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Emerging roles of melanocortin receptor accessory proteins (MRAP and MRAP2) in physiology and pathophysiology

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Abstract

Melanocortin-2 receptor accessory protein (MRAP) has an unusual dual topology and influences the expression, localisation, signalling and internalisation of the melanocortin receptor 2 (MC₂); the adrenocorticotrophic hormone (ACTH) receptor. Mutations in *MRAP* are associated with familial glucocorticoid deficiency type-2 and evidence is emerging of the importance of MRAP in adrenal development and ACTH signalling. Human MRAP has two functional splice variants: *MRAP-α* and *MRAP-β*, unlike *MRAP-β*, *MRAP-α* has little expression in brain but is highly expressed in ovary. *MRAP2*, identified through whole human genome sequence analysis, has approximately 40% sequence homology to *MRAP*. MRAP2 facilitates MC2 localisation to the cell surface but not ACTH signalling. MRAP and MRAP2 have been found to regulate the surface expression and signalling of all melanocortin receptors (MC₁₋₅). Additionally, MRAP2 moderates the signalling of the G-protein coupled receptors (GPCRs): orexin, prokineticin and GHSR1a; the ghrelin receptor.

Whilst MRAP appears to be mainly involved in glucocorticoid synthesis, an important role is emerging for MRAP2 in regulating appetite and energy homeostasis. Transgenic models indicate the importance of MRAP in adrenal gland formation. Like *MC3R* and *MC4R* knockout mice, *MRAP2* knockout mice have an obese phenotype. *In vitro* studies indicate that MRAP2 enhances the MC3 and MC4 response to the agonist αMSH, which, like ACTH, is produced through precursor polypeptide proopiomelanocortin (POMC) cleavage. Analysis of cohorts of individuals with obesity have revealed several *MRAP2* genetic variants with loss of function mutations which are causative of monogenic hyperphagic obesity with hyperglycaemia and hypertension. MRAP2 may also be associated with female infertility. This review summarises current knowledge of MRAP and MRAP2, their influence on GPCR signalling, and focusses on pathophysiology, particularly familial glucocorticoid deficiency type-2 and obesity.

Keywords

MRAP; MRAP2; MC2; MC1R; MC2R; MC3R; MC4R; MC5R; melanocortin; ACTH; Dual topology; Ghrelin; Alpha-MSH; Adrenal gland; Glucocorticoid; Female infertility; Familial

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glucocorticoid deficiency; Melanocortin 2 receptor accessory protein; Melanocortin receptor; Obesity.

1. Introduction

Genetic variants of melanocortin-2 receptor accessory protein (*MRAP*) are associated with severe glucocorticoid deficiency (Metherell et al., 2005). Adrenocorticotrophic hormone (ACTH) is the endogenous agonist for the melanocortin receptor 2 (MC₂), a class A G-protein coupled receptor. MRAP is a small transmembrane protein which promotes the assembly of melanocortin receptor 2 in the endoplasmic reticulum and stabilises MC₂ in the plasma membrane. MRAP influences the binding of ACTH to MC₂ and following agonist stimulation, MC₂ via Gs protein, activates adenylate cyclase generating cyclic adenosine monophosphate (cAMP).

Effective ACTH signalling is vital to produce steroids including glucocorticoid, hence genetic variants of *MRAP* are linked to familial glucocorticoid deficiency. *MRAP* expression is high in the brain and the adrenal gland and MRAP has been demonstrated to affect the differentiation of progenitor cells within the adrenal gland (Novoselova et al., 2018).

MRAP2 shares approximately 40% sequence homology with *MRAP* but unlike MRAP, which facilitates MC₂ localisation and signalling; MRAP2 has less receptor fidelity, influencing the signalling of melanocortin receptors (MC₁₋₅), as well as orexin receptor, ghrelin receptor and growth hormone secretagogue receptor 1a (GHSR-1a) (Srisai et al., 2017). MC₃ and MC₄ both have important roles in appetite control with deficiencies linked to obesity (Chen et al., 2000). The MC₄ agonist setmelanotide is currently in phase 3 clinical trials for rare genetic disorders of obesity caused by mutations in proopiomelanocortin (*POMC*) (Kühnen et al., 2016) and leptin receptor (*LEPR*) (Clément et al., 2018). Evidence from *in vitro* analyses, MRAP2 transgenic animals and *MRAP2* variants in populations with metabolic syndrome (Baron et al., 2019), indicate that MRAP2 has an important role regulating appetite control, with *MRAP2* genetic variants causing obesity.

This article will review the structures of melanocortin receptor accessory proteins, physiological roles, and emerging evidence about the *MRAP* and *MRAP2* genetic variants which shed light upon pathophysiological conditions.

2. Protein structure

MRAP is a hydrophobic single pass transmembrane protein which is encoded for by a single gene located on chromosome 21q22.11 (Xu et al., 2002) composed of 6 exons. There are two functional MRAP splice variants: MRAP-α derived from exon 1–5 which is 172 amino acids (19 kDa) and MRAP-β derived from exon 1–4, 6, which is 102 amino acids (14.1 kDa). These splice variants have conserved N-terminal homology but differ at the C-terminus (Xu et al., 2002; Metherell et al., 2005)

When first described, it was predicted that the MRAP dimer would orientate with intracellular N-terminus and extracellular C-terminus (Metherell et al., 2005). However,

through epitope immunoprecipitation and live cell imaging studies, the quaternary structure of the MRAP was found to form a stable anti-parallel homodimer with N-termini orientating both intracellularly and extracellularly, which is still thought to be unique in eukaryotes (Sebag and Hinkle, 2007).

MRAP is partially glycosylated and this is dependent upon the Asn-X-Ser/Thr motif facing the luminal surface of the endoplasmic reticulum (Sebag and Hinkle, 2007), however, the creation of functional MRAP glycosylation mutants in this study determined that glycosylation was not necessary for dual topology.

A further series of mutational studies identified the N-terminal 31–37 sequence (LKANKHS) adjacent to the transmembrane region to be essential for this dual topology (Sebag, and Hinkle, 2009). Using bimolecular fluorescence complementation, Sebag and Hinkle demonstrated that this dual topology occurs in the endoplasmic reticulum; once formed this stable orientation is retained when the protein is expressed on the plasma membrane (Maben et al., 2016).

The N-terminus and the transmembrane domains of MRAP are conserved between species however, the carboxyl region has greater variation (Agulleiro et al., 2010). The 18–21 LDYI sequence is unique to the MRAP isoform and is required for the binding of ACTH to melanocortin receptor 2 (Sebag and Hinkle, 2009) but MRAP C-terminus truncation experiments suggested this domain has less impact on ACTH signalling (Sebag and Hinkle, 2009). However, truncated MRAP (with the removal of the C-terminal domain), expressed in HEK293/FRT cells, revealed diminished MC₂ surface expression and cAMP production compared to full length MRAP; suggesting that the C-terminus does modulate MC₂ cAMP generation in response to ACTH (Roy et al., 2012). In addition, serine and threonine residues in the C-terminus are necessary for MC₂ internalisation and recycling (Roy et al., 2011). The exact residues necessary for MRAPs to interact with GPCRs have not been fully characterised; however, co-precipitation studies with MC₂ suggest that the MRAP transmembrane domain is necessary (Webb et al., 2009).

3. Discovery, structure and characteristics of MRAP2

As a paralog to *MRAP*, *MRAP2* was identified in the human genome after sequence analysis. The *MRAP2* gene is located at 6q14.3 and encodes for a 205 amino acid protein, the sequence alignment of *MRAP2* indicates approximately 40% homology with *MRAP*. The greatest sequence conservation lies at the N-terminal and within the transmembrane domain, whilst the carboxyl terminal differs greatly between these two proteins (Chan et al., 2009).

MRAP2 is a transmembrane protein that traverses the membrane once and as with the MRAP, MRAP2 could be found both on the surface membrane and subcellularly on the luminal surface of the endoplasmic reticulum (Sebag and Hinkle, 2010). MRAP2 shares the dual orientation of the carboxyl and amino terminal forming an antiparallel homodimer (Fig. 1). Interestingly, MRAP2 has been shown to form a heterodimer with the MRAP (Fig. 1); a

yellow fluorescent protein in bimolecular complementation studies revealed an antiparallel orientation of the MRAP-MRAP2 heterodimer (Sebag and Hinkle, 2010).

4. MRAP gene regulation

MRAP gene expression is upregulated by lipopolysaccharide (Gibbison et al., 2015), ACTH (Liu et al., 2013) and in adipocytes by PPAR γ (peroxisome proliferator-activated receptor gamma) (Kim et al., 2013). Diurnal *Mrap* expression has been observed in rats, with *Mrap* mRNA correlating with the expression of circadian regulating genes (*per1*, *per2* and *CLOCK*) (Spiga et al., 2020). Since the *MRAP* mRNA half-life is shorter than *MC2R*, Clark and Chan, (2019) have proposed a model where MRAP may facilitate the transport of MC₂ to the plasma membrane without necessitating *de novo* *MRAP/MC2R* expression.

Gnomon automated computational analysis of the 21q22.11 region of the GRCh38.p13 (Souvorov et al., 2010) has predicted the presence of a novel *MRAP* antisense RNA 1 (*MRAP-AS1*; XR_937664.1). Whether this non-coding RNA has a biological function and can regulate *MRAP* remains to be determined (Fig. 2).

5. The evolution of MRAPs

Phylogenetic studies have revealed the existence of the *MRAPs* orthologues in different piscine species: zebrafish, tetrapod and some bony fish subspecies (Valsalan et al., 2012). Interestingly, whilst *MRAP2* was found in the sea lamprey and cartilaginous fish, *MRAP* was absent which led to a theory that *MRAP2* gene duplication may have given rise to *MRAP* (Västermark, and Schiöth, 2011). However, cDNA blast analysis from the elephant shark genome project located the *MRAP* sequence (Venkatesh et al., 2014) discredited an *MRAP2* ancestral role. Considering all these findings, the origin of the *MRAPs* genes is thought to be as a result of R2 genome duplication events according to the evolution theory (Ohno et al., 1968); a comprehensive review of melanocortin receptor evolution has been presented by Dore (Dore et al., 2016).

The presence of both *MRAPs* has been described in most mammals and chicken (Valsalan et al., 2012), the dual topology feature of MRAP and MRAP2 was conserved through the appearance of the motifs LKANKH (MRAP) and LKAHKY (MRAP2) in the orthologs of mice, chicken and zebrafish. Additionally, sequence alignment studies of all lineages revealed the MRAP sequence to be shorter than the MRAP2 sequence, with the transmembrane domain being well conserved amongst all species examined.

6. Distribution of the MRAPs

In humans, reverse transcription PCR (RT-PCR) indicated that both isoforms are present at high levels in the adrenals, testis and breasts; with low expression in the digestive system, heart, fat and skin (Metherell et al., 2005). In humans, *MRAP- α* and *MRAP- β* were found expressed in the adipose tissue, breast, testis and adrenal cortex (see Table 1).

Examining the “Human Protein Atlas (HPA)- RNA-seq” data of 95 human samples reveals a wide distribution of *MRAP*, with the highest expression levels observed in the adipose

tissue and the adrenal gland. This is followed by the skin and the male reproductive organs; whilst brain, heart, ovary and the kidney had a lower expression (Fagerberg et al., 2014). Table 1 lists human tissues expressing the *MRAP-α* and *MRAP-β*. Along with the adrenal gland expression, *MRAP2* was expressed in different parts of the brain at high levels, as shown in the HPA-RNA-seq data (Fagerberg et al., 2014). *MRAP2* was also found to be highly expressed in the female and male reproductive systems and stomach whilst endocrine glands, liver and peripheral blood had the lowest *MRAP2* expression (Fagerberg et al., 2014).

As discussed above, the *MRAPs* are present in other species, including chicken, fish and mice (Zhang et al., 2017; Agulleiro et al., 2010; Asai et al., 2013; Valsalan et al., 2012). While the *MRAP* was widely distributed in the tissues of those species, the *MRAP2* expression is mainly localised to the brain.

7. MRAPs and the melanocortin system

7.1. MRAP and MC₂

MC₂ was initially difficult to express on the surface of cells until an adrenal cell line was utilised, revealing that MRAP is an essential accessory protein (Metherell et al., 2005). The MC₂ receptor only responds to ACTH; ACTH activation of MC₂ results in glucocorticoid-, mineralocorticoid- and steroid-genesis in the adrenal gland. Genetic variants of *MC2R* result in severely impaired glucocorticoid production which is discussed in more detail below. MRAP was found to assist in MC₂ transport from the endoplasmic reticulum to the cell surface (Metherell et al., 2005), whilst MC₂ has reported to move unassisted to the cell surface (Roy et al., 2007), this finding has been criticised since extremely low endogenous MRAP2 present in HEK293 cells may facilitate the MC₂ movement (Sebag and Hinkle, 2007). MRAP increases both the density of surface MC₂ and the binding affinity of ACTH to MC₂. Measurement of cAMP in response to Gs activation revealed that MRAP-α elicits a 4-fold greater dose response than MRAP-β but the maximal cAMP response was higher in the MC₂/MRAP-β cells compared to the MC₂/MRAP-α (Roy et al., 2007). The interaction of MRAP and MC₂ also has an effect on receptor internalisation and recycling (Roy et al., 2011).

The three zones of the adrenal cortex synthesise different steroids: zona glomerulosa, mineralocorticoids; zona fasciculata, glucocorticoids and zona reticularis, androgens. The binding of ACTH to MC₂ results in the production of cAMP, that in turn stimulates the protein kinase A (PKA) signalling pathway. In the adrenal gland, this signalling pathway is responsible for the transcription of genes necessary for steroid synthesis, such as steroidogenic acute regulatory protein (*StAR*) (Manna et al., 2003). The interaction of MC₂ with either of the MRAP isoforms and the response variation (Roy et al., 2007) reflects the physiological requirements of these steroids in different physiological conditions, such as the response to stress or blood pressure changes.

In addition to *MRAP*, *MRAP2* is expressed in human and rat adrenal glands (Gorrigan et al., 2011; Fagerberg et al., 2014). The response of MC₂ depends on which MRAP it is coupled with, *in vitro* studies revealed that MC₂/MRAP2 transiently transfected into HEK293 cells

required higher concentrations of ACTH to elicit a response compared to MC₂/MRAP transfected cells (Gorrigan et al., 2011). *MRAP* and *MRAP2* expression has been described in the foetal adrenal gland suggesting a role of MC₂/MRAP and or MC₂/MRAP2 in the adrenal differentiation and proliferation (Gorrigan et al., 2011). This is supported by the phenotype of *MRAP*^{-/-} mice which have disrupted adrenal gland development with no apparent zonation in the adrenal cortex, without *in utero* corticosterone supplementation, the majority of these *MRAP*^{-/-} mice died at birth (Novoselova et al., 2018). *MRAP*^{-/-} mice had disrupted adrenal progenitor cell differentiation due to impaired sonic hedgehog and WNT4/β-catenin signalling (Novoselova et al., 2018).

7.2. MRAPs and other melanocortin receptors

The melanocortin receptors are expressed throughout the body and have established roles in different tissues. There is great interest in the involvement of MRAPs and the surface expression of melanocortin receptors, MC_{1,3-5}. These G-coupled protein receptors are activated by melanocortin peptides: proopiomelanocortin (POMC) and the peptides derived from POMC: adrenocorticotrophic hormone (ACTH), alpha-, beta- or gamma-melanocyte stimulating hormone (α-MSH, β-MSH and γ-MSH). ACTH is further cleaved into: ACTH (1–39), ACTH (1–17), ACTH (1–10), α-MSH and deacetyl α-MSH (Wakamatsu et al., 1997). Unlike MC₂ which only responds to ACTH, these MCs, respond with varying affinity to the endogenous melanocortin peptides and activate different signalling pathways, accounting for MC functional variability. Agouti related protein (AgRP) is an endogenous antagonist of MC₃ and MC₄ (Ollmann et al., 1997; Fong et al., 1997). MRAPs regulate both the expression and the response of the other MCs. Although MRAPs had no effect on the MC₁ and MC₃ cell surface expression in *in vitro* studies with CHO cells; MC₁, MC₃, MC₄ and MC₅ became less sensitive to the synthetic α-MSH analogue, NDP-MSH, when co-expressed with either MRAP or MRAP2 (Chan et al., 2009).

As described above, MC₂ and the MRAPs are important for adrenal gland development. MC₅ expression has been described in the foetal adrenal gland and is suggested to have a role in foetal development (Nimura et al., 2006). The role of MRAP and MRAP2 in regulating adrenal differentiation, may be through interaction with either MC₂, MC₅ or a heterodimer of both, since heterodimerisation of MC receptors has been reported (Mandrika et al., 2005).

MC₁ is well characterised in stimulating melanin synthesis; skin (or retinal) pigmentation occurs through the oxidation of tyrosine resulting in the formation of either eumelanin (brown pigmentation) or pheomelanin (yellowish-red pigmentation). MC₁ is expressed in both melanocytes and keratinocytes, as are POMC, ACTH and the prohormone convertases 1 and 2. α-MSH is the MC₁ agonist, activating cAMP and PKA signalling pathways which ultimately activate the microphthalmia transcription factor (MITF) and tyrosinase transcription (Beaumont et al., 2009). The resultant pigmentation protects the skin from the DNA damaging effects of the ultraviolet radiation. The variability in skin colour is due to loss of function alleles in *MC1R* single gene mutation which also increase the incidence of skin cancer and melanoma (Sánchez-Laorden et al., 2007). Whilst mutations of *MRAP2* do not cause disturbances in skin pigmentation, the phenotypic characteristics

of *MRAP* mutations have included skin hyperpigmentation, as well as high plasma ACTH concentrations (Jain et al., 2011). Expression of MRAP and MRAP2 in skin is very low (Table 1) a lack of functional MC₂ in *MRAP* mutation may result in higher circulating ACTH and consequently MC₁ hyperactivity impacting upon skin pigmentation.

The ratio of MRAP2 to the melanocortin receptor may have an important effect on the melanocortin signalling; a 1:4 ratio of MC₄:MRAP2 gave greatest cAMP response to α -MSH whilst a 4:1 ratio of MC₄:MRAP2 reduced cAMP below mock transfected cells (Schonnop et al., 2016). Further work is required to elucidate the role of MRAP2 on MCs; the *MRAP2* construct design, cell type, melanocortin peptide used to stimulate transfected cells and technique to measure MC activation are important factors to consider in interpreting this complex interaction.

In zebrafish, two MRAP2 isoforms have been reported (*mrapp2a* and *mrapp2b*) and the expression of these varies during development; the expression of *mrapp2a* was found to be present from day 1 of embryogenesis, as was *mc4r*; however, there was little expression of *mrapp2b* (Sebag et al., 2013). Knockdown of *mrapp2a* with antisense morpholinos significantly decreased growth, however, when the *mrapp2a* was knocked down in *mc4r* null fish the effect was less marked; in the embryo, *mrapp2a* inhibits MC₄ signalling. AgRP is an MC₄ antagonist, if both *AgRP* and *mrapp2a* were knocked down there was a significant impairment of growth and they are proposed to collaborate inhibiting MC₄ constitutive activity (Sebag et al., 2013).

In contrast the *mrapp2b* isoform was highly expressed in adult zebrafish, and reduced MC₄ constitutive activity and sensitized MC₄ to the agonist α -MSH; the *mrapp2b* has homology to mouse *MRAP2* (Sebag et al., 2013). It was suggested that this switch in the *mrapp2* expression may facilitate a change in metabolism to enable free-feeding once embryonic development was complete.

8. MRAP and familial glucocorticoid deficiency (FGD)

Glucocorticoids modulate the stress response through the hypothalamic–pituitary–adrenal (HPA) axis and mediate negative feedback control over the corticotrophic releasing hormone (CRH) in the hypothalamus, as well as ACTH release from the pituitary gland. *MRAP2* expression has been reported in hypothalamus, adrenal and pituitary glands (see table 1) alongside evidence of the melanocortin ligands (Chaly et al., 2009). These findings suggest the control of the response may be moderated by the melanocortin receptors, with the assistance of the MRAPs, at all three levels of the HPA axis.

Familial glucocorticoid deficiency (FGD) is characterised by a poor response by the adrenal cells to adrenocorticotrophic hormone (ACTH) leading to cortisol deficiency. As described above, the MRAPs have an important role stabilising the MC receptors and facilitating ACTH signalling. FGD is an autosomal recessive disease with 25% of the cases due to different *MC2R* mutations (FGD-1), 5% of cases relate to the steroidogenic acute regulatory protein (*StAR*) but around 50% of the FGD cases had an unidentified genetic background. Single nucleotide polymorphism (SNP) array genotyping of FGD cases

revealed that 20% were linked to mutations in the *MRAP* sequence (FGD-2) (Metherell et al., 2005). The discovery of MRAP as an essential accessory protein for MC₂ prompted further investigations into the mechanisms underlying FGD-1.

The *MC2R* coding sequence lies predominantly in one exon, therefore splicing mutations are very rare and missense mutations preserve a residual function, thus, they are associated with a milder or later onset of the disease. FGD-1 occurs in early childhood with hypoglycaemia, developmental delay and frequent infections, all due to low cortisol levels. The reduced receptor sensitivity to ACTH leads to increased circulating ACTH concentrations which are associated with hyperpigmentation of the skin and tall stature. These latter signs are thought to result from increased MC₁ stimulation and estradiol synthesis by excessive circulatory ACTH (Imamine et al., 2005).

The cell lines HEK293 and CHO do not express melanocortin receptors and provide a useful *in vitro* model for transfection with the MRAPs and the MCs. Chung and colleagues investigated 24 reported MC₂ polymorphisms with MRAP using cAMP reporter assays and cell surface analyses, they found that two thirds of these SNP mutations impaired MC₂ receptor transport to the cell surface, despite interacting with MRAP, and resulted in a failure of MC₂ receptor to respond to ACTH (Chung et al., 2008).

Unlike *MC2R* mutations, almost all the mutations occurring in the *MRAP* are homozygous non-sense or splice-site mutations which cause severe truncation or ablation of the MRAP protein (Jain et al., 2011), (See table 2). Those mutations were found to be associated with severe glucocorticoid deficiency immediately after birth or in early infancy, with seizures due to hypoglycaemia. Cases of the FGD-2 have been reported where there is a later onset and a milder presentation of the symptoms. Sequencing the genome of individuals with later onset glucocorticoid deficiency revealed a homozygous missense (c.175 T > G) mutation that caused an asparagine to tyrosine substitution at position 59 (N59Y). Whilst another case was reported a missense (c.76 T > C) which led to an alanine to valine substitution at position 26 (V26A) (Hughes et al., 2010).

MRAP^{-/-} mice do not survive after birth without the intervention of *in utero* corticosterone and had impaired adrenal gland development (Novoselova et al., 2018). MRAP is likely to have a major role in adrenal gland development and the severity of the FGD-2 could be due to underdevelopment of this gland. Although *MRAP2* is also expressed in the adrenal gland, it did not seem to compensate for absent or mutated *MRAP* and therefore, might not have a role in the pathogenesis of FGD. However, as there are still about 50% of FGD cases with unidentified mutations, further nucleotide sequencing and genome analysis of FGD individuals may reveal a role of MRAP2.

Whilst *MRAP* genetic mutations leading to glucocorticoid deficiency are rare, the use of synthetic glucocorticoids for inflammatory disorders and autoimmune diseases is widespread. A side-effect of synthetic glucocorticoid treatment is adrenal insufficiency, characterised by the failure of the adrenal gland to respond to ACTH. In a rat model, five day exposure of methylprednisolone reduced ACTH and corticosterone concentrations and downregulated both *MRAP* and *Star* expression (Spiga et al., 2020).

9. MRAP2 and appetite control

Appetite control comprises a complicated neuroendocrine circuit involving several hormones, the sympathetic and parasympathetic nervous system and some elements of the limbic system. MC₄ regulates energy expenditure and appetite control and has been found in areas of the brain associated with energy homeostasis: *MC4R* mRNA has been detected in the paraventricular nucleus (PVN), ventromedial, medial preoptic and the supraoptic nucleus of the hypothalamus; all of which are integrated in controlling feeding behaviour (Adan et al., 2006; Siljee et al., 2013). In the PVN, *MC4R* was detected in transcription factor single minded 1 (*SIM1*⁺) expressing neurones, a population of neurones known to regulate food intake and mediate MC₄ signalling through a glutamatergic pathway (Xu et al., 2013). The MC₄ agonist setmelanotide is in clinical trials for treating severe obesity with underlying genetic causes (Kühnen et al., 2016; Clément et al., 2018). *MC4R*^{-/-} transgenic mice are severely obese and studies with these animals supported the involvement of MC₄ in appetite control, they had hyperphagia and were non-responsive to melanotan II (MTII), an α -MSH analogue (Marsh et al., 1999). α -MSH is released by POMC neurons in the arcuate nucleus in response to sensory input from the periphery, this in turn stimulates PVN-MC₄ signalling leading to reduced appetite. Whilst leptin/hunger signalling to the counteracting agouti-related peptide neurons causes the inactivation of the same PVN, leading to increased appetite. This is a simplified account of how MC₄ is involved in controlling feeding behaviour.

MRAP2 was found to be expressed at high levels in the brain and in areas that are associated with appetite control, where MC₄ has been comprehensively described (Liang et al., 2018; Bruschetta et al., 2018). Similar to transgenic *MC4R*^{-/-} mice, both whole body *MRAP2*^{-/-} and brain targeted *MRAP2*^{-/-} mice were morbidly obese, however, *MRAP2*^{-/-} mice do not show signs of hyperphagia or reduced energy consumption (locomotion and body temperature), they have normal expression of *POMC* but reduced *AgRP* levels; and they respond to MTII treatment (Asai et al., 2013). Deletion of both *MC4R* and *MRAP2* produced mice that were less obese than single deletion *MC4R*^{-/-} mice. Exposure of *MC4R*/*MRAP2* transfected cells with α -MSH increases cAMP production compared to *MC4R* expressing cells, further suggesting an interaction between MC₄ and MRAP2 (Asai et al., 2013). MRAP2 was also found to interact with MC₃, cAMP production increased in *MC3R*/*MRAP2* transfected cells in response to α -MSH compared to *MC3R* expressing cells (Asai et al., 2013).

MRAP2 might be involved in an energy homeostasis/feeding behaviour circuit independently of MC₄. MC₃ is perceived to have a minor role in energy homeostasis, *MC3R* knockout mice have an obese phenotype but with a reduced lean body mass and reduced appetite (Chen et al., 2000). *MC3R* is expressed on the POMC neurons in the arcuate nucleus and thought to be exerting autocrine feedback control (Lee et al., 2008). *MC3R*, *MC4R* and *MRAP2* have been observed in chicken hypothalamus; chicken (*c*) *MRAP* (a homolog of *MRAP2*) was found to enhance the constitutive activity of ACTH treated *c* *MC3R*/*c* *MC4R* transfected cells in a CRE-luciferase reporter assay (Zhang et al., 2017).

10. MRAP2 interactions with non-melanocortin receptors

The role of MRAP2 in mediating energy homeostasis and feeding behaviour extends beyond the melanocortin receptors. *MRAP2* is expressed in the AgRP neurones, aforementioned AgRP is an MC₄ antagonist; MRAP2 may regulate the AgRP neuronal activity, since deletion of *MRAP2* resulted in reduced *AgRP* expression and inhibited AgRP orexigenic activity in response to starvation (Srisai et al., 2017). The MRAP2-AgRP effect may explain the reduced food intake reported in *MRAP2*^{-/-} mice. GHSR-1a (growth hormone secretagogue receptor 1a) is a receptor found in the arcuate nucleus of the hypothalamus and binds ghrelin, which is released by the stomach, sending sensory input to stimulate feeding via AgRP neurones. Srisai et al described MRAP2 to enhance the signalling of GHSR-1a (Srisai et al., 2017), via Gα_{q/11} however, MRAP2 independently inhibits β-arrestin recruitment and signalling (Rouault et al., 2020). Whilst MRAP2 was found to stimulate GHSR-1a and thus feeding, it was also found to induce feeding by a counteracting mechanism, suppressing the prokineticin receptor 1 (PKR-1). *PKR-1* has a wide tissue distribution and mediates several functions including suppressing food intake. Measurement of surface density and surface expression of tagged-PKR-1 in *MRAP2* positive and negative cells revealed lower PKR-1 surface expression in MRAP2 expressing cells; MRAP2 may not contribute to PKR-1 transport to the cell surface (Chaly et al., 2009). In the presence of MRAP2, PKR-1 was found to have reduced signalling, as lower production of cAMP and IP₃ were observed, respectively in the CRE-luciferase and IP-One assays (Chaly et al., 2009).

An MRAP2/orexin receptor (OX1R) interaction has been described, OX1R is a hypothalamic receptor expressed in the PVN which stimulates food intake. MRAP2 was found to downregulate the surface expression of *OX1R* and inhibit OX1R signalling (Rouault et al., 2017). This study mapped the MRAP2 domains and found the C-terminal domain of MRAP2 inhibited signalling of both OX1R and PKR-1. The MRAP2 N-terminal 23–33 domain was involved in inhibiting the OX1R transport to the cell surface; and the N-terminal 34–43 domain was suggested to exert negative regulatory control over the MRAP2 activity (Rouault et al., 2017). A distinct region of the MRAP2 N-terminus, from that required for OX1R and PKR-1, is suggested to regulate GHSR-1a signalling (Rouault et al., 2020).

11. MRAP2 and obesity

Polygenic obesity is affected by genetics, life-style and environmental factors which predispose to increased appetite and reduced energy expenditure; whole genome and exome sequencing have been invaluable to identify genetic factors. Monogenic obesity has a negligible environmental influence, obesity is usually very severe and occurs in early childhood. A role for MRAP2 in the pathogenesis of obesity was suggested following a realisation that it regulates MC₄, which, as discussed above, is essential for energy homeostasis and feeding behaviours. *MRAP2* knockout mice have severe early onset obesity (Asai et al., 2013). Asai et al sequenced the *MRAP2* coding regions and the intron-exon boundaries, in both adults and children with obesity. They discovered four genetic variants, one which resulted in a non-functional MRAP2 (E24X, see Table 3). The other three rare

variants were towards the C-terminus and considered to have less impact on obesity (Asai et al., 2013 - see table 3). The different functional domains in the MRAP2 observed by Rouault et al., (2017), might suggest that these variants could influence interactions of MRAP2/MC₄. Eight more genetic variants of *MRAP2* have been detected (3 intronic; 2 synonymous; 2 coding and one synonymous; see Table 3) following mutational sequencing of the *MRAP2* coding region of extremely obese individuals (Schonnop et al., 2016), which were absent in the control group. This study also reported reduced MC₄ signalling when co-transfected with the nonsynonymous *MRAP2* mutant (p.Gln174Arg) in HEK293 cells (Schonnop et al., 2016). Studies of the 6q14.1–6q16.3 region revealed *SIM1*⁺ and *MRAP2* variants in patients with Prader-Willi like-syndrome (Geets et al., 2016).

The mechanism by which *MRAP2* deletion causes obesity is not clearly understood and predicted to be due to effects on MC₄. However, RT-qPCR analysis of hypothalamus from *MRAP2*^{tm1a/tm1a} (transgenic mice with targeted exon 4 deletion) showed normal *MC4R* PVN content. In contrast, there was a 50% reduction in the *SIM1*⁺ in the PVN and decreased PVN expression of oxytocin, arginine vasopressin, corticotrophin-releasing hormone and thyrotropin-releasing hormone (Novoselova et al., 2016). *MRAP2*^{tm1a/tm1a} mice also had raised cholesterol and lipoprotein (HDL and LDL) concentrations, consistent with observations in MC₄ deficient mice; there is a suggestion that *SIM1*⁺ is either activated downstream of MC₄ (Novoselova et al., 2016) or mediates the MC₄ function within the hypothalamic PVN (Xu et al., 2013).

Although mutations in the *MRAP2* cause an early onset obesity like *MC4R* mutations, Asai et al. (2013) observed no change in the feeding or energy expenditure patterns in the *MRAP2* knockouts compared to the *MC4R* knockouts. The transgenic mice used by Novoselova et al which have a different genetic background, show some changes in the feeding behaviour. These two reports preceded reports that MRAP2 interacts with the non-melanocortin GPCRs: GHSR-1a, ORX1 and PKR-1. This raises a possibility that, the obesity pathologies observed in *MRAP2* transgenic mice could involve pathways outside of melanocortin signalling.

The *MRAP2* exon regions were sequenced in a cohort of 9418 subjects, and 23 rare heterozygous variants were identified which independently increase the risk of obesity (Baron et al., 2019). To elucidate a molecular mechanism these variants were co-transfected in CHO cells with *MC4R* and the cAMP production recorded in response to ACTH or α -MSH. Seven *MRAP2* variants (c.3–7 del, G31V, F62V, F62C, N77S, K102* and P195L) decreased cAMP produced in response to ACTH or α -MSH. The variants C5–5del, c.3–7 del, G31V, F62V, F62C, N77S, K102* and P195L were associated with obesity (table 3), the authors remarked that 75% of the carriers of *MRAP* variants also displayed an abnormal eating behaviour (Baron et al., 2019). *Lep*, *LepR*, *MC4R*, *PCSK1*, *POMC* and *SIM1* are implicated in monogenic obesity, however, individuals with *MRAP2* variants also have hypertension and hyperglycaemia (Baron et al., 2019). As mentioned above, Srisai *et al* reported that MRAP2 enhances the ghrelin signalling through GHSR-1a; ghrelin receptor has been described to affect blood pressure regulation (Mao et al., 2016) and further investigation of MRAP2 and ghrelin signalling is required to understand the hypertensive phenotype described in these individuals.

12. MRAP and insulin signalling

The first description of MRAP came from murine 3T3L1 adipocytes; a novel low molecular weight protein was found in differentiated adipocytes but not pre-adipocytes, and in both in brown and white adipose tissue. Initially named fat tissue low molecular weight protein (FALP) (Xu et al., 2002) it was subsequently renamed MRAP after recognition of the MC₂ interaction (Metherell et al., 2005). PPAR γ , a regulator of adipocyte differentiation, activates transcription of *MRAP* (Kim et al., 2013). MRAP was observed in adipocytes at the *peri*-nuclear membrane, but if adipocytes were exposed to insulin the distribution changed, with discrete MRAP specks distributed throughout the cytoplasm (Xu et al., 2002).

Baron et al reported that siRNA *MRAP2* knockdown in EndoC- β H1 cells reduced insulin secretion (Baron et al., 2019). In adipocytes, MRAP is required for lipolytic responses to ACTH through MRAP interaction with G α s (Zhang et al., 2018). Increased insulin sensitivity in response to a high fat diet has been reported in transgenic mice with *MRAP* overexpression in adipose tissue, correlating with decreased plasma triglyceride and cholesterol concentrations. Insulin resistance and glucose intolerance have also been described in *MRAP2* knockout mice (Rouault et al., 2017). Further research is required to elucidate these pathways.

13. Inflammation

MC₁ and MC₃ signalling through α -MSH and ACTH mediate anti-inflammatory responses downregulating and inhibiting the release of proinflammatory cytokines: TNF- α , INF- γ , IL-6; inducible nitric oxide synthase and the chemokine receptors CXCR1 and 2, whilst stimulating IL-1 release (Catania et al., 1998; Catania et al., 2004; Getting et al., 2003). MC₃ therapeutics targeted for rheumatoid arthritis and other chronic inflammatory conditions report side effects of hyperpigmentation and increased melanoma risk, through hyperstimulation of MC₁. Adrenal inflammation is associated with side effects of synthetic corticosteroids where MRAP is downregulated (Spiga et al., 2020). Additional investigations may determine whether either MRAP isoform has an anti-inflammatory role.

14. *MRAP2*- single nucleotide variations related to cancer

The BioMuta single-nucleotide variation (SNV) and disease database revealed *MRAP2* SNV to be linked to different types of cancers with varying frequencies. High frequency of SNV was seen with melanomas and uterine cancers followed by a lower variation frequency seen in colorectal, lung, stomach and liver cancers (Simonyan, and Mazumder, 2014). As discussed above *MC1R* mutations are associated with melanoma (Sánchez-Laorden et al., 2007), although not affecting MC₁ surface expression, *MRAP2* reduces responsiveness of MC₁ to the synthetic α -MSH agonist NDP-MSH and affects receptor signalling (Chan et al., 2009). Other types of cancer that showed a lower *MRAP2* SNV included breast, thyroid, prostate, pancreas, urinary tract and brain cancers (Simonyan, and Mazumder, 2014). SNV that are linked to *MRAP2* overexpression included liver, lung and pancreatic cancers, however, SNV leading to *MRAP2* suppression included breast, thyroid and prostate cancers. The tissue distribution of *MRAP2* is shown in table 1; *MRAP2* has not been

reported in healthy human liver, thyroid or urinary tract. The association of MRAP2 to a wide variety of cancers is of interest. However, the potential pathogenesis and involvement of the melanocortin receptors or other GPCRs needs to be elucidated.

15. Female fertility

Recently in this journal, MRAP2 was described to be significantly up-regulated in women with unexplained infertility (D'Aurora et al., 2019). *MRAP-α*, *MRAP-β* and *MRAP2* have low expression in the endometrium (Table 1). Hypertrophy of the uterine gland epithelium was observed in women with unexplained infertility. The increased *MRAP2* expression suggests that MRAP2 might influence the stability of the endometrium (D'Aurora et al., 2019); understanding the roles of MRAP and MRAP2 in the uterus and female reproductive axis may reveal a role for melanocortin signalling in infertility.

16. Conclusion and perspectives

In summary, genetic variants of *MRAP* are associated with severe familial glucocorticoid deficiency, however evidence is emerging that synthetic glucocorticoids may also disrupt ACTH and *MRAP* expression resulting in adrenal inflammation. *MRAP2* genetic variants are associated with severe obesity but the phenotypes are subtly different to the phenotypes observed with *MC4R* genetic variants. There is a great deal of interest in the development of melanocortin therapeutics to treat obesity, as evidenced by setmelanotide, and fully understanding the role of MRAP2 and the interactions with both melanocortin receptors and other GPCR would be of great value. The use of the *MRAP2* knockout and transgenic models have aided understanding of interactions with the different MCs in metabolic regulation, feeding behaviour and energy homeostasis. *MRAP* is detected in areas with absent or extremely low MC₂ expression, potentially it also may bind to non-melanocortin receptors. Further studies with transgenic models and insights from studies of genetic variants may provide further insights into the MRAP physiological processes.

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MRAP: https://en.wikipedia.org/wiki/Melanocortin_2_receptor_accessory_protein.

MRAP2: <https://en.wikipedia.org/wiki/MRAP2>.

Abbreviations:

5' UTR	5'- terminal untranslated region
ACTH	adrenocorticotrophic hormone
AgRP	Agouti-related protein

Asn	(Asparagine)-X (any amino acid except proline-Ser (serine)/Thr (threonine))
c-AMP	cyclic-adenosine monophosphate
CHO	Chinese hamster ovary cell line
CRE	c-AMP response element
CRH	corticotrophic releasing hormone
CXC1 and 2	chemokine receptors 1 and 2
ELISA	Enzyme-Linked Immunosorbent Assay
Falp	fat tissue specific low molecular weight protein
FGD	familial glucocorticoid deficiency
GHSR-1a	growth hormone secretagogue receptor-1a
Gnomon	NCBI eukaryotic gene prediction tool
GPCR	guanine protein coupled receptors
HDL and LDL	high and low density lipoproteins
HEK293	Human embryonic kidney 293 cell line
HPA axis	hypothalamic-pituitaryadrenal axis
HPA data	Human Protein Atlas data
IL-1 and 6	interleukin-1 and 6
INF-γ	interferon-gamma
MC	melanocortin receptor
MITF	microphthalmia transcription factor
MRAP	melanocortin 2 receptor accessory protein
MRAP2	melanocortin 2 receptor accessory protein 2
α-/β-/γ-MSH	alpha-/beta-/ gamma-melanocyte stimulating hormones
Myc-tag	c-myc derived polypeptide protein tag
OX1R	orexin receptor
PCR and RT-PCR	polymerase chain reaction and real time- polymerase chain reaction
PKA	protein kinase A
POMC	proopiomelanocortin prohormone

PRK-1	Prokineticin receptor-1
PVN	paraventricular nucleus of the hypothalamus
RNA-seq	RNA sequencing analysis
SIM1⁺	single minded 1 neurones
StAR	steroidogenic acute regulatory protein
TNF-α	tumour necrosis factor-alpha
WNT4	(<i>Wnt</i> family member 4)

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Fig. 1.

Dual topology of MRAP homodimer, MRAP2 homodimer and MRAP/MRAP2 heterodimer; the green transmembrane domain represents the structure and the dual topology with the amino (NH₂)/carboxyl (COOH) both being located extracellularly and amino (NH₂)/carboxyl (COOH) termini also intracellularly. This dual topology allows for homodimer (MRAP/MRAP; MRAP2/MRAP2) or heterodimer (MRAP/MRAP2; MRAP2/MRAP) formation. The LKANKS₃₁₋₃₇ (orange shading) immediately before the transmembrane region is required for dual topology, the LDYI₁₈₋₂₁ (yellow shading) is found only in MRAP and is required for ACTH binding to MC₂.

Fig. 2.

Predicted non-coding RNA, *MRAP-AS1* (XR_937664.1, shown in red) on chromosome 21q22.11 in relation to *MRAP* (blue).

Table 1

Expression of *MRAP-α*, *MRAP-β* and *MRAP2* in human tissues. +++: high expression; +: low expression.

Tissue	<i>MRAP-α</i>	<i>MRAP-β</i>	<i>MRAP2</i>	References
Adrenal gland	+++	+++	+++	Metherell et al., 2005 Chan et al., 2009
Testis	+++	+++	+	Metherell et al., 2005
Adipose	+	+	+	Xu et al., 2002 Metherell et al., 2005 Fagerberg et al., 2014
Breast	+++	+++	–	Metherell et al., 2005
Ovary	+++	+	+	Fagerberg et al., 2014
Endometrium	+	+	+	Fagerberg et al., 2014
Digestive tract	+	+	+	Fagerberg et al., 2014
Liver	+	–	–	Metherell et al., 2005
Heart	+	–	–	Metherell et al., 2005 Fagerberg et al., 2014
Skin	+	+	+	Metherell et al., 2005
Brain:	–	+	+++	Gardiner et al., 2002
Pre-frontal cortex		+	+++	Chan et al., 2009;
Cerebellum		+	+++	Fagerberg et al., 2014;
Hippocampus		+	+++	Chaly et al., 2009
Hypothalamus		–	+++	
Spinal cord		+	+++	
Pituitary gland	+	–	+++	Chaly et al., 2009
Thyroid gland	+++	–	–	Metherell et al., 2005

Table 2
Human *MRAP* mutations which have been described to be associated with familial glucocorticoid deficiency-type 2.

Type of mutation	Codon substitution- Codon number	Amino acid substitution	FGD-2 Mutation phenotype	Reference
Missense	G > A-1	Met1Ile	Disruptive	(Metherell, et al., 2005)
Missense	C > A-11	Tyr11Termination	Disruptive	(Metherell, et al., 2005)
Missense	T > C-26	Val26Ala	Disruptive	(Hughes, et al., 2010)
Missense	T > G-59	Tyr59 Asp	Disruptive	(Hughes, et al., 2010)
Missense	T > C-76	Val76Ala	Disruptive	(Hughes, et al., 2010)
Deletion	c.-17_23del	–	Disruptive	(Modan-Moses, et al., 2006)
Deletion	p.R451W in CYP11A1	–	*Disruptive (Primary adrenal insufficiency)	(Guran, et al., 2016)
Deletion	c.IVS3ds + 1delG	–	Disruptive	(Metherell, et al., 2005)
Deletion	c.130del	Val44insTer	Disruptive	(Metherell, et al., 2005)

* This mutation in *MRAP* sequence causes primary adrenal insufficiency.

MRAP2 mutations associated with obesity in humans. 1 - *MRAP2* heterozygous mutations detected using coding/intron-exon sequencing of *MRAP2* gene in coding exons; 2- two cohort studies with severe obesity; 3- patients with Prader-Willi like phenotype; 4- Genome - wide association study for severe early -onset obesity.

Table 3

Type of mutation	Codon substitution- Codon number	Amino acid substitution	Mutation phenotype	References
Missense	G > A-3	Ala3Thr	Uncertain significance	1 (Baron, et al., 2019)
Missense	G > T-3	Ala3Ser	Uncertain significance	1 (Baron, et al., 2019)
Missense	C > G-13	Gln13Glu	Uncertain significance	1 (Baron, et al., 2019)
Nonsense	G > T-24	Glu24Term	Disruptive	2 (Asai, et al., 2013)
Missense	G > T-31	Gly31Val	Disruptive	1 (Baron, et al., 2019)
Missense	C > T-32	Pro32Leu	Uncertain significance	1 (Baron, et al., 2019)
Missense	G > T-40	Ala40Ser	Non-pathogenic	3 (Geets, et al., 2016)
Missense	T > G-62	Phe62Cys	Disruptive	1 (Baron, et al., 2019)
Missense	A > G-77	Asn77Ser	Disruptive	1 (Baron, et al., 2019)
Missense	A > T-88	Asn88Tyr	Non-pathogenic	2 (Asai, et al., 2013)
Missense	T > C-91	Val91Ala	Uncertain significance	1 (Baron, et al., 2019)
Missense	G > C-99	Glu99Gln	Uncertain significance	1 (Baron, et al., 2019)
Missense	A > T-102	Lys102X	Disruptive	1 (Baron, et al., 2019)
Missense	A > G-113	Arg113Gly	Uncertain significance	1 (Baron, et al., 2019)
Missense	T > G-114	Ser114Ala	Uncertain significance	1 (Baron, et al., 2019)
Missense	C > G-115	Leu115Val	Non-pathogenic	2 (Asai, et al., 2013)
Missense	A > G-121	Asn121Ser	Uncertain significance	1 (Baron, et al., 2019)
Missense	C > T-125	Arg125Cys	Uncertain significance	1 (Baron, et al., 2019)
Missense	G > A-125	Arg125His	Non-pathogenic	4 (Schonnop, et al., 2016)
Missense	C > T-133	His133Tyr	Uncertain significance	1 (Baron, et al., 2019)
Missense	G > A-137	Ala137Thr	Non-Pathogenic	4 (Schonnop, et al., 2016)
Missense	T > C-162	Met162Thr	Uncertain significance	1 (Baron, et al., 2019)
Missense	A > G-174	Gln174Arg	Non-pathogenic	4 (Schonnop, et al., 2016)
Missense	A > G-193	Thr193Ala	Uncertain significance	1 (Baron, et al., 2019)
Missense	C > T-195	Pro195Leu	Disruptive	1 (Baron, et al., 2019)
Missense	G > T-203	Asp203Tyr	Uncertain significance	1 (Baron, et al., 2019)

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Type of mutation	Codon substitution- Codon number	Amino acid substitution	Mutation phenotype	References
Deletion	c.-5_5del	–	Loss of function	1 (Baron, et al., 2019)
Deletion	c.-3_7del	–	Loss of function	1 (Baron, et al., 2019)