Structural determinants regulating cell surface targeting of melanocortin receptors

A R Rodrigues^{1,2}, D Sousa³, H Almeida^{1,2} and A M Gouveia^{1,2,4}

¹Department of Experimental Biology, Faculty of Medicine, University of Porto, Alameda Prof. Hernâni Monteiro, 4200-319 Porto, Portugal

²Instituto de Biologia Molecular e Celular (IBMC), University of Porto, Porto, Portugal

³IPATIMUP, Institute of Molecular Pathology and Immunology, and ⁴Faculty of Nutrition and Food Sciences, University of Porto, Porto, Portugal

Correspondence should be addressed to A M Gouveia
Email
agouveia@med.up.pt

Abstract

Melanocortin receptors (MCRs) belong to the G-protein-coupled receptor family of transmembrane proteins. They recognize specific ligands named melanocortins that are mainly produced in the pituitary and hypothalamus. Newly synthesized MCRs at the endoplasmic reticulum are subjected to quality control mechanisms that screen for the correct structure, folding or processing, essential for their proper cell surface expression. Some motifs, located at the N- or C-terminus or even on transmembrane and in loop regions, have been implicated in these biological processes. This article reviews these specific domains and the role of accessory proteins and post-translation modifications in MCRs' targeting to cell surface. Additionally, promising approaches involving pharmacological stabilization of misfolded and misrouted mutant MCRs, which improve their forward transport, are reported. Understanding the MCRs' structural determinants fundamental for their proper cell surface integration is essential for correcting abnormalities found in some diseases.

Key Words

- ▶ melanocortin receptors
- ▶ anterograde transport
- ▶ cell surface
- ▶ targeting domains

Journal of Molecular Endocrinology
(2013) 51, R23–R32

Introduction

Melanocortin receptors (MCRs) have received increased attention by the pharmaceutical industry due to their involvement in pigmentation, steroidogenesis, energy homeostasis, sexual function, inflammation and exocrine gland regulation. MCRs are a subfamily of G-protein-coupled receptors (GPCRs), a large group of cell surface receptors that is divided into six classes, based on their homology and the affinity for the native ligands. Among these classes, only four are present in multicellular organisms: the rhodopsin/ β_2 -adrenergic-like receptors (class A), the adhesion and secretin-like receptors (class B), the metabotropic glutamate/pheromone receptors (class C) and the frizzled/taste 2 receptors

(class F) (Gether 2000, Schioth & Fredriksson 2005, Latek *et al.* 2012). The class A is the largest one and includes all the MCRs.

The life cycle of the MCRs is still not deeply characterized but, as for all GPCRs, it is assumed to start in endoplasmic reticulum (ER), undergo several post-translational modifications along the secretory pathway (e.g. glycosylation, ubiquitination and oligomerization) and finish at the cell surface. Correct folding and assembly result from the coordinated interaction with several molecular chaperones, heat shock proteins or accessory proteins (Cooray *et al.* 2009, Cooray & Clark 2011, Meimaridou *et al.* 2011). Misfolded polypeptides

are retained in the ER and later degraded by the ubiquitin–proteasome system.

MCRs that correctly reach the cell surface are able to bind extracellular ligands and activate specific signalling pathways, commonly by intermediate of G proteins (Gantz & Fong 2003). Signalling is frequently attenuated by desensitization and/or internalization of receptors (Shinyama *et al.* 2003, Kilianova *et al.* 2006, Benned-Jensen *et al.* 2011, Rodrigues *et al.* 2012), which may recycle and reach the plasma membrane or address to lysosomes for degradation (Jean-Alphonse & Hanyaloglu 2011).

Cell surface targeting of MCRs is crucial for binding to their extracellular ligands and depends on small amino acid domains present at the protein primary sequence, diverse post-translational modifications or protein interactions. This review outlines the current knowledge about the molecular characteristics of these receptors, fundamental to reach the cell surface.

Melanocortin system: receptors, ligands and accessory proteins

MCRs belong to class A of the GPCR superfamily and include a five-member group made up of MC1R, MC2R, MC3R, MC4R and MC5R, named by the order of their cloning. MCRs are activated by a variety of neuropeptides, termed melanocortins, that include the adrenocorticotrophic hormone (ACTH) and α , β and γ -melanocyte-stimulating hormones (MSHs). Melanocortins derive from post-translational processing of the common polypeptide precursor pro-opiomelanocortin, expressed mainly in the hypothalamus and pituitary (Eves & Haycock 2010, Cooray & Clark 2011).

The study of MCRs began in the 1990s with the identification of MC1R, a receptor with high affinity to α -MSH (Chhajlani & Wikberg 1992, Mountjoy *et al.* 1992). This receptor is expressed in different skin cell types, including melanocytes, keratinocytes and epithelial cells. MC1R is directly related to regulation of melanogenesis but also presents anti-inflammatory effects (Garcia-Borrón *et al.* 2005). The ACTH receptor was simultaneously discovered and was termed MC2R (Mountjoy *et al.* 1992). It is abundantly expressed in the adrenal cortex, where it mediates stress responses by regulating steroidogenesis and, consequently, glucocorticoid synthesis and release. Mutations in MC2R are responsible for the human familial glucocorticoid deficiency (FGD), a rare autosomal disease caused by severe adrenocortical failure (Tsigos *et al.* 1993). MC2R is also found in the skin (Slominski *et al.* 1996) and

in mouse adipocytes (Boston & Cone 1996), where it has been suggested to promote lipolysis (Boston 1999, Moller *et al.* 2011) and decrease leptin production (Norman *et al.* 2003). MC3R and MC4R are mainly expressed in the CNS and control energy homeostasis (Mountjoy 2010). This function is illustrated by the severely obese *Mc4r* knockout mice and MC4R human gene mutations that underlie the most prevalent syndrome of monogenic obesity (Huszar *et al.* 1997, Vaisse *et al.* 2000, Lubrano-Berthelier *et al.* 2006). The last MCR to be discovered was MC5R, the most ubiquitous receptor among the melanocortin family with a wide peripheral distribution (Gantz *et al.* 1994, Griffon *et al.* 1994, Labbe *et al.* 1994). MC5R has an important role in the regulation of exocrine gland secretion (Chen *et al.* 1997), fatty acid oxidation in muscle (An *et al.* 2007) and lipolysis and re-esterification in adipocytes (Rodrigues *et al.* 2013).

Similar to other GPCRs, the MCRs' anterograde transport is assisted by accessory proteins. This idea was first suggested by Noon *et al.* (2002), who failed to obtain a correct trafficking of MC2R to the cell surface when the receptor was expressed in cells that lacked endogenous expression of the melanocortin system. Subsequent work revealed that this forward transport is mediated by specific proteins named melanocortin 2 receptor accessory proteins (MRAPs; Metherell *et al.* 2005). Although only MC2R requires MRAPs for proper trafficking to cell membrane, all the other MCRs interact with them (Chan *et al.* 2009, Sebag & Hinkle 2009a).

MRAPs comprise two closely related proteins: MRAP1 and MRAP2. MRAP1 is mainly expressed in the adrenal gland and presents two isoforms, MRAP α and MRAP β , produced by alternative splicing. In addition to MC2R, mutations in MRAP1 are also responsible for FGD (Metherell *et al.* 2005). MRAP2 is found in the adrenal gland and, at high levels, in the hypothalamus (Chan *et al.* 2009). Both MRAP1 and MRAP2 form anti-parallel homodimers and heterodimers that stably associate with MC2R to assist its targeting to the cell membrane, facilitating ER exportation and further post-translational glycosylation at the Golgi apparatus (Sebag & Hinkle 2007, Chan *et al.* 2009). Recent data revealed that MRAP1 α is responsible for the enhancement of MC2R and MC4R asparagine-linked glycosylation (N-linked glycosylation) complexity (Kay *et al.* 2013). Unlike MC2R, all the other MCRs traffic efficiently to the plasma membrane in the absence of MRAPs, but when MRAP1 is present, MC4R and MC5R trafficking is impaired while there is no effect on the cell surface expression of MC1R and MC3R (Sebag & Hinkle 2007, 2009a, Chan *et al.* 2009).

In addition to their role in the anterograde transport of MCRs, MRAPs also regulate the receptors' function. Chan *et al.* (2009) demonstrated that both MRAP1 and MRAP2 enhance MC2R but inhibit MC1R, MC3R, MC4R and MC5R signalling ability. By contrast, Sebag & Hinkle (2009b) showed defects on MC2R signalling when co-expressed with MRAP2 despite its correct trafficking to the cell membrane. These contradictory results may be explained considering the ACTH concentration used in the assays, as only supraphysiological doses of ACTH used by Chan *et al.* (2009) were capable of inducing an activation of MC2R in the presence of MRAP2 (Sebag & Hinkle 2009b). However, MRAP2 does not rescue MC2R function in FGD patients with *MRAP1* mutations, regardless of the high ACTH plasma levels that characterize this disorder. Therefore, MRAP2 does not appear to play a major role in adrenocortical ACTH signalling (Metherell *et al.* 2005). A precise function on MC4R signalling modulation and, consequently, on body weight control, was recently attributed to MRAP2 (Asai *et al.* 2013, Sebag *et al.* 2013). *Mrap2* knockout mice are severely obese and weight gain seems to result, in part, from a reduced function of MC4R (Asai *et al.* 2013). In fact, MRAP2 was found to enhance MC4R signalling in response to α -MSH (Asai *et al.* 2013, Sebag *et al.* 2013), in contrast to the study by Chan *et al.* (2009) that reported a MRAP2 inhibitory effect on MC4R signalling using NDP- α -MSH instead of α -MSH.

Besides MRAPs, other proteins were identified as potential accessory proteins for MCRs, namely attractin, attractin-like protein and mahogunin ring finger 1 (MGRN1). These proteins seem to regulate MC1R and MC4R function (Cooray & Clark 2011) as well as MC2R and MC4R trafficking and/or degradation (Cooray *et al.* 2011, Overton & Leibel 2011). The role of MGRN1 in MCRs' function is thought to occur by ubiquitination, a modification that is required for proteasome-mediated degradation and, as recently recognized, to regulate its own signalling and trafficking (Marchese & Trejo 2013).

MCR structural determinants implicated in cell surface targeting

Human MCRs are constituted by 296–360 amino acids and the corresponding genes contain just one exon coding region. They share the characteristic GPCR structural architecture composed by an extracellular N-terminus, an intracellular C-terminus and seven transmembrane domains (TMs) connected by intracellular and extracellular loops. MCRs are the smallest known GPCRs,

with short amino- and carboxyl-terminal ends and a very small second extracellular loop (loop 4) (Fig. 1). MCRs display other GPCR characteristics, namely the N-linked glycosylation sites in their N-terminal tail and conserved cysteine residues at the C-terminus. Human MCRs exhibit sequence homologies ranging from 67% between MC4R and MC5R to 42% between MC1R and MC2R (Yang 2011). The phylogenetic analysis of the MCRs' family using full-length amino acid sequences of each receptor revealed that MC3R, MC4R and MC5R are more closely related to each other than to the other two MCRs (Schieth *et al.* 2003, 2005).

C-terminal

Several conserved C-terminal motifs seem to be involved in the intracellular trafficking of GPCRs from ER to cell surface, such as the triple phenylalanine motif F(X)₃F(X)₃F identified in the dopamine D1 receptor (Bermak *et al.* 2001) and the di-leucine motif E(X)₃LL of the vasopressin V2 receptor (Schulein *et al.* 1998). Additionally, the di-leucine sequence may form part of a more complex hydrophobic motif, such as FN(X)₂LL(X)₃L or F(X)₆I/LL identified in α 2B-adrenergic, angiotensin II type 1 or vasopressin V1b/V3 receptors (Duvernay *et al.* 2005, Robert *et al.* 2005, Dong *et al.* 2007).

The di-leucine motif with an upstream acidic residue (glutamate or aspartate) seems to be also important in the anterograde transport of MCRs. Plasma membrane expression of the MC4R is dependent on a C-terminal di-isoleucine sequence (I316/I317) (Ho & MacKenzie 1999, VanLeeuwen *et al.* 2003). Single- and double-isoleucine mutants showed reduced ability to target the cell surface (VanLeeuwen *et al.* 2003). This motif is highly conserved at the C-terminus of all MCRs as well as the acidic glutamate that is located eight amino acids upstream (see Fig. 1). This similar motif E(x)₇LL is also present in several other GPCRs such as dopamine and β 3, α 1 and α 2-adrenergic receptors (Schulein *et al.* 1998).

In human MC1R C-terminus, Sanchez-Mas *et al.* (2005) observed that three residues (T314, C315 and W317) were also essential for proper expression on plasma membrane. Regarding MC2R, the loss of the last eight amino acids resulted in impaired cell surface expression (Hirsch *et al.* 2011). Interestingly, co-immunoprecipitation studies of MRAP and this c-terminus mutant showed no alteration in the interaction of the receptor with the accessory protein, suggesting that MRAP itself does not guarantee normal localization (Hirsch *et al.* 2011).

hMC1R	-----MAVQGSQRRLG	13
hMC2R	-----MKHIINSYEN	10
hMC3R	MSIQKTYLEGDFVFPVSSSSFLRTLLEPQLGSALLTAMNASCCLPVQPT	50
hMC4R	-----MVNSTHRGMHTSLHLWNRSSY	21
hMC5R	-----MNSSFHLHFLDLN	13
TM1		
hMC1R	LNSTPTAIPQLGLAANQTGARCLEVSI SDGLFLSLGLVSLVENALVVATI	63
hMC2R	INNNTARNSD-----CPRVVLPEEIFFTISIVGVLENLIVLLAV	49
hMC3R	LPNGSEHLQAPFFSNQSSSAFCEQVFIKPEVFLSLGTVSLLLENILVILAV	100
hMC4R	RLHS--NASESLGKGYSDGGCYEQLFVSPEVFVTLGVI SLLLENILVIVAI	69
hMC5R	LNATEGNLSGPNVKNKSSP--CEDMGIAVEVFLTLGVISLLENILVIGAI	61
Loop 1 TM2 Loop 2		
hMC1R	AKNRNLHSPMYCFICCLALS DLLVSGSNVLETAVI LLLEAGALVARAAVL	113
hMC2R	EKNKNLQAPMYFFICSLAIS DMLGSLYKILENILLI LRNMGYLKPRGSFE	99
hMC3R	VRNGNLHSPMYFFLC SLAVADMLVSVSNALETIMIAIVHSDYLTFFEDQFI	150
hMC4R	AKNKNLHSPMYFFICSLAVADMLVSVSNGSETIVI TLLNSTD-TDAQSFT	118
hMC5R	VKNKNLHSPMYFFVCSLAVADMLVSMSSAWETITIT IYLLNNKHLVIADAFV	111
TM3 Loop3		
hMC1R	QQLDNVIDVITCSSMLSSLCFLGAI VDRYISIFYALRYHSIVTLPRARR	163
hMC2R	TTADDIIDSLFVLSLLGSIFSLSVIAADRYITIFHALRYHSIVTMRRTVV	149
hMC3R	QHMDNIFDSMICISLVASICNLLAIAVDRYVTIFYALRYHSIMTVRKALT	200
hMC4R	VNIDNVIDSVICSSLLASICSLLSIAVDRYFTIFYALQYHNIMTVKRVGI	168
hMC5R	RHIDNVFDSMICISVVASMCSLLAIAVDRYVTIFYALRYHHIMTARRSGA	161
TM4 Loop4 TM5		
hMC1R	AVAAIWVASVVFSTLFIAYYDHVAVLLCLVVFFLAMLVLMVAVLYVHMLAR	213
hMC2R	VLTVIWTFTCTGTGITMVI FSHHVPTVITFTSLFPLMLVFILCLYVHMFL	199
hMC3R	LIVAIWVCCGVCVGVFIVYSESKMIVCLITMFFAMMLLMGTLYVHMFLF	250
hMC4R	IISCIIWAAC TVSGILFIIYSDSSAVIICLITMFFTMLALMASLYVHMFLM	218
hMC5R	IIAGIWAFACTGCGIVFIIYSESTYVILCLISMFFAMFLVSLYIHMFL	211
Loop5 TM6		
hMC1R	ACQHAQGIARLHKRQR-PVHQGFGLKGA VTLTILLGIFFLCWGPFFLHLT	262
hMC2R	ARSHTRKISTLPRAN-----MKGAITLTILLGVFIFCWAPFVLHVL	240
hMC3R	ARLHVKRIAALPPADGVAPQQHSCMKGA VTLTILLGVFIFCWAPFFLHLV	300
hMC4R	ARLHIKRIAVLPGTG--AIRQGANMKGAITLTILIGVFVVCWAPFFLHLI	266
hMC5R	ARTHVKRIAALPGAS--SARQRTSMQGA VTVTMLLGVFTVCWAPFFLHLT	259
Loop6 TM7		
hMC1R	LIVLCPEHPTCGCIFKNFNFLFLALI ICNAIIDPLIYAFHSQELRRTLKEV	312
hMC2R	LMTFCPSNPYCACYSLSLFQVNGMLIMCNAVIDPFIYAFRSPELRDAFKM	290
hMC3R	LIITCPTNPYCICYTAHFNTYLVLIMCNSVIDPLIYAFRSLELRNTFREI	350
hMC4R	FYISCPQNPYCVCFMSHFNLYLILIMCNSIIDPLIYALRSQELRKTKEI	316
hMC5R	LMISCPQONLYCSRFSHFNMYLILIMCNSVMDPLIYAFRSQEMRKTKEI	309
DPxIY E(x)₇LL		
hMC1R	LT-CSW-----	317
hMC2R	IF-CSRYW-----	297
hMC3R	LCGCNGMNLG-----	360
hMC4R	IC-CYPLGGLCDLSSRY	332
hMC5R	IC-CRGFRIACSFPRRD	325

Figure 1

Primary sequence alignment of the five human melanocortin receptors. N-terminal glycosylated amino acids are highlighted in red and residues that interfere in cell surface expression of MCRs are highlighted in blue. Conserved amino acids among the five MCRs are highlighted in green. Two highly conserved motifs important for the proper expression of MCRs at the cell surface are also illustrated. This alignment was performed with

Clustal W2 (Larkin *et al.* 2007), which was analysed with Quick2D (Nugent & Jones 2009) for prediction of transmembrane domains. The GenBank accession numbers for human MCR sequences are as follows: MC1R (AAD41355.1), MC2R (AAI04170.1), MC3R (AAH98169.1), MC4R (NP_005903) and MC5R (NP_005904).

TMs and loops

The presence of ER export signals at hydrophobic domains or in intracellular or extracellular loops was also described in several GPCRs (Dong *et al.* 2007). For example, a three-arginine motif located in the third loop functions as an export signal for α 2B-adrenergic receptor (Dong *et al.* 2012). Such a motif was not described in MCRs, but other residues important to their correct cell surface expression were identified. Five variants of human MC1R (R151C, R160W, T157A, I155T and R162P) located in loop 3 or TM4 of the receptor (Beaumont *et al.* 2005, Sanchez-Laorden *et al.* 2009), and D84E variant located in TM2 (Beaumont *et al.* 2005), presented altered trafficking to cell surface. Confocal laser scanning microscopy co-localization with specific organelle markers revealed that R151C is retained in ER, while R160W continued in the secretory pathway until the *cis*-Golgi region. This evidence is noteworthy because, in human population, this was the first time that a GPCR showed accumulation in a post-ER zone. T157 is located within 157TLPRI60, a protein kinase C target domain, and, in fact, phosphorylation of T157 is a major determinant for MC1R forward trafficking (Sanchez-Laorden *et al.* 2009). We can speculate that the phosphorylation of this residue may be generally important for MCRs' export as it is highly conserved among the five human MCRs (see Fig. 1; Sanchez-Laorden *et al.* 2009).

Several MC2R natural mutations found in FGD patients (L55P, S74I, G116V, S120R, Y129C, I130N, R137W, H139Y, R146W, T152K, T159K, L198P, G226R, A233P, C251F, Y254C, S256F and P273H) are localized in the intra- and extracellular loops and also on TMs. The majority of mutations interfered with the cell surface targeting of the receptor, even though interactions with MRAP were not disturbed (Chung *et al.* 2008). The analysis of chimeric proteins, where regions of MC2R were replaced by homologous segments of MC4R, revealed that TM3 and TM4 were the main determinant domains to the correct MC2R anterograde transport due to their ability to interact with MRAP and mask an ER arrest signal (Fridmanis *et al.* 2010).

Decreased plasma membrane targeting was observed in human MC3R mutations that are associated with childhood obesity, E80D and E92D (TM1) and D158E (TM3) (Wang *et al.* 2008). Additionally, I335 located at the end of TM7 and part of the highly conserved N/DPxxY motif was critical for multiple aspects of the MC3R function, including cell surface expression (Tao 2007). This isoleucine as well as the entire motif (DPxIY) is fully

conserved among all MCRs (Fig. 1). Another study demonstrated the importance of I87 (TM1), M134 (TM2), L249 (fifth loop), T280 and L297 (TM6) from human MC3R for cell surface expression, ligand binding and signalling (Yang & Tao 2012).

The functional study of 20 novel mutations naturally occurring in human MC4R and responsible for the obesity phenotype revealed that I69 (TM1), H76 (first loop), I194 and I195 (TM5), P260 (TM6) and L300 (TM7) are important residues for cell surface expression (Wang & Tao 2011). Other authors analysed in detail the entire TM3 and TM6: they found two residues located in TM6 that regulate MC4R export but none was found in TM3 (Huang & Tao 2012, Mo *et al.* 2012).

N-terminal

Compared with the C-terminus, the domains at the N-terminus that regulate GPCR export to cell surface were less investigated and are more controversial. There are data on the α 2B-adrenergic receptor showing that a tyrosine- and serine (YS)-conserved motif in this extracellular domain was important for export from the Golgi complex (Dong & Wu 2006).

In human MC3R, two natural mutations in residues located at the N-terminus (S69C and A70T) were found to interfere with the correct cell surface expression of the receptor (Yang & Tao 2012). Our own results on MC5R anterograde transport demonstrated that a four-amino acid motif located in the N-terminus was essential for this process (manuscript in preparation). When these residues were mutated to alanine or when GFP or myc tags were added to the MC5R N-terminus, probably masking the sorting sequence, cell surface expression of the receptor was significantly impaired (Rodrigues *et al.* 2009). This seems to be a particularity of MC5R as N-terminal epitope tagging of other MCRs did not affect receptor trafficking or function (Sanchez-Mas *et al.* 2005, Roy *et al.* 2010, Wang & Tao 2011, Yang & Tao 2012).

Asparagine-linked glycosylation The N-linked glycosylation at the consensus sequence NxS/T is a frequent post-translational modification in GPCRs. In some reports, it affected the correct GPCR targeting, whereas in other situations, no significant influence was observed (Dong *et al.* 2007).

MCRs also possess consensus N-linked glycosylation sites at the N-terminus. Analysis of the electrophoretic pattern of different MCRs confirmed this assumption

because it showed several bands representative of glycosylated forms (Sanchez-Laorden *et al.* 2006, Sebag & Hinkle 2009a, Kay *et al.* 2013). Our own experience on the electrophoretic profile of MC5R-tagged forms sustains this observation (A R Rodrigues, D Sousa, H Almeida and A M Gouveia, 2011, unpublished observations).

Bioinformatic analysis indicated that MC1R has two putative N-linked glycosylation targets, 15NST17 and 29NQT31, that were confirmed with endoglycosidase studies (Herraiz *et al.* 2011). While N15 was dispensable for the presence of MC1R at cell surface, N29 played an important role due to its involvement in the forward trafficking as well as in the retrograde uptake of the receptor (Herraiz *et al.* 2011).

Human MC2R N-linked glycosylation in two N-terminal sites (12NNT14 and 17NNS19) seems necessary for MC2R cell surface expression but not for MC2R function (Roy *et al.* 2010). These data agree with the observation that the N-terminus of MC1R, MC3R, MC4R and MC5R, including the N-linked glycosylation sites, is not essential to MCR function (Schioth *et al.* 1997).

Oligomerization and MCR trafficking

For some GPCRs, it is believed that oligomerization occurs in ER and is checked by quality control systems in this organelle (Salahpour *et al.* 2004, Dong *et al.* 2007). Similarly, all MCRs form oligomers, although direct evidence is lacking on where this process takes place and on its importance to cell surface expression. Mandrika *et al.* (2005) concluded that MC1R and MC3R had the capacity to form homodimers and also heterodimers. Moreover, MC1R homodimerization occurred before reaching the plasma membrane, most likely in the ER (Sanchez-Laorden *et al.* 2006, Zanna *et al.* 2008). More recently, MC3R was found to heterodimerize with the ghrelin receptor (GHSR) in the hypothalamus, and this interaction did not increase cell surface expression levels of MC3R (Rediger *et al.* 2011). MC2R was also able to homodimerize but was retained in the ER in the absence of MRAP (Sebag & Hinkle 2007, 2009a). Conversely, MRAP inhibited formation of MC5R dimers and disrupted its trafficking to plasma membrane, suggesting that MC5R monomers may be trapped intracellularly (Sebag & Hinkle 2009a). Different groups also described the ability of MC4R for constitutive homodimerization (Biebermann *et al.* 2003, Elsner *et al.* 2006, Nickolls & Maki 2006).

Strategies for stabilization of MCR mutants

Modifications in protein structure frequently cause a loss of function by interfering with protein synthesis, transport or stability. Frequently, mutated forms of GPCRs are retained in the ER or Golgi, decreasing its expression levels on cell surface (Dong *et al.* 2007). An important issue is to understand how it is possible to reverse the intracellular retention of mutant forms. It is known that chaperones recognize mistakes in protein folding, such as insertion of hydrophilic residues in TMs, immature glycans or unpaired cysteines, and may rescue the function of MCR mutants by increasing their cell surface expression (Ward *et al.* 2012). Three MC4R clinical mutations associated with obesity (S58C, P78L and D90N) demonstrated reduced trafficking to plasma membrane, but this situation could be reversed by the quality control system. In fact, an enhanced cell surface expression of these mutants due to action of the chaperones Hsc70 and Hsp90 in receptor folding was observed (Meimaridou *et al.* 2011). Other obesity-linked MC4R variants, which were retained at the ER, were also rescued to the cell surface by the chemical chaperone 4-phenyl butyric acid (Granell *et al.* 2010) or other pharmacological chaperones (Rene *et al.* 2010, Ward *et al.* 2012). The ability to rescue the function of these mutations could be of significant therapeutic value in the treatment of severe early-onset obesity.

Some other MC4R mutants are retained in the ER because of an increased tendency to be ubiquitinated rather than to misfold. For example, the cell surface expression of the obesity-linked MC4R P272L variant is restored after treatment with inhibitors of ubiquitin-activating enzymes (Granell *et al.* 2012). In this case, a therapeutic strategy to combat obesity may be conveyed by reducing the ubiquitination capacity of the cell, which will increase proper cell surface expression of the receptors and thereby promote its signalling.

Conclusion

The melanocortin system in general and their receptors in particular attracted increasing attention due to their vast physiologic effects, such as exocrine regulation, pigmentation, control of energy homeostasis and sexual function (Ducrest *et al.* 2008, Humphreys *et al.* 2011, Roulin & Ducrest 2011). At the moment, the aim is to understand their dynamics in the intracellular environment focusing on how receptors are directed to the cell surface to exert their physiologic effect. Here, studies describing molecular

mechanisms implicit in such process as well as the structural characteristics of MCRs with important roles therein were reviewed. Structural motifs found in one MCR, although highly conserved among the MCR family, may not be important for the expression, folding, post-translational modification or anterograde transport of others MCRs.

The variety of techniques employed in the assessment of cell surface expression and kinetics of MCRs require a careful appraisal of the data obtained, which is sometimes conflicting. The structural/microscopical approach is most commonly employed to track GPCRs in cells and uses a variety of fluorescent ligands, antibodies, autofluorescent proteins (such as GFP) or peptide tags. The differences in the labelling procedures have to be taken into account for getting reliable results. It is also important to consider the characteristics of the cell lines when performing overexpressing experiments. Additionally, other techniques that allow the quantification of the surface expression of the receptors are also widely used, such as ELISA, in-cell western or flux cytometry detection of the labelled antigenic epitope or biotinylated extracellular domains. The different assays have varying efficiencies and sensitivities to detect cell surface expression of membrane proteins that could justify contradictory results.

The fundamental research on GPCR forward transport is of utmost importance also for therapeutic strategies, which are currently focused on ligand–receptor interaction. Specifically for MCRs, direct targeting may not be the most intelligent therapeutic approach due to their structural high homology and the likelihood for cross-reactions with agonists to occur. Additionally, they are expressed in a large number of different tissues, certainly mediating different cellular functions. Therefore, to specifically target a pathway involving the melanocortin system, it seems fundamental to modulate MCR trafficking and/or downstream targets.

Thus, improving our knowledge on MCR traffic regulation will also elicit the development of new therapeutic perspectives, as already demonstrated by the effect of chaperones or escort proteins that improve the maturation and forward transport of mutated MCRs.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

Funding

A R R was supported by POCI 2010, FSE and 'Fundação para a Ciência e Tecnologia' (SFRH/BD/41024/2007).

Acknowledgements

The authors gratefully thank Sociedade Portuguesa de Endocrinologia, Diabetes e Metabolismo, ABBOTT and Tanita Healthy Weight Community Trust for financial support of this work.

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Received in final form 25 July 2013

Accepted 31 July 2013

Accepted Preprint published online 1 August 2013