The multifaceted melanocortin receptors

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Abstract

The five known melanocortin receptors (MC_s) have established physiological roles. With the exception of MC_2, these receptors can behave unpredictably and since they are more widely expressed than their established roles would suggest, it is likely that they have other poorly characterized functions. The aim of this review is to discuss some of the less well-explored aspects of the four enigmatic members of this receptor family ($MC_{1,3\text{-}5}$) and describe how these are multifaceted G-protein coupled receptors (GPCRs). These receptors appear to be promiscuous in that they bind several endogenous agonists (products of the proopiomelanocortin gene) and antagonists but with inconsistent relative affinities and effects. We propose that this is a result of post-translational modifications that determine receptor localization within nanodomains. Within each nanodomain there will be a variety of proteins, including ion channels, modifying proteins and other GPCRs,that can interact with the MC_s to alter the availability of receptor at the cell surface as well as the intracellular signalling resulting from receptor activation. Different combinations of interacting proteins and MC_s may therefore give rise to the complex and inconsistent functional profiles reported for the MC_s. For further progress in understanding this family, improved characterization of tissue-specific functions is required. Current evidence for interactions of these receptors with a range of partners resulting in modulation of cell signalling suggests that each should be studied within the full context of their interacting partners. The role of physiological status in determining this context also remains to be characterized.

Introduction

Melanocortin receptors (MC_s) are instrumental for a range of clinically-relevant physiological functions. MC_1 mediates pigmentation of both skin and hair, MC_2 is required for adrenal steroidogenesis and therefore the stress response, MC_3 and MC_4 modulate the central control of food intake and satiety and MC_5 regulates sebogenesis. These are essential functions: it might therefore be assumed that the receptors are both structurally and functionally well characterized. The aim of this review is to demonstrate that there are many aspects of the multifaceted MC_s that warrant further investigation.

For each of the 5 receptors identified to date, the primary and secondary structures are well described but the tertiary structures are only recently being revealed for some. Hhow structure relates to function is therefore a work in progress. Except for MC_2, the MC_s bind multiple ligands and this lack of specificity is an unusual feature of a G-protein coupled receptor (GPCR). Another exceptional feature is that some MC_s also have endogenous antagonists. Receptor activation is associated with a range of cellular responses, which at first was attributed to the multiple ligands that can activate the receptors. However, it is increasingly apparent that this explanation is inadequate: the reality is far more complex and context-dependent. MC_s are more widely expressed throughout the body than the functions described in the opening sentences might suggest, albeit in some tissues their expression is very low. Their functions in these other tissues are not well characterized if indeed known. More than one MC type may be expressed in a single tissue and even within the same cell. *In vitro* data suggests that MC_s can form heterodimers which may affect signalling on activation. Two melanocortin receptor accessory proteins, the MRAPs,

interact with the MC_s to influence MC signalling. MC signalling can be further modified by not just the MRAPs and MC interactions with each other but also through specific interactions with some other proteins as well as other GPCRs. Hence multiple factors need to be considered when trying to characterize each of the MC_s before we can further our understanding of the multifaceted MC family.

This review will not consider MC_2: it is the 'black sheep' of the family in that it only binds one of the melanocortin peptides. In the future though, we may learn more about the other MC_s by exploring why MC_2 is different.

Established physiological roles of MC_s and consequences of genetic variation

The complexity of the MC_s and their multifaceted features belies "textbook" views of a simpler range of functions, many of which are underpinned by overt human and mouse phenotypes resulting from mutations: these are described briefly below to put in context the more complex aspects we will describe later.

MC_1

In epidermal and hair follicle melanocytes, MC_1 regulates the synthesis of eumelanin (black/brown) pigments. The MC_1 gene is highly polymorphic in individuals of European ancestry, but not in those of African ancestry, and many of the 80 plus variants identified to date produce a non-functional receptor (1). Loss-of-function in MC_1 results in an increase in the relative amount of phaeomelanin (yellow/red) to eumelanin synthesized. The resulting phenotype is fair skin, freckles and red hair (red hair colour (RHC) variants). An association between fair skin and the incidence

of melanoma has generated interest in these variants. RHC variants are associated with an increased susceptibility to developing both melanoma and non-melanoma skin cancers however not all variants associated with an increase in skin cancer susceptibility are also associated with changes in pigmentation (2). Not all loss-of-function is associated with reduced cyclic AMP (cAMP) activity on receptor activation as some of the variants result in a reduced number of receptors at the cell membrane suggesting dysfunctional receptor trafficking (3).

MC_3

The MC_3 knockout (KO) mouse has reduced lean body mass and increased fat mass resulting in an obese phenotype (4,5). The association between human MC_3 gene variants and obesity is still unclear due to the rarity of such variants (6). The two most common variants, T6K and V81I, have been reported by some as associated with an obese phenotype but by others not: a mouse model with these two variants is obese (6).

Both male and female MC_3 KO mice have impaired linear growth (5). Screening whole-exome sequence data of 200,000 individuals from the UK Biobank revealed over 170 different variants in the human MC_3 gene (7):all are exceptionally rare. The researchers selected the 3 most common variants and did sophisticated analyses using all 500,000 participants in the UK Biobank to demonstrate that these 3 variants were each associated with shorter stature (7). MC_3 co-localizes to GHRH neurones in the hypothalamus and the authors suggest that MC_3 may therefore act at the level of the hypothalamus to regulate height. Rat anterior pituitary somatotrophs both express MC_3 and respond to melanocortins (8,9) so the potential involvement of pituitary function in this phenotype should not be ignored.

MC_4

Like the MC_3 KO, the MC_4 KO mouse is obese, however, there are substantial differences between the two KOs; in particular, the MC_3 KO is hypophagic and has reduced linear growth whilst the MC_4 KO mouse is hyperphagic with increased linear growth (10). Appreciation of a possible role for MC_4 in regulating body weight in the mouse (10,11),prompted a search for variants resulting in obesity in humans. Back-to-back publications reported the identification of two individuals and some of their family members who were heterozygous for a rare frame-shift variant that resulted in a truncated MC_4 and therefore a non-functional receptor: the affected individuals were all obese (12,13). From then, the focus on MC_4 has been mainly on its roles in regulating appetite. Using publicly available data, a number of heterozygous loss-of-function variants in MC_4 with associations to body weight have been identified (14,15). Variants with a loss of function resulting in reduced generation of cAMP on receptor activation are associated with weight gain, whilst gain of function mutations, which result in biased increased beta-arrestin recruitment followed by increased mitogen-activated protein kinase (MAPK) pathway activation, are associated with a lean phenotype (14). Body weight is not just a function of appetite: evidence is accumulating that MC_4 in the dorsal raphe may also have a role in regulating both thermogenesis and locomotion, and hence energy expenditure (16). Both POMC and AgRP neurones in the arcuate have projections to the dorsal raphe (17,18). Within the dorsal raphe, there are both GABAmergic and glutamergic neurones that express MC_4 (18,19). Activation of GABAergic neurones by α-MSH results in decreases in firing rate concomitant with decreases in food intake (19,20). Increased prolylcarboxypeptidase, an enzyme which results in decreases in available synaptic α-MSH (21), was associated with increased thermogenesis and

137 locomotion (20). Arcuate AgRP acts as an inverse agonist on MC_4 expressing-
138 glutamatergic neurones within the dorsal raphe, resulting in activation of a cluster of
139 5HT-neurones also within the dorsal raphe. These serotonergic neurones stimulate
140 thermogenesis without eliciting an effect on food intake (18).

141 To date the focus has remained on centrally expressed MC_4 and their role in body
142 weight regulation, however, there is evidence for MC_4 expression in the periphery
143 (EMBL-EBI gene expression atlas).

144 *MC_5*

145 The only phenotype observed in global MC_5 KOs was that the mice took longer to
146 dry their fur after doing swim tests because of reduced sebogenesis (22). In a study
147 of the human gene, five variants were identified in a small sample of individuals with
148 skin/sebaceous gland disorders, however, these same variants were also found with
149 a similar distribution in individuals of a wide range of ethnicities that were
150 phenotypically normal (23). In mouse models, there are also reports of roles for MC_5
151 in regulating immunological responses in autoimmune disorders of the eye, fatty acid
152 oxidation in skeletal muscle and lipolysis in adipocytes (24-28). To date there are no
153 reported variants in the human gene associated with any of these roles.

154

155 **Tissue distribution of the MC_s**

156 Perhaps based on the results of the studies described above it is generally thought
157 that MC_1 is confined to the integumentary system, MC_3 and MC_4 to the central
158 nervous system (CNS) and MC_5 to exocrine glands. To date, the validity of these

conclusions have been hampered by the inability to specifically identify the different MC_s using immunohistochemical approaches. The commercially available antibodies for the MC_s are not specific (example (29)). Databases such as the EMBL-EBI gene expression atlas suggest that all four receptors are more widely distributed throughout the body and basic searches of available literature identifies multiple reports of expression in other tissues, albeit with varying strength of evidence. Importantly, it is evident that some tissues express more than one type of MC: perhaps even within the same cell (30). *In vitro*, it is known that MC_s can heterodimerize with each other so a better understanding of within-tissue expression is required. The advent of multiplex nucleic acid *in situ* hybridization technologies, like RNAscope©, are enabling better precision in identifying MC expression patterns.

The lack of clarity of the tissue distribution of the MC_s has important consequences for fully understanding the aetiology of some of the phenotypes associated with MC variants, as these may in part be due to dysfunction of the receptor in peripheral tissues. For example, MC_4 is expressed in the heart (EMBL-EBI gene expression atlas, (31)) and therefore some of the associations with cardiovascular dysfunction (32,33) may be due to direct effects on cardiac function and not sequelae of obesity and/or central MC_4 effects. Insulin release is decreased in both lean and obese rats following treatment with NDP-MSH, a synthetic agonist of MC_4 (34). Given that MC_4 is expressed in the pancreas (EMBL-EBI gene expression atlas, (34)), dysfunctional insulin release from the pancreas may contribute to the obesity linked to MC_4.

Why are the MC_s unique amongst GPCRs?

MC ligands

Except for MC_2, which is highly selective for adrenocorticotrophic hormone (ACTH), $MC_{1,3\text{-}5}$ interact with each of the melanocortin proteins derived from the post-translational cleavage products of the proopiomelanocortin (*POMC*) gene. The melanocortin proteins are alpha-, beta- and gamma-melanocyte stimulating hormone (α-, β-, γ-MSH) and ACTH. All the melanocortin ligands, have a conserved HFRW motif (35) with the motif found at the base of the 'U' in their U-shaped three-dimensional structures. The benzene ring of the phenylalanine of the HFRW motif penetrates deeply into the TMD core of the receptor (36-38) and results in the downward movement of two phenylalanines (F257 and F280) in MC_1 and a leucine (L133) in MC_4. The downward movement of these residues in turn pushes on residues (W254 on MC_1 and W258 on MC_4) that act as toggle switches on TMD6. When switched on, TMD6 moves outward and the receptors are activated.

There are also two other gene products that bind to MC_s: an inverse agonist, agouti-related protein (AgRP), that is specific for MC_3 and MC_4 (39,40); and an antagonist of α-MSH, agouti-signalling peptide (ASIP), that competes for binding to MC_1 and MC_4 (41). The ligands can be released into the circulation or act in an autocrine or paracrine way. This ligand diversity and their inconsistent potencies at each MC (described further below) is unique amongst GPCRs.

MC structure

The 5 MC_s identified to date are all members of the α-subfamily of class A (rhodopsin-like) GPCRs and the human receptors share 42-67 % of their amino acid sequences (42). There are strong similarities between the reported tertiary structures of MC_1 and MC_4 (36-38), hence it could be assumed that the other MC_s will also be structurally similar.

The MC_s have several structural features that set them apart from other class A GPCRs. First, the receptors are short (ranging from 297360 amino acids) and compared to other class A GPCRs have relatively short N- and C- termini (42). Secondly, both MC_1 and MC_4 have a wide extracellular opening to the orthosteric ligand binding pocket (36-38). The width is due to an exceptionally short second extracellular loop (ECL2), a lack of the conserved cysteines in transmembrane domain 3 (TMD3) and ECL2 found in other class A GPCRs, and the absence of conserved prolines in TMD2 and TMD5 that are present in other class A GPCRs (42). Extracellular Ca^{2+} has long been recognised as a co-factor for melanocortin binding (43). Within TMD2 and TMD3 are 3 conserved residues unique to the MC_s, which form a Ca^{2+}-binding pocket in conjunction with 3 conserved amino acids in the ligands (36-38). Calcium ion binding is important for agonist interaction but not for that of antagonist (37). To date, no one has reported the tertiary structure in the presence of the MRAPs and/or other GPCRs that the MC_s are known to interact with.

Post-translational modification of MC_s

Several studies have demonstrated the importance of the conserved cysteine(s) in the C-terminus for normal function of the MC_s. These cysteines are sites for post-translational modification by palmitoylation, which involves the enzymatic addition and removal of a palmitic acid to the cysteine.

Lack of palmitoylation of C315 in the cytoplasmic tail of MC_1 prevents proper receptor function. The zDHHC-protein acyl transferase (zDHHC-PAT), zDHHC-PAT13, responsible for palmitoylation of MC_1 is phosphorylated by UVB light (44). Increasing the interaction of MC_1 with phosphorylated zDHHC-PAT13, results in

greater MC_1 activation as seen by increases in palmitoylation, cAMP production, DNA repair and decreases in cell senescence (44). In the presence of a mutated palmitoylation site, no rescue was achieved with increased phosphorylation and/or increased amounts of ZDHHC-PAT13 (44).

In humans, two cysteine residues in the cytoplasmic tail of MC_4 have been identified as predicted sites for palmitoylation (https://swisspalm.org/), which has been confirmed in studies by Moore and Mirshahi (45). This group has also suggested a functional consequence of loss of palmitoylation. MC_4 variants that result in a truncation of the region of the cytoplasmic tail that is palmitoylated leads to loss of receptor function and is associated with altered BMI: the authors speculate that palmitoylation stabilises receptor localisation at the cell surface. Further analysis is required to establish the consequences of MC_4 palmitoylation and identify the ZHHCs, as well as specific depalmitoylating enzymes, regulating this post-translational modification.

Both MC_3 and MC_5 have two cysteines in their cytoplasmic tails, which are predicted to be palmitoylated (https://swisspalm.org/): at C315/C317 and C311/312, respectively. To date there are no reports that describe these cysteines in any detail, however, mutation of the residue separating C315/C317 (pG316D) in MC_3, has been reported to result in a lean phenotype (6). We predict that this amino acid change is sufficient to prevent palmitoylation and hence anchoring of the cytoplasmic tail to the cell membrane and that a similar mechanism may be essential for the normal function of several MC_s. Diet, in particular fatty acids, have been shown to modulate palmitoylation (46) therefore one might speculate that MC function may also be modified by diet.

Not all MC signalling is mediated through cAMP

Canonical MC signalling

Initially descriptions of MC activation concurred that all MC_s are $G\alpha_s$–coupled, activating adenylyl cyclase, which in turn catalyzes the conversion of ATP to cAMP. cAMP is a second messenger and will initiate an intracellular cascade, often through activation of protein kinase C (PKC). MC activation and signalling is terminated by recruitment of beta-arrestin, which traffics the receptor back to endosomes.

The affinities and potencies reported for the different ligands at each MC are highly variable between studies. To understand this variability we systematically reviewed the literature reporting the cAMP response to different ligands for $MC_{1,3\text{-}5}$. We found 100-fold differences in the published EC50s (potencies) for $MC_{1,3\text{-}5}$ in response to the same ligand (Figure 1). These fold differences were even found in data published by the same laboratories. As will be reviewed below, the cAMP response to different MC ligands is complex and context-dependent. We suggest that the significant range of cAMP responses measured is a function of receptor interaction with other proteins and/or receptors.

Evidence for signalling through other G protein alpha (Gα) subunits

Some of the MC_s may interact with other Gα subunits: $G\alpha_i$ and/or $G\alpha_{q/11}$ (MC_3 (47); MC_4 (48)). In neuronal cell culture, it has been demonstrated that activation of MC_4 by α-MSH in neurones of the paraventricular nucleus (PVN) results in activation of $G\alpha_{q/11}$ and not $G\alpha_s$ (49). What has yet to be determined are the mechanisms that switch a MC from interacting with $G\alpha_s$ to $G\alpha_i$ or $G\alpha_{q/11}$.

Gα independent coupling with Kir7.1

279 Kir7.1 is an inwardly rectifying K^+ channel and coupling with MC_1 and MC_4 has been
280 demonstrated (50). In the PVN, the depolarization and hyperpolarization induced by
281 α-MSH and AgRP, respectively, occurred independently of $G\alpha$ pathways
282 downstream of MC_4 (50). MC_4 appears to be unusual among the GPCRs assessed
283 to date, in that it does not modulate the activity of Kir7.1 via glycosylation (51).
284 Targeted deletion of Kir7.1 in MC_4 expressing cells of the PVN, resulted in the failure
285 of α-MSH to activate these MC_4 neurones and blocking of associated phenotypes
286 (52). By contrast, the phenotypes associated with the activation of MC_4 by AgRP
287 were not blocked. Recent tertiary structural analysis suggests that MC_4 signalling
288 associated with coupling to Kir7.1 also requires Ca^{2+} binding (53). It is not yet known
289 if coupling to Kir7.1 is a generic property of the MC_s or unique to MC_4 and possibly
290 MC_1.
291 *Constitutive activity of* MC_s
292 MC_s appear to have constitutive cAMP-generating activity: the evidence for MC_1 and
293 MC_4 are the most compelling though. *Pomc* KO mice maintain normal coat colour
294 even in the absence of endogenous ligands, whilst MC_1 knockout mice are yellow
295 (phaeomelanin), suggesting that the MC_1 constitutive activity in the absence of
296 endogenous ligands is sufficient to maintain coat colour (54). MC_4 has some
297 constitutive activity and AgRP is able to act as an inverse agonist in the presence of
298 this activity (55).
299 Whether MC_3 is constitutively active is debatable: some report that the human MC_3 is
300 not (56,57) whilst others report some basal activity as measured by cAMP. The
301 constitutive activity may therefore be species and context-dependent (58). A mutant
302 form of MC_3 (F347A) is constitutively active (58): its basal cAMP activity is about 7-
303 fold greater than that for wild type MC_3. Even in the absence of ligand, mouse or

304 human MC_5 stably transfected into B16/G4F melanoma or HEK293 cells,
305 respectively, produced cAMP (40,59) although others have not detected this (57).
306 In the late 1990s, it had been concluded that the N-terminus could be removed from
307 all four receptors without effects on receptor function (60). However, later work on
308 MC_4 showed that the N-terminus acts as a tethered ligand and is responsible for the
309 constitutive activity of the receptor (55,61). MC_4 constitutive activity has been shown
310 to be augmented in the presence of human MRAPa (the long isoform of human
311 MRAP1) and may be due to human MRAPa enhancing N-linked glycosylation of the
312 N-terminus of MC_4 (57,62). Constitutive activity may provide tone to a signalling
313 pathway; that is, the ability to move in either direction from a set point.
314 *Biased signalling*
315 The early understanding of the role of beta-arrestin in GPCR signalling was that it
316 terminated the intracellular signalling cascade initiated by the $G\alpha$ subunit. It is now
317 understood that beta-arrestin can initiate its own signalling and this activity can occur
318 once the activated receptor has been internalized and is in the early endosome.
319 Regardless of the usual $G\alpha$ subunit a GPCR normally activates, its associated beta-
320 arrestin can also recruit $G\alpha_i$ to form a complex that interacts with the ERK1/2
321 pathway (63). Others have previously demonstrated that MC activation can
322 upregulate the MAPK ERK1/2 pathway independent of cAMP but dependent on PI3K
323 (MC_1 (64,65); MC_3 (66); MC_4 (67); MC_5 (68,69)) and inhibit the MAPK c-Jun N-
324 terminal kinase (JNK) pathways (MC_4 (70); MC_5 (71)).
325 Biased agonism by AgRP binding to MC_3 and MC_4 has been demonstrated (72,73):
326 activation of either receptor with AgRP independently stimulates the ERK1/2
327 pathway whilst decreasing cAMP activity. Whether any of these pathways are beta-
328 arrestin dependent is unknown, as is the extent of biased signalling.

Modulators of MC expression and activation

There is accumulating evidence that several proteins can interact with the MCs to modulate their activation; including the MRAPs,membrane bound attractin, mahogunin ring finger and defensin. The latter three proteins will not be discussed in this review. MC activation may also be modulated by whether the receptor is acting as a monomer, homodimer or heterodimer with other MCs or other GPCRs. What is not yet clear is how these putative modulators of MC activation exert their effects. It is possible that these modulators result in biased signalling and/or regulate the number of MCs presenting at the cell membrane.

MRAPs modulate MC activity

MRAP1 and MRAP2 interact with and regulate the function of all members of the MC family (74) as well as other GPCRs (75-77). While MRAP1 is present as antiparallel homodimers at the plasma membrane (78), MRAP2 can also form parallel homodimers as well as higher order oligomers (79). Both MRAPs are widely expressed in tissues, including the brain, pituitary, adrenal gland, testis, ovary, lung and heart (74,75,80), which in part overlaps with expression of MCs. Co-expression of MRAP2 with MC_3 and MC_4 in the same cells has been demonstrated at the RNA level (81).

The complexity of the MC family is further evidenced by the contradictory influence of MRAPs on MC function, MC_3 being a prime example. Co-expression of human MRAP2 and MC_3 has been shown to either reduce (81) or have no influence (74) on MC_3 surface expression. Human MRAP1 and MRAP2 increased MC_3 cAMP signalling in response to α-MSH (57,81), whereas human MRAP2 inhibited and MRAP1 did not significantly influence the MC_3 cAMP response to NDP-α-MSH in

another study (74). Chicken MRAP2 produced a 9-fold increase of the potency of chicken ACTH(1-39) at MC_3 (82), whereas co-expression of chicken MRAP2 and MC_3 had no effect on the potency of human ACTH(1-24) (83). Zebrafish MRAP2a or MRAP2b had no significant effect on α-MSH-induced MC_3 cAMP signalling at a ratio of 1:5 of receptor to MRAP2 (84), whereas MRAP2 of the related channel catfish inhibited the cAMP response of MC_3 to α-MSH at the same receptor to MRAP2 ratio (85).

The divergent effects of the interaction between MRAPs and MC_s highlight the influence of context on MC function. The concentration of MRAPs appears to be one of the context-dependent factors that influence MC activity, as MRAP2 alters receptor function differently depending on the expression ratio of MRAP2 to MC (81,85,86). What underlies the dose-dependent effects is unclear, however, the ability of MRAP2 to form different homo-oligomeric conformations, each with a potentially different effect on receptor function, may play a role (79,86). As MRAP2 is differentially expressed across zebrafish development (84) and in the endometrium during different stages of the human menstrual cycle (87), altering the cellular concentration of MRAP2 may be an additional mechanism used by organisms to fine-tune MC signalling.

MC homo- and heterodimerization

All 5 MC_s have the ability to homodimerize (88-91). While the exact cellular ratio of receptor monomers to homodimers is unclear, the prevalence of homodimers can be regulated by ligands and interacting proteins with potential functional consequences. ACTH binding increases MC_2 homodimerization (92), MRAP1 reduces the plasma membrane concentration of MC_5 by inhibiting MC_5 homodimerization (91) and disruption of MC_4 homodimers increases receptor-mediated cAMP accumulation

(93). Furthermore, MC_4 has two tandem binding sites with different ligand binding affinities and kinetics, likely corresponding to sites on receptor homodimers (94). Homodimerization may therefore produce additional MC states with new functional properties and distinct interactions with other membrane proteins.

Bioluminescence resonance energy transfer and co-immunoprecipitation assays have provided evidence for physical association between different MC_s. Heterodimerization between flounder MC_1 and MC_5 (95), human MC_1 and MC_3 (88) and mouse MC_3 and MC_4 (96) has been demonstrated in transfected cells. The receptor pairs are also co-expressed *in vivo*: MC_1 and MC_5 in flounder melanophores (97), MC_1 and MC_3 in alveolar macrophages (98) and MC_3 and MC_4 in the murine hypothalamus (81). Studies of the functional significance of MC interactions to date, indicate that any effects are highly ligand dependent. The efficacy of α-MSH in cells co-transfected with flounder MC_1 and MC_5 was significantly lower than in cells transfected with either MC_1 or MC_5, whereas the efficacy of desacetyl-α-MSH was significantly increased in double transfected compared to single transfected cells (95). Co-expression of mouse MC_3 and MC_4 had no significant effect on the potencies of α-MSH, NDP-α-MSH or melanotan II, whereas the potency of bivalent ligand CJL-1-87 was moderately increased in cells expressing both MC_3 and MC_4 compared to a mixture of cells expressing MC_3 and MC_4 separately (96).

The interaction partners of MC_s are not limited to members of the MC family

Recently, Li et al. provided a significant advance towards characterizing the protein interactomes of MC_3 and MC_4 by identifying 23 and 32 GPCRs, respectively, that physically associate with the two MC_s *in vitro* (99). The functional consequences of receptor co-expression were diverse, with inhibition, potentiation and no effect on MC_3 and MC_4 signalling observed depending on the GPCR partner present.

Previous studies have also described interactions between various GPCRs and MC_3 and MC_4 (100,101). Despite the attested ability of the receptors to heterodimerize at the membrane, the reported effects of receptor co-expression on signalling activity may also arise due to crosstalk between signalling pathways. Such a mechanism may account for the combined effects of α-MSH and endothelin-1 on melanocyte function (102) and the signalling crosstalk between MC_3 and the GH secretagogue receptor (103).

Future perspectives

Understanding MC signalling

As described above, there are many examples of non-canonical MC signalling and therefore understanding what factors determine MC signalling is critical for optimizing the selectivity and efficacy of pharmacological interventions. Recently, setmelanotide received FDA approval for chronic weight management for patients with, in effect, genetic ablation of POMC, PCSK1 (proprotein convertase subtilisin/kexin type 1: responsible for cleavage of POMC resulting in α-MSH and ACTH) or LEPR (leptin receptor). A series of clinical studies demonstrated significant weight loss in these patients and chronic treatment was not associated with the negative side-effects seen with the use of other agonists (104-106). Its use has not been without off target effects though. Individuals with genetic ablation of POMC and PCSK1 are characteristically fair with red hair. After extended treatment with setmelanotide, the hair colour of these individuals became brown demonstrating that the setmelanotide is also acting on MC_1 (107). This is not surprising since

setmelanotide is known to also interact with both MC_1 and MC_3 albeit with lower potencies (108). Setmelanotide has biased $G\alpha_{q/11}$ signalling and binds to MC_4 differently to α-MSH (36). Although the identification of a highly selective agonist or antagonist for any of the MCs remains elusive, compounds like setmelanotide have already and will continue to provide insight into the MC_s's structure and function as well as having therapeutic use.

Understanding GPCR crosstalk

To date, research on GPCR crosstalk has mostly been limited to interactions between two partners due to the lack of techniques for detecting large multi-member protein oligomers (109) and the challenge of untangling the complex functional effects caused by interplay between several proteins. New techniques have generated demonstrations of the formation of higher-order receptor oligomers (109-112). Such "receptor mosaics" (113) may not only be composed of several different GPCRs but also of accessory proteins, ion channels and other types of receptors which together determine the functional properties of the larger unit (114).

The diverse interaction profiles of the MC_s suggest that the receptors participate in larger heteromeric complexes. The GPCRs interacting with MC_3 and MC_4 are all expressed in the hypothalamus, many of them in the same cells (99). Given that several of the GPCRs heterodimerize with each other in addition to interacting with MC_s, the number of possible oligomeric complexes that may form is staggering. Which of these interactions occur *in vivo* and what determines the oligomeric species present at any one time remains unclear, however, the cellular context is likely to have a major influence. Complex interactions between MC_s and the many different proteins that make up the cellular environment may therefore give rise to context-

dependent functional units, which each respond to ligands in a unique manner. Differential expression of some of these functionally diverse MC complexes between different cell lines and cell states may help explain the variable potencies reported for MC_s.

What is the role of nanodomains in producing context-dependent functional units? Within a cell, there is the possibility of a variety of nanodomains (115): a localized membrane environment that may contain “receptor mosaics”, hetero- or homodimers of the MC_s along with different G proteins, beta-arrestins and accessory proteins. MC_3 transfected into a mouse neuronal cell line localizes to lipid rafts (116), one type of nanodomain. Organization of different MC oligomers into distinct nanodomains could provide spatial separation of signalling responses and contribute to the diverse MC responses observed since the distribution and makeup of lipid rafts is heterogeneous between and within cell types (117,118). The importance of MC compartmentalization has already been shown for MC_4, which requires MRAP2-mediated trafficking into primary cilia in the PVN for its anorexigenic effect (119,120). The presence of MC_4 on primary cilia may also be indicative of another role; that is, that the MC_s may be involved in volume transmission (121).

Understanding how these different membrane proteins are compartmentalized to different nanodomains, and the potential role for post-translational modifications such as palmitoylation, is going to be essential to understanding the diversity of responses following MC activation.

Do specific MC_s have roles in a broader range of tissues?

In order to advance our understanding of MC biology, further research into their exact cellular localization throughout the body is required. The development of a 'rainbow' mouse expressing each of the MC_s tagged with a different fluorophore might therefore be useful. Determining the exact cellular localization of each of the potential interactors with the MC_s is also required and whether physiological status changes the combinations of interactors. In the absence of specific antibodies for each of the MCs, this may be possible by multiplex RNAscope© combined with tissue optical methods (example, (122)). The interactions of MC_s with other GPCRs suggests they may act as the conductor of an orchestra by monitoring the activity of these other GPCRs – perhaps through oligomerization - to regulate their signalling and therefore cellular responses. Further structural studies to determine the tertiary structures of each of the MC_s with and without either MRAP as well as with and without different receptor dimers are also required. So many facets of MC_s remain to be fully explored and understood.

Conclusions

The focus of a wide body of research on specific roles for the MC_s attests to their importance in physiology, however, their potential importance in the function of a range of other tissues is currently unclear. This has implications for both understanding mechanisms leading to disease as well as their characterization as therapeutic targets. Study of these other roles, as well as those that are well-established, will require a more complete understanding of their multifaceted biology and how this relates to ligand specificity, as well as modulation of signalling from these GPCRs in the context of their interacting partners.

Data availability

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

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Legends

Figure 1. Reported potencies (EC50) of endogenous and exogenous melanocortin ligands for the melanocortin receptors in the published literature. To obtain the values, Web of Science was searched for “potenc*” or “ec50” and the names of the receptors using their various naming conventions. Only values obtained with

890 the following methodologies were included: untagged receptor constructs
891 transfected into a cell line and receptor activity measured in a cyclic AMP or cyclic
892 AMP response element (CRE) based assay. Values from literature reviews were
893 excluded. All values given for melanocortin receptor 1 (MC_1) are for the MC_1a
894 isoform only. Abbreviations: MC (melanocortin receptor), MSH (melanocyte-
895 stimulating hormone), ACTH (adrenocorticotrophic hormone), MTII (melanotan
896 II), NDP ([Nle4, D-Phe7]).