates that eumelanin synthesis in humans has no absolute requirement for melanocortin peptides and indicates that, just as in mice, additional genetic factors are likely to contribute to how the index monogenic defect is reflected in the final phenotype (Smart and Low, 2003).

This aside, the first report of a mouse model with disruption of both alleles of the POMC gene (Yaswen et al., 1999) essentially recapitulated the phenotype seen in humans, indicating that melanocortin pathways in humans and rodents subserve very similar physiological functions. We have also extensively studied a second, independent line of mice lacking all POMC-derived peptides that are also markedly obese, hyperphagic and have both altered pigmentation and adrenal insufficiency (Fig. 1). This increase in weight is as a result of an increase in both fat and lean mass. Additionally, Pomc null ($Pomc^{-/-}$) mice have a lower basal metabolic rate than their wild type litter mates, which may be due in part to reduced activity of the hypothalamic-pituitary-thyroid axis. More recently, Xu et al. have generated a further corticosterone-deficient mouse model of POMC deficiency by deletion of the critical mitochondrial transcription factor, Tfam, from POMC expressing cells (Xu et al., 2005). These mice exhibited a progressive adult-onset obesity with an increase in both fat and lean mass.

3. POMC and dietary fat

We have used *Pomc* null mice to determine how *Pomc* haploinsufficiency might interact with changes in dietary composition. On standard chow, only homozygous mutant mice became obese, with mice heterozygous for the mutant allele ($Pomc^{+/-}$) achieving an adult weight similar to that of wild type mice. However, with high fat feeding (45% fat), $Pomc^{+/-}$ mice also became obese. At 6 months of age they weighed 20% more than wild type littermates, who failed to develop obesity on high fat diet. The development of obesity in heterozygous mice was the result of increased energy intake, with $Pomc^{-/-}$ and $Pomc^{+/-}$ eating 40% and 18% more than wild type mice, respectively. Thus under certain environmental conditions, a single functional copy of the Pomc gene is not sufficient to maintain normal energy homeostasis. Additional evidence for this hypothesis comes from the findings that none of the 10 adults heterozygous for *POMC* mutations reported by Krude et al. had a low-normal BMI value, with body weight in heterozygous mutation carriers shifted to the high-normal +1 BMI-SDS or even overweight range of +2 BMI-SDS (Krude et al., 2003). The availability of a large extended pedigree related to the Turkish *POMC* null proband highlighted above provided the opportunity to address whether loss of one copy of the POMC gene was sufficient to alter obesity risk. Twelve relatives were heterozygous for the mutation and 7 were wild type. Of the 12 heterozygotes, 11 were obese or overweight compared with only 1 of the 7 wild type relatives. The mean BMI SD score was 1.7 ± 0.5 in heterozygotes and 0.4 ± 0.4 in the wild type relatives (Farooqi et al., 2006). Thus *POMC* haploinsufficiency may indeed shift the individual body weight to higher normal or mildly obese level.

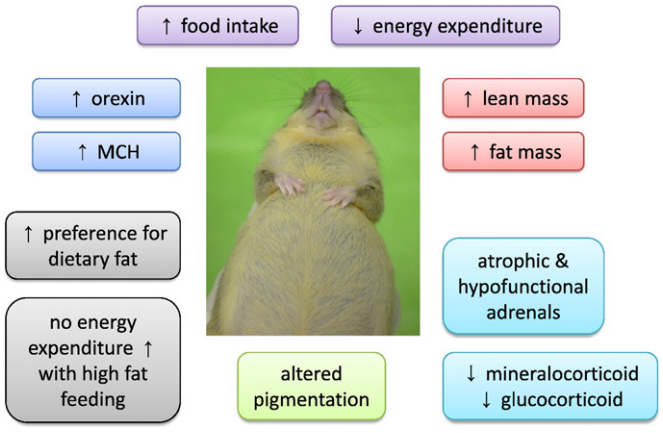

Fig. 1. Consequences of POMC deficiency.

There are also data suggesting that perturbations in melanocortin signaling can impact upon feeding behavior by modulating macronutrient choice, with pharmacological antagonism increasing fat consumption (Hagan et al., 2001; Koegler et al., 1999; Samama et al., 2003). We have recently undertaken analysis of the macronutrient preference of POMC insufficient mice. When mice had ad libitum access to a three-choice buffet of food pellets containing 10, 45 or 60% fat, both *Pomc*$^{+/-}$ and *Pomc* null mice preferentially consumed diet with a higher fat content (Tung et al., 2007). In a further buffet test using three foodstuffs of near pure macronutrient content (fat, protein or carbohydrate) there was a clear gene dosage effect on fat consumption with homozygous null and heterozygous mice eating 45% and 98% more fat than wild type mice, respectively.

Pomc null mice also showed a markedly different response in energy expenditure when challenged with a high fat diet. On a 60% fat diet, wild type mice increased their ambulatory activity by an additional 50% compared to their activity levels seen on standard chow. In contrast the activity levels of Pomc null mice remained entirely unchanged from those seen on standard chow. Thus POMC-derived peptides appear to have influences on multiple aspects of the organism's response to the external diet, including a major influence on the dietary preference for fat. The cumulative impact of these perturbations results in substantial weight gain when a high fat diet is available, highlighting an important gene–environment interaction and further supporting the hypothesis that genetic variation around the POMC locus confers a risk for developing obesity (Comuzzie et al., 1997).

4. Melanocortin hierarchy

The melanocortin peptides share a core peptide motif—Histidine–Phenylalanine–Arginine–Tryptophan—which is critical for interaction at melanocortin receptors. To begin to determine whether melanocortins have unique, overlapping or redundant roles in the control of food intake we compared the effects of melanocortins given intracerebroventricularly (i.c.v.) to *Pomc* null mice (Tung et al., 2006). Of the peptides administered, α-MSH brought about the biggest reduction in food intake. Furthermore, this melanocortin was the only one able to reduce the excess fat and lean mass found in *Pomc*$^{-/-}$ mice. On the basis of pair-feeding experiments, these effects of α-MSH were mediated primarily through a reduction in food intake.

However, one problem with mice is that rodents lack the N-terminal cleavage site necessary for the generation of β-MSH. As this melanocortin is not an endogenous ligand, murine studies are likely to be biased to favoring α-MSH above β-MSH in the melanocortin hierarchy. Indeed, two recent human genetic studies have indicated that β-MSH is likely to play a critical role in the hypothalamic control of body weight in humans. Lee et al. (2006) screened the POMC gene in 538 patients with severe, early-onset obesity and identified five unrelated probands who were heterozygous for a rare missense variant in the region encoding β-MSH, whereby a tyrosine at position 221 is changed to cysteine. The variant co-segregated with obesity in affected family members. Of note, it was also found in 1/300 non-obese UK Caucasian adults and also found in 3 of 4852 subjects from an unselected UK Caucasian population. However, this was still at significantly lower frequency than in the cohort with early-onset obesity, and in vitro studies of the mutant β-MSH demonstrated that it had impaired ability to bind to and activate signaling at the melanocortin MC_4 receptor. Biebermann et al. also reported the same missense mutation within POMC (Biebermann et al., 2006). The Y5C-β-MSH mutation was also found in obese family members of the index child but because other family members who did not carry the mutation were overweight, it is likely the mutation was not the only cause for obesity in the studied family. They also used immunohistochemistry to demonstrate that β-MSH was expressed in POMC neurons in the human arcuate nucleus, adding a detailed distribution pattern of β-MSH in human hypothalamus that had not been previously reported.

5. Adrenal phenotype

Pomc null mice are rare among animal models of obesity in that increased fat mass and hyperphagia develop despite circulating glucocorticoid being below the detection level of standard assays. The lack of glucocorticoid is no surprise given that POMC-derived ACTH is the primary ligand required for melanocortin MC_2 receptor activation within the adrenal cortex. Perhaps more surprising was the initial phenotypic analysis of *Pomc*$^{-/-}$ mice generated by Yaswen et al. They reported that 6 month old *Pomc*$^{-/-}$ mice not only lacked macroscopically visible adrenal glands but also that the rudimentary tissue seen microscopically had no clearly discernible cortical and medullary zones (Yaswen et al., 1999). Further, neither circulating corticosterone nor aldosterone was detectable in these mice. Given that the major regulators of aldosterone production from the mature adrenal are not POMC-derived, the failure to detect aldosterone was taken by these authors as support for the concept that mice congenitally deficient in POMC fail to develop adrenal glands.

However, since this initial report Hochgeschwender and colleagues have published data re-examining the adrenal phenotype of their *Pomc* null mouse (Karpac et al., 2005). They report that *Pomc* null mice are indeed born with adrenal glands and, in accord with our data, have demonstrated that mice with POMC deficiency do have adrenal glands, albeit markedly atrophic with disrupted cortical architecture (Challis et al., 2004; Coll et al., 2004). Data from both models now also agree that *Pomc* null mice have detectable but reduced levels of circulating aldosterone. This is in accordance with data from humans affected by congenital POMC deficiency who are hypocortisolaemic but have normal aldosterone levels (Krude and Gruters, 2000), indicative of a functioning zona glomerulosa. Further, post-mortem studies in such subjects have revealed structurally intact zona glomerulosa and adrenal medulla but an absence of zona fasciculata and reticularis (Krude and Gruters, 2000). Thus, the presence of discernible adrenal tissue with a disordered cortex but a clear cortical/medullary demarcation and detectable mineralocorticoid suggest that the structural and functional sequelae of congenital POMC deficiency seen in the knockout model resemble closely those seen in humans. Furthermore, data from two independent models are in agreement that is it possible to develop an adrenal gland in mice lacking all endogenously derived POMC peptides indicating there is unlikely to be a developmental period during which the creation of a functional adrenal cortex is dependent on exposure to POMC-derived peptides.

5.1. ACTH and adrenal function

Of the POMC-derived peptides, ACTH is the classic adrenocorticotropic hormone and is the most important pituitary derived peptide controlling steroidogenesis in the adult adrenal. The crucial role of ACTH is highlighted by the findings in humans affected by familial glucocorticoid deficiency (Clark and Weber, 1998) in whom there is a loss of function mutation in the melanocortin MC_2 receptor. This mutation in the endogenous ACTH receptor results in adrenal unresponsiveness to ACTH and severe glucocorticoid deficiency (Clark and Weber, 1998). Affected adrenal glands are atrophic and have a disordered zona glomerulosa with no evidence of fasciculata or reticularis cells within the adrenal cortex. Furthermore, the *Mc2r* null mouse has close phenotypic similarities, with disrupted architecture of the adrenal cortex and undetectable plasma corticosterone (Chida et al., 2007).

Two other groups have looked specifically at the response of *Pomc*$^{-/-}$ to peripherally administered ACTH. Smart and Low back-crossed the Pomc mutant allele from the 129/SvEv strain used by Yaswen et al. on to a C57BL/6 background (Smart and Low, 2003). These mice had undetectable corticosterone but did have identifiable, severely hypoplastic adrenals. When these mice were treated with 1 μg of $ACTH_{1-24}$ twice daily by intraperitoneal injection for two weeks there was no increase in

corticosterone production. This result led these authors to conclude that a functionally competent adrenal cortex may require exposure to POMC-derived peptides other than ACTH. Similarly on the basis of a failure of *Pomc*$^{-/-}$ mice to produce corticosterone 1 h after a single 1 μg dose of ACTH, Karpac et al. concluded that the presence of other POMC peptides would be required to permit ACTH to elicit corticosterone production in these animals (Karpac et al., 2005). In contrast, we have demonstrated that administration of a highly selective ACTH analogue (Depot Synacthen) to *Pomc*$^{-/-}$ mice for 10 days transformed previously dysmorphic, hypofunctional adrenals into glands that were not only indistinguishable from wild type in terms of size and morphology and but which also were now able to synthesize glucocorticoid (Coll et al., 2004). The differences are likely, in part, to be as a consequence of the ACTH being administered at a higher dose and in the form of a subcutaneous depot preparation. In particular, it is plausible that in the setting of complete and chronic loss of corticotroph function, a short stimulation test (as used by Kapac et al.) is insufficient to assess the true synthetic potential of the adrenal. For example, in patients with congenital corticotroph deficiency due to mutations in the TPIT gene, cortisol levels do not increase with acute ACTH but can respond to prolonged ACTH administration (Vallette-Kasic et al., 2004).

Histological and immunohistochemical analysis show this response to ACTH as being primarily hypertrophy rather than hyperplasia, indicative of a role for ACTH in adrenocortical differentiation of cells already present in the *Pomc* null adrenal. Of note, Karpac et al. have recently looked at the growth potential of Pomc null adrenals using cross-transplantation experiments (Karpac et al., 2005). At postnatal day 9, *Pomc*$^{-/-}$ adrenals were implanted into adrenalectomized wild type littermates. By two months the transplanted mutant adrenals were producing expected levels of both corticosterone and aldosterone in the wild type recipients. At three months, the mice were sacrificed and the adrenal glands removed. The transplanted *Pomc* null adrenals had considerably enlarged and developed a typical cortical architecture although no other measure of cell proliferation was made. Of course this procedure results in prolonged exposure to all circulating POMC-derived peptides, including ACTH, and from these data alone it is impossible to discern which POMC product is wholly necessary or sufficient for proliferation and maintenance of adrenal structure.

One other result worthy of further comment is the fact that ACTH administration did not correct the deficiency in mineralocorticoid production and indeed dramatically reduced aldosterone levels in wild type mice. Previous reports using rats have also described that treatment with high doses of ACTH result in a significant suppression of aldosterone and a markedly different response within cortical regions with zona glomerulosa cell undergoing transformation into a zona fasciculata-like form (Abayasekara et al., 1989; Muller, 1978; Rebuffat et al., 1991)

5.2. Non-ACTH peptides and adrenal function

A long-standing area of controversy within adrenal biology is whether POMC-derived peptides other than ACTH are involved in the control of adrenal growth and development (Estivariz et al., 1982, 1988a,b; Fassnacht et al., 2003a; Lowry et al., 1983). In particular, a sizeable body of evidence proposes that the highly conserved 16 kDa amino terminal region sequence of POMC contains a smaller fragment with adrenal mitogenic activity.

Both γ-MSH and 1–52 POMC have been purported to fulfill such a role. Although γ-MSH is not recognized as a circulating melanocortin peptide and cannot stimulate steroidogenesis directly, it has been reported to be able to augment ACTH induced glucocorticoid synthesis by activating adrenal hormone sensitive lipase. In doing so, stored cholesterol esters are hydrolysed into free cholesterol thereby increasing the flux of cholesterol though the steroid synthetic pathways. However, three different species of γ-MSH are described and it remains to be resolved which exact molecular form activates hormone sensitive lipase in vivo. The putative receptor through which γ-MSH species may exert this action also remains unresolved.

The idea that 1–52 POMC might be an adrenal mitogen came from work carried out by Bicknell et al. in 2001. This paper outlined an important new mechanistic aspect of N-POMC related function by identifying a protease in rats that cleaves circulating 1–76 POMC to smaller peptides at the level of the adrenal (Bicknell et al., 2001). This novel adrenal secretory protease thus described was upregulated during compensatory adrenal growth following unilateral adrenalectomy, appeared to only be expressed in the adrenal cortex and was able to confer responsiveness to 1–76 POMC when expressed in a murine adrenocortical cell line which remained otherwise unresponsive to this peptide if lacking Adrenal secretory protease. By bringing about cleavage between valine and methione at positions 52/53 (two residues away from a dibasic cleavage site), the action of adrenal secretory protease would then reveal the biologically active fragment 1–52 POMC. A single transmembrane domain 80 kDa receptor has been reported in abstract form to be the putative receptor for 1–52 POMC but this remains to be fully characterized (Bicknell, 2002).

These findings fueled the hypothesis that by regulation of adrenal secretory protease expression adrenal glands had the ability to control its tonic state independently of the steroidogenic stimulus elicited by ACTH. However, more recent data has emerged arguing against adrenal secretory protease having either a physiological or pathological role in controlling adrenocortical growth.

Although upregulation of adrenal secretory protease expression in the remaining adrenal gland upon unilateral adrenalectomy has been demonstrated also in the mouse (Beuschlein et al., 2002) there are marked differences in levels of adrenal expression between rats and mice with high levels of expression in many extra-adrenal sites (Hansen et al., 2004). Indeed, it is now clear that adrenal secretory protease is a secretory isoform of the transmembrane airway trypsin-like protease (Hansen et al., 2004). Furthermore, in humans the short isoform of the airway trypsin-like protease that would correspond to adrenal secretory protease does not exist and the long isoform is expressed only with very low abundance in both normal adrenal tissue and adrenocortical tumors in humans (Hahner et al., 2005).

The highly conserved amino terminal sequence of POMC, 1–28 POMC, has also been mooted to have a role in adrenal biology. Originally isolated from a large scale purification of human pituitary glands and considered to be an extraction artifact rather than a physiologically formed product (McLean et al., 1981), Estivariz et al. nevertheless demonstrated that purified 1–28 POMC could elicit a dose-dependent increase in incorporation of thymidine into DNA of dispersed rat adrenal cells (Estivariz et al., 1982). Further, they reported that continuous administration of purified 1–28 POMC to female rats by subcutaneous pump over seven days (3 μg per day) resulted in a significant increase in adrenal weight and mitotic index. To determine whether these effects may have been in part due to untoward contamination with other POMC-derived peptides, the same group also looked at adrenal response to synthetic 1–28 POMC. Estivariz et al. delivered 8 μg of either purified or synthetic 1–28 POMC to intact female rats using osmotic minipumps (Estivariz et al., 1988b). Both forms of peptide significantly increased mitotic activity in the adrenals of treated animals compared with saline treated controls, although neither produced a change in plasma corticosterone. Furthermore, in vitro both purified and synthetic 1–28 POMC significantly stimulated [^{3}H] thymidine incorporation into DNA of adrenal cells in a dose dependent manner, although the synthetic peptide was somewhat less potent than the purified peptide in this assay. Lowry and colleagues demonstrated that 3 μg of 1–28 POMC caused a significant increase in adrenal weight and mitotic index when given to wild type rats (Estivariz et al., 1982). Estivariz et al. studied the effects of POMC peptides on adrenal cellular proliferation following adrenal enucleation and hypophysectomy and found that 5 μg 1–28

POMC post hypophysectomy caused an increase in mitotic activity (Estivariz et al., 1988a). More recently, Fassnacht et al. demonstrated that 1–74 POMC, 1–48 POMC and 1–28 POMC were all capable of stimulating proliferation of both adrenocortical tumor cells and primary cell cultures of bovine adrenals in vitro (Fassnacht et al., 2003b). The proliferation seen with each peptide was of a similar magnitude indicating that mitogenic activity encoded within the N-POMC peptide appeared to be dependent upon the extreme N terminus 1–28.

Yet despite these data, we found that administration of synthetic 1–28 POMC to *Pomc* null mice, at a similar dosing route and regimen to that of ACTH, has no measurable effect upon adrenal function (Coll et al., 2006). This lack of biological activity of 1–28 POMC may be as a result of inadequate amounts of active peptide entering the circulation or that 1–28 POMC might exert a mitogenic activity only in a paracrine fashion and that administered peptides were degraded in the periphery. However, the debate appears to be on-going, with a recent study of hypophsectomised rats indicating that synthetic modified N-POMC (1–28) can influence *in vivo* proliferation and block apoptosis in rat adrenal cortex (Torres et al., 2010).

6. Corticosterone supplementation

Glucocorticoids have profound and widespread effects on metabolism with many features of the *Pomc* null phenotype potentially influenced by the lack of circulating glucocorticoid. To further investigate the interaction between glucocorticoids and the melanocortin system we therefore re-analysed the phenotype of POMC deficient mice that had received corticosterone-supplemented drinking water for 10 days (25 μg/ml final concentration, "CORT"), comparing the effects to those seen in wild type mice. Pomc deficient animals appeared hypersensitive to the adverse metabolic effects of corticosteroroids (Coll et al., 2005). Corticosterone supplementation of $Pomc^{-/-}$ mice from weaning lead to a severe metabolic phenotype with mice developing hyperglycaemia, ketonuria and hepatic steatosis by 8–12 weeks Acute exposure also has adverse effects, with 10 days of corticosterone treatment enough to significantly food intake and body weight as well as increasing plasma insulin. A similar finding was independently observed by Smart and colleagues in 2006. A 'neuronal specific' *Pomc* null mouse was derived whereby a transgene was introduced to selectively restore peripheral (pituitary) melanocortin and corticosterone secretion in a $Pomc^{-/-}$ mouse. This mouse was found to have increased body weight, adiposity, insulin resistance and food intake as well as decreased energy expenditure when compared to global *Pomc* null mice indicating that peripheral restoration of *Pomc* accentuates the metabolic disturbances seen in global deficiency (Smart et al., 2006).

In trying to determine what changes may have been brought about with glucocorticoid treatment we looked at both peripheral and hypothalamic tissue from glucocorticoid treated *Pomc* deficient mice. Corticosterone treatment markedly increased 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) expression in the adipose tissue of $Pomc^{-/-}$ mice and, consistent with the exaggerated accumulation of fat in these mice, caused a marked increase in the expression of lipoprotein lipase mRNA in all fat depots (Michailidou et al., 2007).

Analysis of orexigenic peptide expression within the hypothalamus revealed striking differences in expression levels of AgRP. Corticosterone-deplete $Pomc^{-/-}$ mice had less than half the expression level seen in wild type mice yet were still more hyperphagic. Furthermore, although glucocorticoid treatment resulted in higher absolute expression levels of AgRP in CORT-treated WT mice, glucocorticoid treated $Pomc^{-/-}$ mice had a significantly higher food intake, suggesting that glucocorticoid deficiency may afford *Pomc* null animals some protection from the full adverse consequences of melanocortin deficiency, potentially through a mechanism involving suppression of AgRP by the hypoadrenal state (Coll et al., 2005).

7. Agouti and Agrp

As the class name of the peptides indicates, there is a well-recognized association between melanocortins and skin pigmentation. Decades before any link with hypothalamic functioning was made, melanocortins had a clearly defined role in influencing melanocyte function. Yet it was the study of the Agouti mouse, a naturally occurring obese mouse with a stark yellow pigmentation phenotype, which proved to be invaluable in contributing to our understanding of the role of melanocortins in energy homeostasis system. Agouti is a protein involved in regulating hair pigmentation (Wolff et al., 1999). It is secreted within the hair follicles to act in a paracrine fashion, antagonizing the action of α-MSH at melanocortin MC_1 receptor expressed on the surface of melanocytes. Agouti induces a switch in pigment production from brown-black eumelanin to yellow-red phaeomelanin. A number of dominant Agouti alleles, such as A^y and A^{vy}, result in widespread ectopic expression of the Agouti protein, giving rise to a distinct neuroendocrine phenotype of obesity, hyperphagia, hyperinsulinemia, hyperglycemia in males, increased linear growth, and yellow coat color (Wolff et al., 1999). The potential mechanism of how a peptide that regulates hair color could be linked to an obesity syndrome was made clearer when Agouti was found to antagonize melanocortin MC_4 receptor as well as melanocortin MC_1 receptor. Additional insights came with the identification in rodents and humans of a second 132-amino acid peptide expressed predominantly in the hypothalamic arcuate nucleus and the adrenal gland. This peptide showed high sequence homology with Agouti and was therefore named Agouti-related protein (AgRP) (Barsh et al., 1999). Transgenic mice ubiquitously expressing human AGRP developed hyperphagia and an obesity phenotype indistinguishable from that of the Agouti mouse without an effect on pigmentation (Graham et al., 1997; Ollmann et al., 1997). Thus, the obesity seen in the Agouti mouse was explained as being the result of aberrant antagonism of hypothalamic melanocortin receptors with AgRP exerting its orexigenic effects by antagonizing α-MSH at central melanocortin receptors.

AgRP is expressed in the adrenal gland and the hypothalamus with hypothamamic expression predominantly in the arcuate nucleus and median eminence (Ollmann et al., 1997; Shutter et al., 1997). AgRP neurons project to many of the same post-synaptic cells as POMC indicating an interaction between these neuronal populations (Broberger and Hokfelt, 2001). Also, just like POMC, AgRP is a pro-peptide which undergoes processing by prohormone convertase 1 (PC1) to produce smaller, more biologically active peptide (Creemers et al., 2006). For example, the carboxyl terminal fragment of AgRP, $AgRP_{83–132}$, is six times more potent than full length AgRP at melanocortin MC_4 receptors in vitro (Creemers et al., 2006).

Fasting up-regulates AgRP mRNA expression with leptin replacement to pre-fasting levels normalizing AgRP expression (Mizuno et al., 1998, 1999; Mizuno and Mobbs, 1999). AgRP expression is also upregulated in ob/ob and db/db mice (Shutter et al., 1997). Furthermore, leptin regulates AgRP and POMC neurons independently of transcriptional effects by hyperpolarising (inhibiting) AgRP neurons and depolarising (activating) POMC neurons (Elias et al., 1999). This also leads to reduced GABA release from AgRP/neuropeptide Y neurons which removes tonic inhibition of POMC neurons by GABA and increases POMC neuron action potential frequency (Cowley et al., 2001).

Finally, central administration increases food intake (Rossi et al., 1998) and blocks the decreases in food intake and body weight seen with leptin administration (Ebihara et al., 1999).

Data from these studies would suggest an important role for AgRP in energy homeostasis; however a full understanding of the biology of this sometimes enigmatic peptide AgRP remains elusive. The phenotype of the Agrp null mouse illustrates this well. Initial reports were of an unremarkable phenotype with normal locomotor activity, growth rates, body composition and food intake as well as normal responses to starvation, diet induced obesity and exogenous leptin

administration (Qian et al., 2002). However, a later study identified a very modest late-onset lean phenotype with reduced body weight and adiposity after 6 months of age which associated with increased metabolic rate, body temperature, and locomotor activity and increased circulating thyroid hormone (T4 and T3) and BAT UCP-1 expression (Wortley et al., 2005).

To identify whether compensatory mechanisms are involved in this paradoxical mild phenotype in *Agrp* null mice, a number of studies sought to ablate AgRP neurons in adult mice and found that postembryonic ablation led to a more robust phenotype characterized by leanness and hypophagia (Bewick et al., 2005; Gropp et al., 2005; Luquet et al., 2005). Although this may be taken to confirm the critical role of AgRP in the regulation of food intake, deletion of a neuronal population is a bigger insult than removing a single peptide from the same neuronal population. AgRP neurons also express neuropeptide Y and GABA and the loss of these or other, as yet uncharacterised, neurotransmitters from AgRP neurons may be playing a part in the dramatic result observed as much as the lost AgRP peptide. Indeed recent evidence suggests that the starvation is independent of melanocortin signaling (Wu et al., 2008) and is primarily driven by loss of GABA signaling to the parabrachial nucleus (Wu et al., 2009).

7.1. Beyond melanocortin MC_4 receptor

There are also some intriguing reports about both the time course and site of action of Agrp. It has been reported to be able to be still antagonize α-MSH even when administered 9 h previously (Rossi et al., 1998) with a number of studies hinting that the long term orexigenic effects of $AgRP_{83–132}$ might involve mechanisms other than melanocortin receptor blockade (Hagan et al., 2000). For example, in 1999 Marsh and colleagues reported that centrally administered AgRP administered into the dorsal third ventricle was able to increase food intake in $Mc4r^{-/-}$ mice over 24 h in a dose dependent manner (Marsh et al., 1999). Of note, the dose of AgRP shown to be orexigenic in wild type mice when administered into the lateral ventricle has more recently been shown to have no effect in Mc4r null mice (Fekete et al., 2004).

7.2. Inverse agonist action?

There is a growing body of evidence suggesting that AgRP acts as an inverse agonist (Adan, 2006). Data from *in vitro* studies has shown that transfection of cells with melanocortin MC_4 receptor leads to an increase in cAMP activity that is clearly associated with receptor density, suggesting that melanocortin MC_4 receptor has constitutive activity (Nijenhuis et al., 2001). This activity has been shown to be dose dependently suppressed by AgRP suggesting that AgRP has inverse agonist properties at constitutively active melanocortin receptors (Haskell-Luevano and Monck, 2001). Furthermore, this effect of AgRP is blocked by the non-selective melanocortin MC_4 receptor antagonist SHU9119 (Nijenhuis et al., 2001).

More recent in vivo studies would seem to support these findings; while i.c.v. AgRP administration was found to have no effects in mice with a global *Pomc* deficiency, in a transgenic model with a neural specific *Pomc* deficiency, AgRP induced delayed and long-lasting effects on energy balance by decreasing oxygen consumption, increasing the respiratory exchange ratio, and increasing food intake. This suggests that AgRP may be able to modulate energy balance in the CNS independently of melanocortin MC_3/MC_4 receptor competitive antagonism and supports an inverse agonist mode of action for AgRP *in vivo*, although once again the involvement of receptors distinct from MC_3/MC_4 receptor remains possible (Tolle and Low, 2008).

Furthermore, the precise mechanisms by which inverse agonism would work at the melanocortin MC_4 receptor remain unclear. One potential mechanism may involve a portion of the N-terminal domain of the receptor. A study of naturally occurring melanocortin MC_4 receptor mutations from obese patients identified a cluster of mutations in the N-terminal domain which were shown to affect the constitutive activity of the receptor (Srinivasan et al., 2004). Thus, the N-terminal domain may act as a tethered partial agonist that generates the constitutive activity of the melanocortin MC_4 receptor. A later study used chimeric forms of the melanocortin MC_4 receptor in which regions of the receptor were replaced with the corresponding regions of melanocortin MC_1 receptor. This data showed that the N-terminus, transmembrane domains 2 and 7 and the distal C-terminus may all involved in AGRP inverse agonism, with the transmembrane domains also involved in NDP-MSH mediated receptor activation (Chen et al., 2006). Another study that functionally characterized clinically reported melanocortin MC_4 receptor third intracellular loop mutants. Their results suggested that this loop is essential for both the functional activity and also the maintenance of constitutive activity of melanocortin MC_4 receptor in association with G-protein coupling (Kim et al., 2008).

8. Pomc/Agrp double null mouse

Despite great advances in the field in recent years, many important questions still remain unanswered, specifically (1) is the phenotype of the *Pomc* null mouse due to a lack of melanocortin peptides, unopposed action of AgRP or both?, (2) is the severe metabolic phenotype of corticosterone treated Pomc null mice due to a mechanism involving AgRP and (3) is AgRP acting as an antagonist or as an inverse agonist in vivo? To further investigate the role of AgRP in energy homeostasis, we have developed a double knockout mouse model lacking both *Pomc* and *Agrp*. Initial data from studies of a mouse on a mixed genetic background suggested that the double knockout mouse may have a phenotype intermediate to that of the *Pomc* null and *Agrp* null mice (A.P Coll, unpublished observations). Ongoing studies in this model suggest that Agrp is in fact acting as an antagonist *in vivo*.

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