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SANTA VEIKŠINA

Development of assay systems for characterisation of ligand binding properties to melanocortin 4 receptors

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Development of assay systems for characterisation of ligand binding properties to melanocortin 4 receptors

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Institute of Chemistry, Facultry of Science and Technology, University of Tartu, Estonia

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LIST OF ORIGINAL PUBLICATIONS

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II Kopanchuk, S., **Veiksina, S.**, Mutulis, F., Mutule, I., Yahorava, S., Mandrika, I., Petrovska, R., Rinken, A., Wikberg, J.E. (2006) Kinetic evidence for tandemly arranged ligand binding sites in melanocortin 4 receptor complexes. *Neurochemistry International*, 49(5):533–542.

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Paper II: The author participated in planning and performing of ligand binding experiments as well as in writing of the manuscript.

Paper III: The author was main person responsible for the paper – planning and performing of experiments as well as writing the manuscript (except of the data analysis part).

Paper IV: The author was main person responsible for the paper – planning and performing of experiments as well as writing the manuscript (except of the data analysis part).

ABBREVIATIONS

AC	adenylate cyclase
ACTH	adrenocorticotropin
AGRP	agouti-related protein
ALP	attractin-like protein
ASIP (ASP)	agouti protein or also named agouti signalling protein
ATP	adenosine triphosphate
BBV	budded baculovirus
BRET	bioluminescence resonance energy transfer
BSA	bovine serum albumin
cAMP	cyclic 3',5'-adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CRE	cAMP-response element
CREB	cAMP-response element binding protein
DMEM	Dulbecco's modified Eagle's medium
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FA	fluorescence anisotropy
FBS	foetal calf serum
FCS	fluorescence correlation spectroscopy
FLIM	fluorescence lifetime imaging microscopy
FRET	Förster/fluorescence resonance energy transfer
G protein	guanine nucleotide-binding protein
GAP	GTPase-activating protein
GDP	guanosine diphosphate
GHSR	growth hormone secretagogue receptor (named also ghrelin receptor)
GPCRs	G protein-coupled receptors
GTP	guanosine triphosphate
HTS	high-throughput screening
IP_3	inositol 1,4,5-triphosphate
Jak	Janus kinase
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MC_1-MC_5	melanocortin receptor subtypes 1 to 5
MGRN1	mahogunin ring finger 1 (formerly known as mahoganoid)
MITF	microphthalmia-associated transcription factor
MRAP	melanocortin receptor accessory protein
MSH	melanocyte-stimulating hormone
PDE	phosphodiesterase
PKA	protein kinase A
PLC	phospholipase C

POMC	pro-opiomelanocortin
RGS	regulator of G protein signalling
SAR	structure-activity relationship
SPA	scintillation proximity assay
STAT	signal transducer and activator of transcription
TR-FRET	time-resolved-FRET
VLP	virus-like particle

INTRODUCTION

Physiological processes in the human body are regulated by complex communication networks between cells – a wide variety of different signals are "recognised" and transmitted via receptors to provide certain effects and regulate certain functions. Diseases can be treated by addressing different receptors and influencing their functions. Members of one of the largest classes of cell-membrane receptors, so-called G protein-coupled receptors, play an important role in these regulatory processes. Thus, G protein-coupled receptors are of particular interest to drug developers/pharmaceutical companies as drug targets for the treatment of different diseases. Among nearly a thousand G protein-coupled receptors identified, the family of melanocortin receptors with five receptor subtypes is one of the most diverse in terms of physiological effects regulated. The developmental process of new drug discovery ranges from seeking, synthesis and *ex vivo* evaluation of pharmacological properties of biologically active molecules, to pre-clinical in vivo characterisation and finally clinical trials on humans. An assay in pharmacological research plays a very important role, as the ability to see the effect of the drug depends on the assay "eyes" through which it is monitored. The profile of drug actions can be very complex, so it is very important to understand the mechanism of receptor-ligand interaction. The main tools that would allow us to shed light on these mechanisms are: drug candidates and targets, assays providing experimental data concerning their interactions, and models describing interaction mechanisms. Thus, the development of new methods, assay systems and model improvements would have an impact on all these components of the drug discovery process.

The current thesis describes the progress of our studies of melanocortin receptors as drug targets starting from searching for drug candidates from a newly synthesised library of compounds, to the development of different new fluorescence anisotropy-based assay systems and new data analysis approaches. This allowed us to characterise the receptor-ligand interaction mechanisms in the melanocortin system, and enhanced the general understanding of the functioning of G protein-coupled receptors.

1. MELANOCORTIN RECEPTORS AS G PROTEIN-COUPLED RECEPTORS

The family of melanocortin (MC) receptors consists of 5 members that belong to class A (rhodopsin/β_2-adrenergic-like family) of the large superfamily of G protein-coupled receptors (GPCRs). GPCRs are cell surface receptors with sequence lengths ranging from about 300 to more than 3300 amino acid residues (Latek et al., 2012); they consist of seven α-helical transmembrane domains that are interconnected by three alternating extra- and intracellular peptide loops; they provide signal transduction from outside the cell to a variety of downstream effector molecules inside the cell by catalysing the GDP/GTP (guanosine diphosphate/guanosine triphosphate) exchange on heterotrimeric G proteins (Gα-$\beta\gamma$). 948 genes encoding GPCRs have been identified from the human genome sequence (Takeda et al., 2002), which corresponds to about 5% of all human genes, but the deorphanisation or discovery of endogenous ligands for receptors is still in progress (Civelli et al., 2013). Due to their wide range of physiological functions regulated, GPCRs comprise the main target group for the treatment of different diseases. That is also why melanocortin receptors, and GPCRs in general, are of particular interest to drug developers/pharmaceutical companies as well as academic researchers.

1.1. Melanocortin receptor subtypes

Via activation or inhibition of the MC receptors a wide variety of different physiological effects can be regulated, the most well-known among them being skin pigmentation changes, anti-inflammatory and antipyretic actions, influence on sexual behaviour, food intake regulation, and influence on learning, attention and memory.

The first two melanocortin receptor subtypes (MC_1 and MC_2) were cloned in the year 1992 by two independent groups (Mountjoy et al., 1992; Chhajlani and Wikberg, 1992), and to date five MC receptor subtypes (MC_1-MC_5) have been identified. MC receptors comprise high sequence homologies, ranging from 67% identity between MC_4 and MC_5 receptors, to 42% between MC_1 and MC_2, and 38% homology between MC_2 and MC_4 receptors (Cone, 2000; Yang, 2011). Phylogenetic analysis of the MC receptors' family using full-length amino acid sequences of each receptor revealed that MC_3, MC_4 and MC_5 receptors are more closely related to each other than to the other two MC receptors (Schiöth et al., 2003a, 2005). MC receptors with about 300 amino acid residues in their sequences are one of the smallest GPCRs known. They share common structural characteristics like short N- and C- terminal ends and a very small second extracellular loop, and also conserved cysteine residues in their C-terminus, which may serve as sites for fatty acid acylation anchoring the C-terminus to the plasma membrane (Wikberg et al., 2000).

The human **MC_1 receptor** is a 317-amino acid protein. It was first detected in malignant melanoma cells, but primarily MC_1 receptors are expressed in melanocytes and involved in the regulation of pigment production. The activation of the MC_1 receptors causes the formation of an increased proportion of black/brown eumelanin, whereas decreased activity results in the formation of a greater proportion of red/yellow phaeomelanin. The first MC_1 receptor mediated effects were observed already in 1912 by Fuchs when he described a darkening of frog skin caused by a pituitary extract (Eberle, 1988); this can be considered as the beginning of history of melanocortin receptors and made a contribution to subsequent studies and the discovery of melanocortin hormones and their receptors. Subsequently the MC_1 receptor has also been found to be expressed in immune/inflammatory cells (e.g. neutrophils, monocytes, macrophages), human dermal microvascular endothelial cells, Sertoli cells in the testes, and in the brain in neurons of the periaqueductal grey matter. Because of the wide distribution, in addition to pigmentation, the MC_1 receptor has been shown to be involved in a number of other biological functions including anti-pyretic and anti-inflammatory actions (Wikberg et al. 2000; Abdel-Malek 2001), and regulation of pain and analgesia (Sandkühler, 1996; Mogil et al., 2003).

The human **MC_2 receptor** consists of 297 amino acids and it is expressed in the adrenal cortex *zona reticularis* and *zona fasciculate*, where it regulates the production of cortisol (Mountjoy et al., 1992). Besides the expression in the adrenal glands, the MC_2 receptor has also been found to be expressed in skin (Slominski et al., 1996) and adipocytes (Boston and Cone, 1996). This receptor is unique among other MC receptors because it can be activated only by adrenocorticotropin (ACTH) and has no significant response to any other melanocyte-stimulating hormone (MSH) peptides. Additionally, in 2005 an accessory protein (named melanocortin-2 receptor accessory protein, MRAP) was identified that is required for functional expression of the MC_2 receptor and critical for ACTH signalling (Metherell et al., 2005; Hinkle and Sebag, 2009).

In 1993, two independent groups reported the cloning and characterisation of the rat (Roselli-Rehfuss et al., 1993) and human (Gantz et al., 1993a) **MC_3 receptor** genes. The human MC_3 receptor is a 360-amino acid protein; it is predominantly expressed in the brain, but also in the peripheral nervous system, in the placenta, in several human gut tissues including the stomach, pancreas and duodenum, in the heart, in human monocytes, testis and ovary (Cone, 2000; Abdel-Malek, 2001). The MC_3 receptor binds all melanocortins with similar affinity but is the only MC receptor to be activated by γ-MSH. The MC_3 receptor has been found to play a role in modulation of autonomic functions, energy homeostasis, feeding and inflammation, as well as appears to be involved in the regulation of sexual behaviour (Gantz and Fong, 2003; Catania et al., 2004; Wikberg et al., 2000; Martin and MacIntyre, 2004).

The **MC_4 receptor** was also cloned in 1993 (Gantz et al., 1993b) and it is a 332-amino acid protein. It is widely expressed in the central nervous system,

including the cortex, thalamus, hypothalamus, brainstem, and spinal cord, but it is not found in the periphery (Wikberg et al., 2000; Abdel-Malek, 2001; Catania et al., 2004). It has been found to be involved in controlling food intake and energy expenditure, as well as pain sense, to exhibit anti-inflammatory effects, to increase systolic blood pressure, and to modulate erectile function and sexual behaviour (reviewed by Catania et al., 2004; Wikberg and Mutulis, 2008; Cooray and Clark, 2011), which have made it very interesting for many pharmaceutical companies.

The 325-amino acid protein of the human **MC_5 receptor** was cloned in 1993 (Chhajlani et al., 1993) and it is the last of the MC receptor family receptors known to date. This receptor is ubiquitously expressed in peripheral tissues, including the adrenal glands, fat cells, kidney, liver, lung, lymph nodes, thymus, mammary glands, testis, ovary, stomach, skin, skeletal muscle and exocrine glands, such as lacrimal, sebaceous, prostate, seminal, pancreatic *etc*., and plays an important role in the production and/or secretion of the major products in these glands (Wikberg et al., 2000; Abdel-Malek, 2001; Catania et al., 2004). Expression of the MC_5 receptor has also been detected in the brain and in B- and T- lymphocytes (Taylor and Namba, 2001), so it plays a role in immune regulation as well.

1.2. Natural melanocortin receptor ligands

The MC receptor system is unique among the other GPCRs in terms of having both naturally occurring activators and inhibitors. Two MC receptor antagonistic compounds, the agouti protein (also-called the agouti signalling protein, ASIP or ASP) and agouti-related protein (AGRP), are the only two endogenous antagonists of GPCRs identified to date (Lu et al., 1994; Ollmann et al., 1997; Catania, 2010).

1.2.1. Melanocortin receptor activators

The melanocyte stimulating hormones (MSHs) were among the first biological peptides to be purified and sequenced in the 1950s (Cone, 2000). Extensive studies in subsequent years revealed that MC receptor specific ligands, also called melanocortins, are peptide hormones formed in post-translational processing of a common precursor protein named pro-opiomelanocortin (POMC). The full amino acid sequence of this protein was not known until the cloning of its cDNA in 1979 by Nakanishi and colleagues (Nakanishi et al., 1979, when it became as the first example of a prohormone precursor encoding a variety of different neuropeptides and peptide hormones. Proteolytic cleavage of 241 amino acid polypeptide POMC generates α-, β-, and γ-melanocyte-stimulating hormones (α-, β-, γ-MSH) and adrenocorticotropic hormone (ACTH; corticotropin) as well as opioid peptides, corticotrophin-like

intermediate lobe peptide (CLIP) and several other physiologically active peptides. Amino acid composition of these linear peptides can vary between different vertebrates but they all share the conserved tetrapeptide core sequence **His-Phe-Arg-Trp** in their structures, which is crucial for their biological activity. Melanocortins and their precursor POMC have been identified in the pituitary gland, the brain and various peripheral tissues of all classes of vertebrates.

ACTH	Ser-Tyr-Ser-Met-Glu-**His-Phe-Arg-Trp**-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-**His-Phe-Arg-Trp**-Gly-Lys-Pro-Val-NH_2
β-MSH	Asp-Glu-Gly-Pro-Tyr-Arg-Met-Glu-**His-Phe-Arg-Trp**-Gly-Ser-Pro-Pro-Lys-Asp-OH
γ_1-MSH	Tyr-Val-Met-Gly-**His-Phe-Arg-Trp**-Asp-Arg-Phe-NH_2
γ_2-MSH	Tyr-Val-Met-Gly-**His-Phe-Arg-Trp**-Asp-Arg-Phe-Gly
γ_3-MSH	Tyr-Val-Met-Gly-**His-Phe-Arg-Trp**-Asp-Arg-Phe-Gly-Pro-Arg-Asn(glycosyl)-Ser-Ser-Ala-Gly-Gly-Ser-Ala-Gln
δ-MSH	Asp-Gly-Lys-Ile-Tyr-Lys-Met-Thr-**His-Phe-Arg-Trp**-NH_2

Figure 1. Amino acid sequences of natural melanocortin peptides.

The sequence of **ACTH** for mammalian and non-mammalian vertebrates contains 39 amino acid residues (Fig. 1). For most of the mammalians region 1–24 of the ACTH is identical, whereas region 25–39 remains highly variable. ACTH is the only natural activator for the MC_2 receptor (Fig.2).

The sequence of **α-MSH** contains the first 13 N-terminal amino acid residues of ACTH and it is almost identical in all species from which it has been isolated. The N-terminal serine residue of α-MSH is N-acetylated in many species and the C-terminal valine almost always contains a carboxamide group. These structure modifications guarantee stability of the α-MSH molecule against exopeptidases and increase the potency of the peptide (Abbott et al., 2000). In addition to the abovementioned core tetrapeptide His-Phe-Arg-Trp also Tyr^2 and Pro^{12} residues are common for α-MSH peptides in all species (Fig. 1). α-MSH is a nonselective agonist for melanocortin receptors MC_1 and MC_3-MC_5 (Fig. 2).

The **β-MSH** peptide usually consists of 18 amino acid residues, but the structure of this peptide is more variable between different species; however, besides core tetrapeptide Tyr^5 and Pro^{15} residues are also conserved (Fig. 1).

γ-MSH peptides are endogenously present in three pharmacologically active forms named **γ_1-**, **γ_2-** and **γ_3-** melanocyte-stimulating hormones (Fig. 1). γ_1-MSH contains 11 amino acids and has an amidated C-terminal; γ_2-MSH has additional Gly residue instead of an amidated C-terminal. γ_3-MSH has an additional 11 C-terminal amino acid residues as it is formed by cleavage at the next dibasic amino acid cleavage site; in some species it may also contain

N-terminal Lys. γ-MSH peptides have been detected in the pituitary and plasma, brain, vascular system, the bronchi and kidneys, however, in some vertebrate species γ-MSH sequences have not been found in the structure of POMC (Cone, 2000). γ-MSH can be considered as an MC_3 receptor specific activator as this receptor subtype is the only among other MC receptors with a higher binding affinity for γ-MSH than other subtypes (Fig. 2).

In the POMC structure of some cartilaginous fish like the dogfish, stingray, sharks and rays the sequence of **δ-MSH** has been found, which is the most recently discovered melanocortin peptide (Dores et al., 2003).

Receptor subtype	Ligand affinity
MC_1	α-MSH ⩾ β-MSH = ACTH >> γ-MSH
MC_2	ACTH
MC_3	γ-MSH = β-MSH = ACTH ⩾ α-MSH
MC_4	β-MSH ⩾ α-MSH = ACTH >> γ-MSH
MC_5	α-MSH ⩾ β-MSH = ACTH > γ-MSH

Figure 2. Subtype selectivity of melanocortin peptides.

Recent studies have revealed a new class of the MC receptor ligands – β-defensins (Kaelin et al., 2008). The β-defensins are a class of small, cationic proteins first recognised as antimicrobial components of the innate and adaptive immune system. More recently it has been discovered that β-defensins function as MC_1 and MC_4 receptor ligands, capable of blocking the action of either stimulatory α-MSH or endogenous inhibitory ligands such as ASIP and AGRP (see next chapter 1.2.2. Melanocortin receptor inhibitors) (Nix et al., 2013; Beaumont et al., 2012), but some uncertainties still remain concerning β-defensins' biophysical and pharmacological basis of action.

1.2.2. Melanocortin receptor inhibitors

As mentioned previously, the melanocortin system is the only one known to date among other GPCRs to be regulated by endogenous ligands exhibiting not only agonistic but also antagonistic behaviour. The **agouti protein** (also-called agouti signalling protein, **ASIP**) and **agouti-related protein** (**AGRP**) are paracrine signalling molecules exhibiting antagonistic activity at the MC receptors (Lu et al., 1994; Ollmann et al., 1997). ASIP was cloned from the mouse in the early 1990s (Bultman et al., 1992; Miller et al., 1993). It was found that this protein acts in a paracrine manner to promote biosynthesis of red/yellow pheomelanin production over black/brown pigment eumelanin by affecting the MC_1 receptor and antagonising the action of α-MSH. Expression of ASIP normally takes place in the skin, but its neuropeptide homolog AGRP is expressed in the *arcuate nucleus* of the hypothalamus, the subthalamic

region, and the adrenal cortex, and with slight expression in the lung and kidney (Gantz and Fong, 2003). AGRP is a potent orexigenic (appetite-stimulating) factor that acts as a competitive antagonist and blocks the action of the melanocortin agonists at the MC_3 and MC_4 receptors. On the other hand, an increasing number of GPCRs have been shown to exhibit basal/constitutive activity *in vitro* supporting the idea that a receptor can produce a signal without agonist intervention and that several antagonists can act as repressors of such spontaneous activity resulting in inverse agonism and negative efficacy (Chai et al., 2003; Coll, 2013). Several studies have demonstrated that in certain conditions the MC_1 and MC_4 receptors exhibit significant basal activity in the absence of α-MSH, accordingly – AGRP acts as an inverse agonist at the MC_4 receptor (Haskell-Luevano and Monck, 2001; Nijenhuis et al., 2001) as well as ASIP may act as an inverse agonist at a constitutively active MC_1 receptor (Siegrist et al., 1997). In summary, ASIP has a nanomolar affinity for the MC_1, MC_2 and MC_4 receptors, a lower affinity for the MC_3 receptor, but is not effective at the MC_5 receptor, whereas AGRP has a nanomolar affinity for the MC_3 and MC_4 receptors and very little affinity for the MC_1, MC_2 and MC_5 receptors (MacNeil et al., 2002). However, the mechanism of molecular interactions between MC receptors and their antagonists is still not clear and requires additional investigation.

To add to the complexity, perhaps the physiological relevance of melanocortin antagonists are even wider than commonly considered as it has been reported that AGRP is able to induce β arrestin-mediated endocytosis of the MC_3 and MC_4 receptors (Breit et al., 2006). If so, it can be hypothesised about the duality of the controlling role of this protein – probably AGRP possesses both rapid-acting, direct, ligand-receptor action and a slower-acting regulatory effect when the amount of the MC receptors located at the plasma membrane is temporarily reduced via endocytosis (Coll, 2013).

Structurally both ASIP and AGRP are relatively small, easily diffusible 132 residue glycoproteins with cysteine-rich C-terminal domains – within 40 amino acid residues of the C terminal, there are 10 cysteines, which form a network of five disulphide bonds. The cysteine-rich region contributes to the stability of these proteins, but also determines their subtype selectivity (Bures et al., 1998; McNulty et al., 2005; Chai et al., 2005). Moreover, both proteins contain a conserved tripeptide motif Arg-Phe-Phe in their cysteine-rich regions, which is closely related to the melanocortin core sequence and was proposed to be essential for binding to the MC receptors (Chai et al., 2005).

1.3. Accessory proteins in melanocortin system

Growing evidence shows that GPCRs may interact with several accessory proteins in the cell – some of them are implicated in the correct folding and trafficking of the GPCRs to the cell surface, while others appear to participate in ligand binding or found to be associated throughout the life cycle of the receptor (Cooray and Clark, 2011).

There are also several accessory proteins found in the melanocortin system, which are involved in the MC receptor function. In the 1960s spontaneous mutations gave rise to two mouse mutants known as *mahogany* and *mahoganoid* (Lane, 1960; Lane and Green, 1960; Phillips, 1963; Cooray 2011); subsequent studies led to the identification of corresponding proteins – mahogany (now known as attractin) and mahoganoid (now known as mahogunin or mahogunin ring finger 1; MGRN1). Similarities in the phenotypic characteristics of both mouse mutants (dark coat colour and lean body mass) led to the suggestion that attractin and MGRN1 may act as accessory proteins for both MC_1 and MC_4 receptor signalling (Cooray and Clark, 2011). **Attractin** encodes a type 1 transmembrane domain protein of 1428 amino acids with a large extracellular domain and a relatively short cytosolic tail of 128 amino acids. It was shown that attractin acts as a co-receptor for ASIP by assisting in the stability of the interaction between the C terminus of ASIP and MC_1 receptor. However, attractin does not bind to the AGRP and do not interact with the MC_4 receptor (He at al. 2001). Subsequently **Attractin-like protein** (**ALP**) was discovered as interacting partner for MC_4 receptor – it is a transmembrane domain protein consisting of 1371 amino acids and its cytosolic tail retains high sequence homology with attractin (Haqq et al., 2003). It was shown that specific regions of the C terminus of ALP interact with the C terminus of the MC_4 receptor suggesting that ALP acts as co-receptor for AGRP. However, it is still unclear whether ALP would interact with any of other MC receptors. Differently from attractin and ALP, **mahogunin** (**MGRN1)** encodes a ring domain-containing cytosolic E3 ubiquitin ligase (He et al., 2003) that is involved in regulation of energy balance and pigmentation via MC_1 and MC_4 receptors. Different models have been suggested describing the action of MGRN1 (Phan et al., 2002), while recent studies revealed that mahogunin decreased MC receptor signalling by competing with $G\alpha_s$ proteins to bind to the receptor-ligand complex (Perez-Olivia et al., 2009). Although it was shown that MGRN1 and MC_2 receptors co-localise, more investigations are needed to clarify the possible involvement of MGRN1 in the function of the other three MC receptors (Cooray et al., 2011).

To add complexity to an already complicated tale, **syndecan-3** was identified as another co-receptor for melanocortin antagonists (Reizes et al., 2001; Bellin et al., 2002). Syndecan-3 is expressed in several hypothalamic nuclei that are known to play important roles in food intake and energy balance. It was shown that syndecan-3 binds AGRP and facilitates antagonism of α-MSH activity at

the MC_3/MC_4 receptors, thus modulating feeding behaviour and energy balance (Bellin et al., 2002; Reizes et al., 2001). The syndecans are a family of heparan-sulphate proteoglycans (HSPGs) that exist in a membrane-bound and a soluble, shedded form. Membrane-bound syndecans facilitate ligand-receptor interactions and act as co-receptors being so involved in several physiological processes (Reizes et al., 2006). Cleaved from the cell surface however, they can inhibit that ligand-receptor interaction in a separate paracrine fashion.

Recently, a family of novel **melanocortin receptor accessory proteins** (**MRAPs**) has been discovered (Metherell et al., 2005; Roy et al., 2007). MRAPs are small single transmembrane domain proteins and they act as MC receptor signalling modifiers (reviewed by Hinkle and Sebag, 2009; Webb and Clark, 2010). In humans they comprise two splice variants, MRAPα and MRAPβ, and a second gene (orthologue), MRAP2 protein. MRAPα encodes a protein of 172 amino acids, whilst MRAPβ encodes a protein of 102 amino acids. Both isoforms share identical N termini and transmembrane domains but differ at the C termini. MRAPs are beneficial for the MC_2 receptor as they are required for trafficking of the MC_2 receptor from endoplasmic reticulum (ER) to the cell surface and for receptor function in the adrenal cortex. Moreover, MRAPs are also essential for efficient MC_2 receptor heterologous expression at the cell surface. However, they are not essential for the functional expression of other MC receptors – moreover, the MC_1, MC_3, MC_4 and MC_5 receptors suffer from the co-expression with MRAPs as they inhibit the efficacy of the MC receptors to stimulate cAMP (cyclic 3',5'-adenosine monophosphate) accumulation in response to melanocortin peptide binding (Chan et al., 2009; Sebag and Hinkle, 2009). MRAP2 was also found to assist MC_2 receptor trafficking to the cell membrane; however it was not needed for the signalling of this receptor (Sebag and Hinkle, 2009). MRAPs are expressed in the brain as well as in the periphery but the mechanism of interactions with MC receptors is not fully clear at present (Cooray and Clark, 2011).

1.4. Signal transduction

The common signal transduction mechanism shared by the majority of GPCRs is the mediation of intracellular signalling through coupling to specific heterotrimeric guanine nucleotide-binding proteins (G proteins). Receptor-activated G proteins are bound to the inside surface of the cell membrane and they consist of three subunits – the α subunit and a complex of tightly associated heterodimeric βγ subunits (Hepler and Gilman, 1992). GPCRs act as guanine nucleotide exchange factors for the heterotrimeric G proteins. In the traditional view of heterotrimeric G protein activation the agonist binding to the receptor causes its activation by inducing conformational changes, which leads to the release of bound GDP from the associated Gα subunit and subsequent binding of GTP (Iiri et al., 1998). Binding of GTP to the α subunit induces

further conformational changes causing dissociation of the α subunit from the βγ complex and from the receptor. Both the released GTP-bound Gα subunit as well as the Gβγ complex can then activate or inhibit a variety of downstream effectors and different signalling cascades (or second messenger pathways) in the cell (Neer, 1995). At least 27 distinct α subunits (including splice variants), 5 β subunits and 14 γ subunits have been identified so far (Hepler and Gilman, 1992; Downes and Gautam, 1999; Landry et al., 2006; Cabrera-Vera et al., 2003) accounting for a vast variety of different combinations. The generally accepted G protein classification is based on the structural similarities of the Gα subunits and their mediated responses. According to this classification G proteins have been divided in four major subfamilies: $G\alpha_s$ family – stimulates the membrane-associated enzyme adenylate cyclase (AC) and activates the cAMP-dependent pathway by stimulating the production of cAMP from ATP (adenosine triphosphate); $G\alpha_i$ family – inhibits AC; $G\alpha_q$ family – increases intracellular Ca^{2+} concentration via stimulating membrane-bound enzyme phospholipase Cβ (PLCβ); and $G\alpha_{12}$ family – involved in regulation of Rho-family guanine nucleotide-exchange factor signalling (so-called small or monomeric G proteins) (Landry and Gies, 2002; Elefsinioti et al., 2004). A comprehensive overview of effectors activated by various G-protein isoforms can be found in (Kristiansen, 2004; Landry et al., 2006; Birnbaumer, 2007).

1.4.1. cAMP pathway

It has been established that all melanocortin receptors are coupled with $G\alpha_s$ protein and accordingly act as AC activators. Activated AC catalyses the conversion of ATP into cyclic 3',5'-adenosine monophosphate (cAMP) leading to the increase of intracellular cAMP concentration. The cAMP is a small, diffusible molecule; it was discovered by Earl W. Sutherland and his colleague Theodore W. Rall and was the very first second messenger identified (Sutherland and Rall, 1958; Rall and Sutherland, 1958). As a second messenger, cAMP has a fundamental role in the generation of a wide variety of cellular responses by activating different effectors. The primary target of cAMP is the cAMP-dependent protein kinase A (PKA) that is a tetrameric complex of two catalytic and two regulatory subunits. cAMP activates PKA by binding to the regulatory subunits after which activated catalytic subunits are released from the complex. Catalytic subunits then modify the activities of a variety of target enzymes by phosphorylating them at the specific serine and threonine residues (catalytic subunit can even translocate to the cell nucleus where it phosphorylates the family of cAMP-response element binding protein (CREB) transcription factors). Thus, this pathway is involved in enzyme activation as well as regulation of gene expression – the activation of pre-existing enzymes is a much faster process in comparison with the regulation of gene expression, which can take even up to hours. Afterwards, the inherent GTPase activity of the $G\alpha_s$ subunit slowly catalyses the hydrolysis of bound GTP to GDP, thereby

directly deactivating the $G\alpha_s$ subunit and terminating its signalling (as well as terminating $G\beta\gamma$ signalling through the re-association with the GDP-bound $G\alpha_s$ subunit). Besides that, the signal of the pathway may also be terminated by other proteins – i.e. binding of RGS (family of regulators of G protein signalling proteins that serve as GTPase-activating proteins (GAPs)) to $G\alpha_s$ subunit facilitates the hydrolysis of bound GTP to GDP. There is also evidence for direct interaction between RGS proteins and AC. The signalling can be also deactivated downstream by dephosphorylating the proteins phosphorylated by PKA, or the receptor can be desensitised (deactivated) (Sunahara and Taussig, 2002; Ligeti et al., 2012).

As an example, the control of skin pigmentation via the MC_1 receptor is realised by the cAMP signal transduction pathway. The expression of POMC, production and secretion of MSH peptides in keratinocytes and subsequent increased expression of the MC_1 receptors in melanocytes is induced by UV-light exposure. Following agonist binding to the MC_1 receptor in melanocytes causes activation of $G\alpha_s$/AC mediated elevation of intracellular cAMP, which in turn activates PKA. Upon its activation, PKA translocates to the nucleus where it phosphorylates the CREB family of transcription factors. Phosphorylated CREBs then induce the expression of genes containing CRE (cAMP-response elements) consensus sequences in their promoters, such as the transcription factor MITF (microphthalmia-associated transcription factor), which in turn activates genes producing the enzyme tyrosinase (TRP-1 and TRP-2), which are involved in melanogenesis by catalysing the synthesis of black/brown eumelanin from L-tyrosine, whereas inhibition of the MC_1 receptor results in the synthesis of red/yellow phaeomelanin to a greater extent (Slominski et al., 2004).

The multitude of different downstream effectors regulated (and accordingly a multitude of different functions regulated by MC receptors) arise after the elevation of cAMP and are primarily dictated by the individual melanocortin ligands, the receptor subtype and the tissue in which that receptor is expressed (Eves and Haycock, 2010). For example, α-MSH acts as an anti-inflammatory and immunomodulatory agent via the MC_1 and MC_3 receptors in peripheral tissues and by the MC_4 receptor in the CNS (Lasaga et al., 2008); this action is thought to be realised via the activation of the cAMP pathway and subsequent inhibition of NF-κB, a transcription factor that exists in almost all cell types and plays a critical role in the immune system by regulating the expression of about 150 genes, including several proinflammatory cytokines, cytokine receptors and adhesion molecules (Baeuerle and Henkel, 1994; Manna and Aggarwal, 1998). The discovery that melanocortins can control NF-κB infers that any gene under the control of NF-κB is under potential melanocortinic regulation. It is also reported that the activation of cAMP via the MC_4 receptor is followed by phosphorylation of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) or attenuation of p38 and JNK (c-Jun N-terminal kinase) MAPKs phosphorylation, which subsequently also results in

the inhibition of NF-κB (Lasaga et al., 2008). However, although it has been shown that MAPKs participate in anti-inflammatory biology of α-MSH, the exact mechanism still remains unclear.

1.4.2. Alternative signalling pathways

However, there is a growing number of reports suggesting that signalling pathways other than $G\alpha_s$ might contribute to the physiological effects of the MC receptors. For example, it has been shown that the MC_4 receptor endogenously expressed in GT1-1 cells (immortalised hypothalamic neuronal cells) is able to activate the $G\alpha_q$/PLC/Ca^{2+} pathway (Newman et al., 2006). Similarly, the data on human melanoma cells and keratinocytes suggest that although α-MSH predominantly acts through $G\alpha_s$/AC/cAMP pathway, but under conditions where this pathway was inhibited, $G\alpha_q$ mediated Ca^{2+} signalling came up instead. This suggests that in fact all five MC receptor subtypes would also be linked to the $G\alpha_q$/PLC/Ca^{2+} signalling pathway (Elliott et al., 2004; Hoogduijn et al., 2002). There are also data available suggesting coupling of the MC_4 receptor to members of the $G\alpha_{i/o}$ family, thus strengthening the idea of dual coupling of the MC_4 receptor to $G\alpha_s$ and $G\alpha_{i/o}$ proteins (Büch et al., 2009). Besides that, there are also data showing the connection of MC_5 receptor signalling to the Jak/STAT pathway (Janus kinase/signal transducers and activators of transcription) probably via activation of the $G\alpha_q$ protein (Buggy, 1998). Moreover, a new theory concerning a model where an agonist can induce different conformational changes of one particular receptor and each of conformations can be coupled with certain Gα protein subtype leading thus to the activation of different signalling pathways (also referred to as ligand-biased signalling) meets with approval on an example of the MC_4 receptor – studies indicate that the MC_4 receptor can couple to all three major classes of G proteins ($G\alpha_s$, $G\alpha_{i/o}$ and $G\alpha_q$) and can signal via different second messengers and activate different pathways (Yang, 2011). Moreover, it was shown that synthetic peptide and nonpeptide MC_4 receptor agonists differ in their ability to induce different receptor conformational states and to couple the receptor to different G proteins (Yang, 2011).

This all together shows that the melanocortin system operates through very intertwining molecular mechanisms; however it should be kept in mind that cellular context, receptor expression level and alternate active receptor conformations all contribute to the data concerning MC receptor alternative G protein coupling and signalling (reviewed in Breit et al., 2011; Coll, 2013).

Besides that, growing evidence indicates that many GPCRs are also able to transduce their signals through G protein independent pathways, e.g. via adapter proteins like arrestins, but not only (Woehler and Ponimaskin, 2009; Rajagopal et al., 2005; Shukla et al., 2011). Initially arrestins have been shown to terminate G protein-dependent signalling after agonist binding to the receptor;

however, arrestins may also activate cellular effectors like ERK-1/2 in a G protein independent manner (Shukla et al., 2011). As it has been shown that agonist binding to the MC_4 receptor could mediate ERK-1/2 activation (Breit et al., 2011) and AGRP and α-MSH induce interactions between MC_4 receptor and arrestins (Breit et al., 2006), these effects probably contribute to G protein independent signalling. However, there are no clear data confirming MC receptor G protein independent signalling yet.

1.5. Oligomerisation

It is well reported and accepted that many GPCRs possess a common structural and functional feature to form homo- and heterodimers or even higher order oligomers (Bouvier, 2001; George et al., 2002; Milligan, 2009; Palczewski, 2010). There are several suggestions but no clear answers concerning the functional significance and mechanism of receptor oligomer formation, as well as no direct evidence on where this process takes place and on its importance to cell surface expression (Terrillon and Bouvier, 2004). However, it has been proposed that one receptor molecule alone may not be sterically able to provide simultaneous interaction with all the heterotrimeric G protein subunits (Bulenger et al., 2005). A detailed molecular mechanism needs to be elucidated, but continuously accumulating data suggest that there are many modulation or cross-talk possibilities between receptor subunits, as well as between the receptor and G proteins, and receptor and ligands. This indicates that organisation of GPCR signalling appears to be more complex than conventionally proposed (reviewed by Rozenfeld and Devi, 2011).

Numerous studies have shown that all MC receptors are also able to form oligomers. For example, MC_4 receptors can form constitutive homodimers (Biebermann et al., 2003; Elsner et al., 2006; Nickolls and Maki, 2006; Kopanchuk et al., 2005, 2006; Chapman and Findlay, 2013), which exist in different dynamic conformational states depending on their association with the G protein and the agonist (Chapman and Findlay, 2013; Breit et al., 2011). Some data suggest that MC_4 receptors can form even higher order oligomers (Chapman and Findlay, 2013). It has been shown that homodimerisation of the MC_1 receptors occurs already before reaching the plasma membrane during their biosynthesis (Sanchez-Laorden et al., 2006; Zanna et al., 2008). Besides that, MC_1 receptors form heterodimers with the MC_3 receptor, while both of them can form homodimers as well (Mandrika et al., 2005). Additionally, accessory protein MRAP also plays a role in MC receptor oligomerisation and cell surface expression (Rodrigues et al., 2013). It was shown that although MRAP does not influence MC_2 receptor homodimer formation, but it is required for their cell membrane expression, as in the absence of MRAP MC_2 receptor homodimers were retained in the ER (Sebag and Hinkle, 2009). On the contrary, MRAP was found to inhibit MC_4 and MC_5 receptor cell surface

localisation, and disrupted MC_5 receptor dimer formation (Sebag and Hinkle, 2009; Chan et al., 2009). Moreover, recently it was found that hypothalamic GPCRs involved in body weight maintenance and regulation of food intake interact with each other via the heterodimer formation. For example, MC_3 receptor heterodimerises with the ghrelin receptor (also known as the growth hormone secretagogue receptor, GHSR) resulting in a modulation of functions of both receptors, whereas the MC_4 receptor forms heterodimers with G protein-coupled receptor 7 (GPR7) (Rediger et al., 2009, 2012).

It is supposed that cross-talk between both, homomer and heteromer, receptor subunits may be characterised by some level of co-operativity within the complex, where binding of a single ligand to one monomer can change properties of the other (Biebermann et al., 2003; Kopanchuk et al., 2005. 2006; Rediger et al., 2012; Chapman and Findlay, 2013). Thus, understanding the physiological role of oligomer formation between different types of GPCRs and identification of regulatory mechanisms of these homo- and heteromers may be a prerequisite for highly specific drug development via discrete sets of signalling effectors (a phenomenon termed "heteromer-directed signalling specificity" (Rozenfeld and Devi, 2011).

1.6. Melanocortin receptor active synthetic compounds

The development of novel and selective peptic agonists and antagonists for MC receptors has closely followed the identification of various MC receptor subtypes. These receptors participate in regulation of a vast variety of different physiological functions, including, energy balance and feeding behaviours (obesity, anorexia), pigmentation, sexual behaviour (erectile dysfunction, sexual motivation), temperature control, pain and inflammatory and immune responses. There is a need for potent, biologically stable, certain MC receptor subtype selective ligands, both agonists and antagonists, that would have or have not, if desired, the ability to cross the blood-brain barrier. This explains why MC receptors and their ligands have become objects of interest for much research both from academic and industrial laboratories.

As native MSH peptides are biologically unstable, being easily hydrolysed by proteases and easily oxidised, the first attempts towards new MC receptor active synthetic compounds were related with modifications of the core sequence and stabilisation of natural MSH peptides. Introduction of the pseudoisosteric amino acid norleucine (Nle) into position 4 of α-MSH prevents oxidation of methionine (Met^4). Enzymatic stability could be increased by replacing phenylalanine at the position 7 (Phe^7) with unnatural $DPhe^7$. The resulting α-MSH peptide analogue [Nle^4, $DPhe^7$]-α-MSH (shortly, NDP-α-MSH or named also melanotan-I (MT-I); Fig. 3) was the first synthetic superpotent and stable agonistic peptide for all the MSH-binding melanocortin receptors

($MC_{1,3–5}$) with highly prolonged biological activity (Sawyer et al., 1980). Radiolabelled derivatives of NDP-α-MSH have been extensively used for studies of biological functions of the MC receptor both *in vitro* and *in vivo.*

Subsequent modifications in the melanocortin "advanced" core tetrapeptide sequence His-DPhe-Arg-Trp on the basis of computational studies and extensive structure-activity relationship (SAR) studies (Hruby et al., 1984) led to the discovery of melanotan-II (MT-II; Ac-Nle^4, c[Asp^5, DPhe^7, Lys^{10}]-α-MSH[4-10]-NH_2; Fig. 3), which is a truncated cyclic lactam analogue of α-MSH. MT-II has even higher agonistic potency on all the MSH-binding MC receptors ($MC_{1,3–5}$) than NDP-α-MSH. It possesses high *in vivo* stability and prolonged activity, and it can cross the blood-brain barrier (Al-Obeidi et al., 1989; Hadley et al., 1989).

Subsequent studies by using the MT-II cyclic template as a starting point led to the discovery of the first potent synthetic antagonists for MC receptors (Hruby et al., 1995). Minimal changes in the structure of MT-II by substitution of only one amino acid (DPhe^7 with more bulky aromatic naphthylalanine DNal(2')^7) resulted in a compound named SHU9119 (or [DNal(2')^7]-MT-II; Fig. 3) with a completely different bioactivity profile – it demonstrates potent and selective antagonistic activity for MC_3 and MC_4 receptors, but also acts as a partial agonist at the MC_1 and MC_5 receptors.

Seeking for subtype selectivity led to the one of the first subtype selective agonists found MS05 (Fig. 3), which is a peptide ligand possessing more than 1200-fold higher affinity for the MC_1 receptor over all other MC receptor subtypes (Szardenings et al., 2000). It has been proposed for usage as an anti-inflammatory agent.

Another MC_4 receptor-specific antagonist, HS131 (Schiöth et al., 2003b), has an 18-fold preference for the MC_4 receptor over the MC_3 receptor and 290- and 410-fold over the MC_1 and MC_5 receptors, respectively.

NDP-α-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-**His**-DPhe-**Arg-Trp**-Gly-Lys-Pro-Val-NH_2
MT-II	Ac-Nle-c[Asp-**His**-DPhe-**Arg-Trp**-Lys]-NH_2
MS05	Ser-Ser-Ile-Ile-Ser-**His-Phe-Arg-Trp**-Gly-Lys-Pro-Val-NH_2
SHU9119	Ac-Nle-c[Asp-**His**-DNal(2')-**Arg-Trp**-Lys]-NH_2
HS131	Ac-c[Cys-Gly-DNal(2')-**Arg**-**Trp**-Cys]-NH_2

Figure 3. Structures of synthetic melanocortin peptides.

In spite of the remarkable success towards improvement of biological properties of native melanocortins, generally, peptides are regarded to be unsuitable for drug development due to their low enzymatic stability, poor oral bioavailability and high cost of production. However, other than per oral drug administration possibilities can be applied for synthetic peptide drugs, e.g. injections, intranasal administration, subcutaneous drug delivery, etc., can be used. As an example, PT-141 (now named as Bremelanotide) (Molinoff et al., 2003) is an

activator of the MC_1 and MC_4 receptors introduced by Palatin Technologies. However, intranasal drug administration for the treatment of male and female sexual dysfunction was discontinued due to arisen side effects of increased blood pressure. Nevertheless, at the moment new clinical trials of this compound are in progress (human Phase 2B studies) by using a new subcutaneous drug delivery system that appears to have less effect on blood pressure (Palatin Technologies, 2013). To obtain substances suited for medical use there is an interest in highly selective non-peptide compounds imitating the most important structural features of the natural melanocortins. Early studies led to the assumption that the β-turn structure of melanocortins' core region provides a conformation essential for biological activity (Hruby et al., 1984). Since then, a wide variety of different scaffolds connected with side-chains containing structurally different pharmacophoric groups has been examined to mimic the "biologically active" spatial conformation of melanocortins. For example, extremely simple scaffolds, such as tertiary amides (Mutulis et al., 2007), or quite complicated tri-substituted nine-membered heterocycles (Haskell-Luevano et al., 1999) correspond to this spatial conformation and have been used to generate libraries of synthetic MC receptor active ligands. The majority of small molecule non-peptide and peptoid melanocortin agonists introduced by different pharmaceutical companies are derivatives of piperidines or piperazines (Pfizer, Amgen, Bristol-Myers Squibb, Eli Lilly, Merck, Neurocrine, Taisho, Procter and Gamble) or substituted guanidines (Chiron, Melacure) (Bednarek and Fong, 2004; Wikberg and Mutulis, 2008). These compounds have been proposed (some of them have undergone different phases of human clinical trials as well) for the treatment of obesity and/or erectile dysfunction as well as for treatment of anxiety and depression (reviewed by Wikberg and Mutulis, 2008; Boughton and Murphy, 2013; King et al., 2007). As an example, THIQ (Fig. 4), highly potent and selective MC_4 receptor agonist, is a small molecule piperidine derivative discovered by Merck research group (Sebhat et al., 2002). THIQ has been studied in detail in rat models and shown to increase erectile activity (Martin et al., 2002), but no human trials have been performed. However, this ligand and its radioactive analogue (Mutulis et al., 2003) have been used in scientific research – in studies of the MC_4 receptor ligand binding mechanism (Kopanchuk et al., 2006) and signalling mechanism (Cai et al., 2004). Results suggested that non-peptide and peptide agonist ligands have different ligand binding as well as signalling mechanisms.

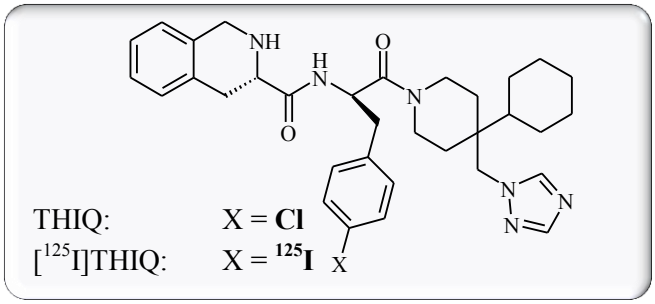


Figure 4. Structure of selective MC_4 receptor agonist THIQ (N-[(3R)-1,2,3,4-tetrahydroisoquinolinium-3-ylcarbonyl]-(1R)-1-(4-chlorobenzyl)-2-[4-cyclohexyl-4-(1H-1,2,4-triazol-1-ylmethyl) piperidin-1-yl]-2-oxoethylamine) and its radiolabelled analogue [^{125}I]THIQ.

Along with a greater understanding of the complexity of the melanocortin receptor system, over the past decade or so it has become widely appreciated that melanocortin receptors possess functional allosteric binding sites. Allosteric modulators bind receptor domains topographically distinct from the orthosteric ligand binding site (binding site on a receptor for the native ligand), altering the biological activity of the orthosteric ligand by changing its binding affinity, functional efficacy, or both. For example, set of ligands that are allosteric activators and inhibitors of the melanocortin receptors has been identified on the basis of modelling the conformation of MT-II (Cain et al., 2006). Allosteric ligands may provide additional possibilities for regulation of receptor functions as they not only affect receptor's orthosteric ligand binding site, but can also modulate signalling pathways (Hruby et al., 2011; Yang, 2011).

Multivalent ligands (multimeric analogues of naturally occurring ligands) that simultaneously bind to multiple receptors can be used for obtaining enhanced specificity and higher affinity in comparison with corresponding monovalent ligands due to multivalent interactions via statistical and/or cooperative effects on binding. Melanoma is a type of skin cancer that develops in melanocytes, and MC_1 receptor is known to be overexpressed at the melanoma cell surface. Therefore, targeting receptor overexpression via the use of multivalent interactions provides an alternative way to enhance selectivity toward these cancer cells (Brabez et al., 2013; Hruby and Cai, 2013). Furthermore, bivalent and heterobivalent ligands that interact with receptor homo- or heterodimers, respectively, could be highly specific and effective tool for specific modulation of receptor signalling capacity – as an example, for patients suffering from obesity or cachexia, targeting of homo- and/or heterodimerised MC_3, MC_4 and GHSRs could represent a valuable tool to modulate food intake (Rediger et al., 2009; 2012). Similarly, feasibility of heterobivalent targeting approach has been shown on tumour cells expressing MC_1 and cholecystokinin-2 receptors (CCK2R) and with synthetic ligands that contain both, MSH and cholecystokinin pharmacophores (Xu et al., 2012).

Radiolabeled heterobivalent peptidic ligands have been proposed to be a valuable tool for in vivo imaging and therapy of malignant diseases (Fischer et al., 2013).

Although much has been learned from the various structure-activity relationship studies of the MC ligands since of the first studies regarding MC system, it still remains difficult to design ligands *de novo* with specific activities at specific MC receptors. However, seeking new ligands has helped to improve the general understanding about the functioning of the MC system and about different opportunities to treat diseases and to maintain human health (Irani et al., 2004; Holder and Haskell-Luevano, 2004; Hruby et al., 2011; Yang, 2011). In fact, melanocortin ligands with multiple unique biological activities have led to clinical trials for pigmentation, melanoma, feeding behaviour, and sexual behaviour and function (Hruby and Cai, 2013; Boughon and Murphy, 2013). For example, Rhythm, a biotechnology company developing peptide therapeutics, in September 2013 announced the initiation of the first of a series of clinical trials with RM-493, the company's novel MC_4 receptor small-peptide agonist, for the treatment of obesity in individuals with a genetic deficiency in the MC_4 receptor pathway (Rhythm Pharmaceuticals, 2013).

2. METHODS FOR G PROTEIN-COUPLED RECEPTOR STUDIES

GPCRs can be studied from different points of view, by using different strategies and methods, but among the most used ones are assays assessing the receptor-ligand binding affinity (ligand binding assays) and efficacy (functional assays). Ligand bound GPCRs are activated to change the conformation and transduce an extracellular signal into the cell. The majority of GPCRs are normally present at very low concentrations in native tissues. That is why highly sensitive methods are required for their detection and characterisation.

2.1. Ligand binding assays

Receptor-ligand binding assays can be used to characterise receptor and its ligands' interactions, giving parameters of intrinsic affinity of ligands to the receptor, association/dissociation rates, and the density of receptor in tissues, cells or receptor preparations.

2.1.1. Radioligand binding

Radioligand binding was implemented as a method for the detection of receptors in 1965 by Paton and Rang (Paton and Rang, 1965). Shortly after that this method was also used in melanocortin system studies, where binding of ^{125}I-labelled ACTH to its adrenal binding sites was demonstrated (Lefkowitz et al., 1970a, 1970b, 1970c). The high sensitivity and selectivity of the method allowed for performing experiments in preparations from native tissues as well as transfected cells thus becoming conventional or so-called "classical" method for molecular pharmacology. This method also allows for characterisation of properties of nonradioactive compounds by their ability to displace the binding of a radiolabelled molecule to the receptor (orthosteric agonists/antagonists) or to modulate the affinity of a radiolabelled ligand for the binding to the receptor (some allosteric modulators). It has made this method indispensable for a long time for the discovery of new drugs targeting specific receptors (Chen et al., 2012).

In this method the receptor-bound radioligand and free radioligand are separated by filtration through glass fibre filters, gel-filtration columns or by centrifugation. The first radioactive MC peptides were ^{125}I-, ^{3}H-, ^{14}C-, or ^{35}S-labelled ACTH and α-MSH. The low specific radioactivity of ^{14}C (63 mCi/mmol per atom) in spite of its long half-life (5760 years) restricts its use in ligand binding assays. Tritium ^{3}H (specific radioactivity = 29.6 Ci/mmol per atom and half-life = 12.26 years) suits well for receptor binding assays and also for physiological experiments, but the relatively high price of ligands' ^{3}H labelling limits its usage. ^{35}S has a much higher specific radioactivity than

tritium (1500 Ci/mmol per atom), but also a much shorter half-life of only 87.2 days. It would be an ideal label for radioligands, but usually there are no direct ways to insert ^{35}S into structure of compounds of interest. ^{125}I has a specific radioactivity of about 2200 Ci/mmol per atom but comparatively short half-life of 60 days, which demands that radioligand should be used within 5–6 weeks after preparation. In spite of this limitation ^{125}I is the most frequently used label for peptide ligands as it can be relatively easily introduced in peptide molecules. The most frequently used radioligand for characterisation of the MC receptors is [^{125}I]-NDP-α-MSH (Eberle, 1988).

Although the radioligand binding method is powerful and still frequently used, it also has several limitations, such as heterogeneity, which requires an additional separation step for the bound and unbound ligand and causes nonequilibrium in the system. Moreover, a lot of important information concerning ligand binding mechanism could be lost during this cumbersome separation step when rapid reactions and/or weak receptor/ligand complexes are investigated. Besides that, safety, waste and cost problems associated with radioactive ligands also must be kept in mind.

Another radioisotopic technique, scintillation proximity assay (SPA), makes it possible to use radioisotopes for monitoring binding reactions continuously in homogeneous conditions without the need to separate free from bound components (Udenfriend et al., 1987; Glickman et al., 2008; Berry et al., 2012). This technology exploits "SPA beads" or scintillating fluomicrospheres into which scintillant is incorporated. Besides that, "SPA beads" are functionalised with affinity tags in order to bind GPCRs, for instance. If a radioalabelled tracer molecule is bound to the receptor in close proximity of the fluomicrosphere (depending on isotope used, the distance from 1 to 125 μm), energy transfer occurs by photons being given off from the radioactive molecule; it stimulates the scintillant to emit light, which can then be detected. Since the development of SPA technology it has been essentially improved (including miniaturisation and automation) and has become a powerful tool for high-throughput screening (HTS) (reviewed by Glickman et al., 2008). However, the main problem in using SPA technology for HTS of GPCR ligands is demand of high-quality receptor preparations in large enough quantities for immobilisation purposes. Typically, either recombinantly expressed whole cells or purified cell membranes are used along with "SPA beads". Thus, issues like signal-to-noise ratio, signal stability, relatively high costs of production of "SPA beds" as well as hazards and restrictions concerning exploitation of radioisotopes still are important reasons for developing alternative homogeneous, non-radioactive methods.

2.1.2. Fluorescence methods

Fluorescently labelled ligands are being used increasingly as an alternative to radiolabelled ligands allowing monitoring and visualisation of ligand binding to the receptor (and other interactions/events with participation of fluorescently labelled partners) in living cells. Novel fluorescent probes stand out with high molecular brightness, low photon-bleaching and insensitivity to solvent polarity and pH changes. These qualities have contributed to the development of a set of alternative methods to radioligand binding assays that exploit different intrinsic fluorescence aspects/properties of the fluorophore (intensity, wavelength, life-time, anisotropy). These parameters can be measured independently or in combination, thus providing more detailed information about certain interaction processes studied. Moreover, fluorescence measurements possess a very high sensitivity, providing the opportunity to observe biological mechanisms on a molecular level. Thus the versatility of fluorescence phenomena can be exploited for GPCR studies both in bulk solution and in the single molecule level. Fluorescence techniques are compatible with live cell experiments, making these advantageous over radioligand binding assays. Fluorescent ligands have been used for GPCR studies ranging from microscopic detection of receptor localisation in tissues and cells and demonstration of the ligand-bound receptor internalisation, to spectroscopic ligand binding assays and to sophisticated studies of receptor interaction mechanisms with different partners (ligands, other receptors, G proteins, etc.). Among ligand binding methods, fluorescence polarisation-based fluorescence anisotropy (FA), Förster/ fluorescence resonance energy transfer (FRET) and time-resolved-FRET (TR-FRET), fluorescence correlation spectroscopy-based assays (FCS) and others have found wider recognition and applications in GPCR studies (Chen et al., 2012; Sridharan et al., 2014). Each fluorescence technique has several advantages, as well as certain limitations.

2.1.2.1. Fluorescence anisotropy

The fluorescence anisotropy (FA) method is based on the phenomenon that the population of fluorescent probes emits light with a certain degree of polarisation when excited by plane polarised light. The fluorescence lifetime refers to the average time the molecule stays in its excited state before emitting a photon. Thus, the polarisation extent of emitted light of fluorophore activated by polarised light depends on pluorophore's freedom of movement within its lifetime. The binding of fluorescent ligands to bigger and more massive receptor proteins constrains their freedom of movement resulting in a greater extent of polarisation of the emitted fluorescence. Emitted fluorescence changes can be detected as a change in emitted fluorescence intensities parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the plane of excitation plane, respectively, and used for calculation of FA signal. Changes in FA signal can be followed in real-time

without of any separation step requirement. FA signal at time t after the initiation of the binding reaction can be defined as parameter $r(t)$ and calculated as following:

$$r(t) = \frac{I(t)_{||} - I(t)_{\perp}}{I(t)_{||} + 2I(t)_{\perp}} \quad (1)$$

Thus, a simple principle, homogenous system and possibility of continuous on-line monitoring of the receptor-ligand interaction dynamics, as well as moderate requirements for equipment makes the polarisation-based FA assay quite attractive for the assessment of GPCR-ligand binding properties. Although several small-molecule fluorescent ligands for GPCRs are available (Vernall et al., 2013), in general, peptide ligands are rather suited for labelling with fluorescent probes than small-molecule compounds as conjugation with fluorophore more probably does not affect the biological properties of the peptide. It has been shown that NDP-α-MSH also retains its activity after labelling with fluorescent dyes (Prystay et al., 2001; Do et al., 2006; Nosjean et al., 2006; Nicholson et al., 2006; Veiksina et al., 2010, 2014) However, although the FA method looks very promising, it still has not been widely adopted in fundamental receptor-ligand binding interaction studies as well as for screening a wide range of receptors from different sources. Some of the obstacles affecting an assay's signal-to-noise ratio are the availability of suitable fluorophore-coupled ligands and receptor preparations with sufficiently high receptor concentration. As this method is ratiometric, changes in the FA signal can be detected only if the ratio of bound and free fluorescent ligand portions is altered (Nosjean et al., 2006). It means that concentrations of receptor and ligand used have to be comparable. It is different from classical radioligand assays, where the used ligand concentration is much higher than receptor concentration ([L] ≫ [R]) and reactions are carried out under pseudo first-order conditions). In other words, in FA assays reactions are performed under second-order conditions ([L] ≈ [R]), where ligand depletion has to be taken into account thus adding to the complexity of data analysis (Roehrl et al., 2004; Veiksina et al., 2014). Moreover, if the total emission intensity (expressed as $I_{||} + 2I_{\perp}$) changes during the binding reaction (reflects the changes of fluorophore's quantum yield), the data analysis becomes even more complicated (Jameson and Mocz, 2005).

2.1.2.2. Other fluorescence techniques

The sensitivity of fluorophore's fluorescent properties from changes in its environment opens other opportunities for assessment of binding of a fluorescently-tagged ligand to the receptor. Thus, ligand binding can be assayed by determination of changes in fluorescence emission spectrum (wavelength) or intensity (quantum yield) upon the binding process. As fluorescence intensity is

linearly dependent on the number of fluorophores in a sample, it provides a basis for quantitative measurements. Binding can be measured in a solution containing receptors (maintained in a homogeneous suspension) and a ligand without the need to separate the bound from free ligand. This approach relies on the ability to distinguish specific binding-dependent fluorescence changes from fluorescent background contributions arising from unbound ligand, cellular autofluorescence and non-specifically bound ligand (Sridharan et al., 2014).

In 1948 Theodor Förster first described the molecular process of energy transfer between two molecules (Förster, 1948). This approach, fluorescence/ Förster resonance energy transfer (FRET) between two fluorophores, where the emission from one overlaps the excitation of the other can be used for monitoring of ligand binding to a receptor. Usually two proper fluorophores are used, where one is coupled with a ligand and the second with a receptor. There is strong distance dependence for resonance energy transfer to occur. By donor and acceptor molecules coming into close enough proximity, FRET can be detected either via enhancement of the acceptor fluorophore's fluorescence emission (with a longer wavelength) or via loss of emission from the donor fluorophore (with a shorter wavelength). In the latter case, even the non-fluorescent acceptor can be used. The assay is carried out in a "mix and measure" format without the separation requirement of bound and unbound ligands. This allows for determination of ligand binding kinetics, but the FRET approach can also be used for assessment of receptor oligomerisation, ligand-dependent conformational changes in GPCRs, as well as in functional receptor activity assays. The main limitations of these FRET-based assays are related to the availability of good FRET donor/acceptor pairs, labelling of GPCRs and ligands without the loss of their properties and expression of tagged-receptors in cells. Moreover, frequently there is a very low signal-to-noise ratio because the fluorescence emission wavelengths of many organic molecules and the cell culture medium overlap those of the common FRET donors. The influence from photobleaching, crosstalk (direct excitation of the acceptor by the wavelength of light used to excite the donor) and bleedthrough (overlap of the donor and acceptor emission spectra) have to be taken into account, which may considerably complicate the interpretation of FRET signal (Chen et al., 2012; Zhang and Xie, 2012; Goddard and Watts, 2012; Sridharan et al., 2014).

Some of these issues can be eliminated and better sensitivity can be achieved by using the time-resolved mode of FRET measurements (TR-FRET). Here, unique properties of lanthanides like a long-lived excited state (up to milli-second range), large Stokes' shift between absorption and emission maximums and narrow emission peak can be utilised by using lanthanide-containing FRET donors. Thus, several orders of magnitude longer fluorescence lifetime of donor molecule over the non-specific background fluorescence enables the specific signal to be measured after a defined delay period when the background fluorescence has already diminished (Chen et al., 2012; Zhang and Xie, 2012;

Goddard and Watts, 2012). Commonly used lanthanides in this type of assays are europium (Eu), terbium (Tb), samarium (Sm) and dysprosium (Dy). Lanthanide-labelled melanocortin ligands (like Eu-labelled NDP-α-MSH) have been used in studies of the MC receptors within the TR-fluorescence approach (Handl et al., 2004) and for new multivalent MC receptor ligand evaluation for treatment of melanoma (Brabez et al., 2013). However, challenges concerning synthesis of lanthanide-labelled ligands have also been shown (Alleti et al., 2013).

Fluorescence lifetime changes also can be exploited in receptor-ligand binding studies. Fluorescence lifetimes for commonly used fluorophores usually are in the nanosecond range. Measuring the fluorescence intensity with time resolution below 10^{-9} seconds (modulated excitation) gives access to fluorophore lifetimes. These measurements can be performed in two ways – under frequency domain or under time domain (Turconi et al., 2001). The lifetime is characteristic for a fluorophore and depends on its local environment, thus allowing monitoring of interactions of molecules or changes in the immediate surroundings of the fluorophore. Fluorescence lifetime measurements can be applied for studies of interactions in solution as well as in single molecule level (fluorescence lifetime imaging microscopy, FLIM). However, the most common use of this technique is the determination of FRET – because in the presence of FRET the donor molecule has an additional pathway over which it can de-excite, its lifetime is shortened and this effect can be measured. The advantage of FLIM compared with conventional FRET is that its signal is more sensitive and precise since it is independent of the fluorescence intensity and artefacts due to scattering and absorption within the sample. In FLIM the fluorescence signal can be recorded with high temporal and spatial resolution allowing its application in combination with other fluorescence techniques and thus giving access to a wider range of physical parameters that make interpretation of results more detailed, easier and more reliable (Liu et al., 2008).

Fluorescence correlation spectroscopy (FCS) is based on detection of changes in a fluorescent ligand's spatial mobility. Time-dependent fluorescence emission fluctuations are measured from a small illuminated volume (confocal volume) during diffusion of fluorescently labelled molecules within this volume. Autocorrelation analysis of the fluctuations in fluorescence intensity provides information about the diffusion time and number of fluorescent molecules in the observation volume. In this way, processes can be accessed on the single molecule level, but at the same time many single events are statistically evaluated via the correlation function. Thus, FCS provides the ability for simultaneous tracking of free and bound ligand populations in the same solution (without the need for physical separation of these ligand fractions) and for measuring of ligand binding in a time-dependent manner with

single-molecule sensitivity (Briddon and Hill, 2007; Jakobs et al., 2012; Sridharan et al., 2014). The most common parameters extracted from FCS are the diffusion coefficient and the concentration of fluorescent particles in the observation volume, which allow quantification of ligand-receptor interactions by estimation of receptor-bound ligand fraction. Besides that, it allows for assessment of the localisation of receptors within the cell and in different membrane domains, as well as the aggregation state of the receptors. It may provide us with information about the activation and signal transduction mechanisms; furthermore, as receptors reside in their natural environment, results are more likely to represent physiologically relevant interactions (Liu et al., 2008). Moreover, FCS with fluorescence-tagged receptors can also be used for detection of receptor oligomers and for determination of receptor monomers in an oligomeric complex (Herrick-Davis and Mazurkiewicz, 2012). Recently, FCS extension with two-focus and dual-colour detection mode opened new possibilities for in vivo detection of receptor-ligand interactions (Ries et al., 2009).

There has been remarkable progress made in the last years in field of single molecule detection methods based on the fluorescence (Hell, 2009). The single molecule assays permit detection of events often masked in averaged ensemble measurements as they allow for simultaneous detection of different molecular components from many single molecule reactions occurring in parallel in a bulk solution. For example, single-molecule microscopy studies using fluorescent ligands have shown dynamic dimerisation of M1 muscarinic and N-formyl peptide receptors (Hern et al., 2010; Kasai et al., 2011). Fast-developing instrumentation possibilities allow for new techniques to become more available to a wider range of scientists. Furthermore, due to the small volume of the sample needed and the short time required for sample measurement, single molecule assays have the potential for miniaturisation and HTS (Christensen et al., 2013). These considerations would make single molecule multiparameter assays (simultaneous collection and integration of intensity, anisotropy, lifetime, FRET, etc., data) favourable from future perspectives as wide variety of processes, including dynamic binding and conformational dynamics of proteins, could be studied (Kudryavtsev et al., 2007; Felekyan et al., 2012).

2.2. Functional assays

Upon ligand binding, GPCRs change their conformation and activate coupled G proteins, which subsequently promote second messenger production via downstream effectors. Although ligand binding assays can provide us with information about binding affinity and in some cases about binding kinetics and/or oligomerisation, they do not tell us whether the compound is an agonist or an antagonist, or allow assessment of the overall physiological potency of the compound. Functional assays are aimed to assess receptor activation and

signalling by measuring either G protein activation or G protein mediated events (including second messenger generation and reporter gene transcription in response to receptor activation). Since GPCR signalling consists of a series of spatial and temporal events, an important consideration is whether to measure a proximal or distal signalling step after GPCR stimulation. Measurement of events proximal to receptor activation will reduce the incidence of false positives. However, moving down the signal transduction cascade will enhance the signal-to-noise ratio due to signal amplification (Zhang and Xie, 2012), but from the other hand it also increases the possibility of non-selective or overlapped signal detection as many GPCRs may activate multiple signalling pathways (Denis et al., 2012).

2.2.1. cAMP assay

All the MC receptors are considered to be coupled with $G\alpha_s$ protein and signalling via second messenger cAMP. Conventional cAMP assays measure changes in intracellular cAMP levels after stimulation or inhibition of cAMP production. $G\alpha_s$ protein-coupled receptors positively stimulate the activity of AC, thus increased cellular cAMP levels can be measured straightforward, whereas it is more challenging to test compounds (especially antagonists) for $G\alpha_{i/o}$ protein-coupled receptors because of the requirement pre-stimulate AC with forskolin (Zhang and Xie, 2012). Additionally, since the intracellular cAMP concentration is regulated by the balance between the production rate by ACs and degradation rate by phosphodiesterases (PDE), an inhibitor of PDE (e.g. 3-isobutyl-1-methylxanthine; IBMX) might be required in the system during assay performance. However, despite the seemingly simple detection of the $G\alpha_s$ protein's activation, one should be careful as an almost 10-fold abundance of the $G\alpha_o$ protein (member of $G\alpha_i$ family) over $G\alpha_s$ protein has been shown in cells possibly leading to hindered $G\alpha_s$-signalling in heterologous systems (Landry et al., 2006). Usually, levels of cellular cAMP are measured in a competition assay with labelled cAMP, where they both compete for binding to an anti-cAMP antibody or cAMP binding protein (Nordstedt and Fredholm, 1990). Radioactive isotope-labelled cAMP is still frequently used in conventional ligand-binding and SPA assay formats. Several commercial fluorescence-based assays for cAMP detection are available as well – e.g. based on fluorescence polarisation, TR-fluorescence, etc., approaches (Zhang and Xie, 2012; Chen et al., 2012; Denis et al., 2012). Moreover, FRET and BRET (bioluminescence resonance energy transfer) biosensor-based assays have been developed for real-time detection of intracellular cAMP accumulation or turnover in live cells. Biosensor-based approaches rely on the use of genetically encoded fluorescent reporters using cAMP binding properties of cAMP downstream effectors, PKA and Epac (exchange proteins directly activated by cAMP). This non-lytic, live-cell assay format is based on the conformational changes of the biosensor upon cAMP binding and subsequent FRET signal

changes. High sensitivity of biosensor-based assays allows also the detection of $G\alpha_i$-coupled receptor active compounds without an artificial forskolin stimulation (Zhang and Xie, 2012; Chen et al., 2012). This type of functional assay has also been applied for detection of the MC_4 receptor allosteric modulators (Pantel et al., 2011), as well as for studies of the Ca^{2+} and Mg^{2+} influence on ligand binding to MC_1 receptor endogenously expressed in B16F10 murine melanoma cells (Mazina et al., 2012).

Additionally to "conventional" signalling, the phenomenon of ligand-biased signalling has been observed for MC receptors, as well (see section 1.4.2. Alternative signalling pathways). As ligand-biased signalling implies that a single receptor has pleiotropic signalling properties and crosstalk of signalling pathways may occur at different levels downstream from the receptor, it becomes challenging to evaluate functional activities of certain ligands and receptors. A lot of effort has been made during the last decades to find strategies that would overcome challenges concerning biased ligands (Denis et al., 2012); however, it still remains to be solved.

2.2.2. Other assays

An early cellular process after ligand binding to the GPCR is G protein activation, which results in the exchange of a guanine nucleotide and the dissociation of the Gα subunit from the G protein complex. Thus, another type of functional assays (popularly known as GTPγS binding assays) directly measure the guanine nucleotide exchange, which is not subjected to amplification or regulation by other cellular processes (Milligan, 2003). Shortly, these assays employ nonhydrolysable GTP analogues (e.g. [^{35}S]GTPγS (from here the unofficial name) or non-radioactive, europium-labelled GTP-Eu) and measure their agonist-dependent accumulation. Typically assays are performed on the plasma membranes prepared from cells expressing GPCRs of interest. The GTPγS binding assays allow for distinguishing full or partial agonists from neutral antagonists, inverse agonists, and allosteric regulators (Harrison and Traynor, 2003; Zhang and Xie, 2012).

The assessment of $G\alpha_q$ (but also $G\alpha_i$) protein activation can be realised via different assays that measure activities of downstream effectors and/or generation of second messengers. As an example, in response to $G\alpha_q$ protein activation and subsequent PLC activation, second messenger inositol 1,4,5-triphosphate (IP_3) is produced in cytoplasm. IP_3 then binds to IP_3 receptors in ER thus inducing transient Ca^{2+} efflux from the internal stores to the cytoplasm. Both, radiometric (filtration and SPA assays) and fluorescence-based (including biosensor systems), assay formats are used to measure concentrations of intracellular Ca^{2+} or inositole phosphates (IP_3/IP_1) reflecting to PLC activity (Denis et al., 2012; Chen et al., 2012; Zhang and Xie, 2012). Due to the complexity and transient nature of calcium signalling sometimes it would be difficult to properly quantify experimental data in order to avoid misinter-

pretation of pharmacology of novel receptor ligands (Charlton and Vauquelin, 2010). A number of studies have also shown MC receptors providing their effects via $G\alpha_q$/PLC/Ca^{2+} pathway (see section 1.4.2. Alternative signalling pathways).

Reporter-gene assays are another alternative for detecting GPCR activity. Activation of GPCRs downstream may lead to initiation of the expression of genes via responsive elements for second messengers like CRE (cAMP-response element) and others. The transcription of a reporter gene is initiated by a transcription factor binding to its designated promoter region; e.g. CRE promoter is activated as downstream effect of the $G\alpha_s$/AC/cAMP pathway. As this assay observes events downstream from second messenger production, it may even take several hours to allow transcription of the reporter gene (Hill et al., 2001). For example, melanogenesis in melanocytes is realised by the MC_1 receptor's activated $G\alpha_s$/AC/cAMP signal transduction cascade, and subsequent CRE activation which leads to expression of genes and activation of enzymes involved in dark pigment eumelanin production (Slominski et al., 2004). Commonly used reporter-gene assays use reporter-enzymes with activities linked to a variety of colorimetric, fluorescent or luminescent readouts, such as luciferase, β-galactosidase, β-lactamase and others (Zhang and Xie, 2012). The first colorimetric assay applied in MC receptor studies utilised a β-galactosidase (lacZ) gene fused to the CRE for assessment of the MC_5 receptor activation (Chen et al., 1995).

Besides the abovementioned other possibilities for evaluation of GPCR activation exist. To mention only some of them – fluorescence-based imaging assays for monitoring β-arrestin recruitment or receptor trafficking (events involved in receptor desensitisation); or even label-free, whole cell assay technologies presenting different strategy in measuring signal transduction (integrated or cumulative responses are detected rather than the resolution of individual events) and employing biosensors that convert the summation of ligand-induced changes to optical, electrical, calorimetric, acoustic, magnetic or other quantifiable signals (Chen et al., 2012; Zhang and Xie, 2012).

2.3. GPCR preparations in assay systems

GPCRs can be studied from different aspects and with the aid of a wide variety of different methods and instrumentations. Depending on the study aims, the chosen analysis method, signal outputs and other considerations, GPCR can be studied on different receptor sources starting from live organisms to purified engineered GPCR proteins reconstituted into artificial membranes (Lundstrom, 2013). From a pharmaceutical point of view the main challenge for new drug discovery is to detect, characterise and optimise the biological activity of active molecules found. Depending on the assay used one is able to see some certain effects of the compound and/or receptor studied through the "eyes" of the assay,

whereas the type of receptor source used determines which (or the absence of which) signal will be found and in which extent. These aspects should be kept in mind when experimental results are interpreted and a signal-producing event is characterised.

Historically ligand-induced events have been studied on isolated tissues (organs) and whole-animal assays. For example, the first melanocortin receptor mediated effects were observed in 1912 on frog's skin when pituitary extract caused its darkening (Eberle, 1988). Such types of assays have their own specific applications and character, but one of the advantages is that these assays can be conducted in real time and with receptor systems in a natural environment. Usually tissues isolated from non-human sources are used; however, some differences in drug activity in human and animal tissues may exist. A shortcoming of isolated tissues is that they are not compatible with high-throughput assays where a large number of compounds should be tested. Besides that, from tissue experiments we can get only an overall drug effect, but the mechanism of drug action cannot be studied. Further technological progress and development of radioligand binding assays (Paton and Rang, 1965), and implementation of receptor sources form recombinant cell lines from human and other species (whole cells or cell membrane preparations) provided increased assays' throughput and assay miniaturisation possibilities.

As most of the GPCRs are normally present at relatively low concentrations in native tissues, very sensitive methods are required for their detection, or receptors from overexpressed or concentrated preparations have to be used. There are a wide range of methods and expression systems available for the heterologous production of recombinant GPCRs and other membrane proteins. Choosing a receptor model system is an essential question, since all of them have their own characteristics as well as advantages and disadvantages for applicability. Traditionally, binding studies have been conducted by employing mammalian cells – both transient and stable cell lines. Mammalian cells provide the most sophisticated protein processing (proper protein folding and post-translational modifications when compared to other expression systems), but the glycosylation profile may vary to a great extent between different mammalian cell types (Hossler et al., 2009). The expression levels from mammalian cell lines are considered to be relatively low; however, improvements in this area have been achieved (Geisse and Fux, 2009).

Higher expression levels (with lower costs of maintenance) can be achieved with insect cell lines. Although glycosylation in insect cells is similar but not identical to that in mammalian cells (Katoh and Tiemeyer, 2013), the posttranslational processing machinery in many aspects is very similar to mammalian cells, which allow for producing highly authentic end products of mammalian origins (Makela and Oker-Blom, 2008). Besides that, efforts have also been achieved in new insect cell line generation with a "mammalianised" protein glycosylation pathway (Aumiller et al., 2012). Insect cells are one of the most widely used expression systems for structural and ligand-binding studies,

but functional responses can also be studied on co-expressed cells (Masuda et al., 2003; Sakihama et al., 2008; Mitsui et al., 2012). We have used membrane preparations from baculovirus infected Sf9 cells (insect cells isolated from the pupal ovarian tissue of the fall armyworm *Spodoptera frugiperda*) in FA-based assay for characterisation of the dynamics of ligand binding to MC_4 receptors (Veiksina et al., 2010). Insect cells are essentially free from endogenous GPCRs, which make them valuable for studies on orphan GPCRs and receptor dimerisation. Moreover, as the baculovirus/insect cell system allows simultaneous co-expression of multiple proteins of interest, it provides the possibility to engineer cells for studying a certain process with a particular emphasis and under an improved signal-to-noise ratio (Schneider and Seifert, 2010).

Besides that, it has been shown that membrane proteins expressed on the surface of Sf9 cells also bud with baculoviruses and remain on their surface (Loisel et al., 1997). Thus, budded baculoviruses (BBV) represent more or less a "one size fits all" solution – viruses that are used for the delivery of genetic information into the cells and for the expression of receptors are also used for the exposure of those receptors on their own surfaces and can be used as a source of GPCRs (for more details see Box 1 in Results and Discussion part). Additionally, baculovirus vectors with mammalian cell-active cassette can be used in the so-called BacMam system for functional GPCR, signalling, reporter and other protein expression in mammalian cells (Kost et al., 2005; Davenport et al., 2009; Mazina et al., 2012). Virus vectors from another, alphavirus family (typically, the Semliki Forest virus, SFV) can also be used for recombinant expression of GPCRs in mammalian cells, which allow for achieving very high expression levels and can be adapted to large-scale production (Hassaine et al., 2006). The SFV/mammalian cell system also allows co-expression of multiple targets simultaneously (Lundstrom, 2003).

Bacterial- and yeast-based GPCR production has been utilised quite a lot as well, but generally these systems are considered to be less adequate platforms (especially for membrane proteins) due to decreased likelihood of proteins' proper folding and posttranslational modifications (Lundstrom, 2013).

Besides that, different artificial GPCR sources (including cell-free expression systems) have found acceptance in a variety of continuously developing specific new applications for receptor studies on the molecular level. For example, for structural and functional studies of GPCRs and other membrane proteins, they may be solubilised in the presence of detergent from their parent systems, purified and then reconstituted into artificial hydrophobic membrane systems like different types of lipoparticles, micelles or nanodiscs (Klammt et al., 2007; Junge et al., 2011). However, the proper folding and functionality of reconstituted membrane proteins remains challenging issue within these methodologies since different naturally present essential components could be eliminated. Porous beads (Roizard et al., 2011), "blebbing" structures or (sub-)micrometre sized cell derived vesicles (Grasso et al., 2013) and surface-

immobilised proteins (Früh et al., 2010) are only a few of the recently developed, interesting and "advanced" carriers of GPCRs.

Basically, introduction of radioligand binding assays pushed the "traditions" of GPCR research from whole-system kinetic approaches towards reductionist, non-natural recombinant system approaches with simplified read-outs (Kenakin, 2009). However, now, when very sensitive and versatile analysis methods are available, again more integrated approaches can be applied for studies of systems in a complex instead of studies of isolated system components. Considering the complexity of GPCR signalling machinery (including different receptor conformations, allosteric modulators, biased signalling, cell type-related functional selectivity, interactions with other signalling proteins like β-arrestins as well as scaffolding proteins) the main shortcoming of recombinant systems would be related to the absence of the whole complex of components required for characteristic therapeutic phenotypes. This could be overcome by the implementation of versatile multiparameter assay systems with cells containing a full complex of signalling reactants and monitoring signalling events as much as possible. To mention only a few, simultaneous detection of several Ca^{2+}-dependent signalling events (Piljic and Schultz, 2008), or simultaneous detection of spatially colocalised changes in Ca^{2+}, cAMP and PKA activities (Woehler, 2013). From the future perspective, multiparameter assay systems could be considered advantageous since a more likely response will be detected, including "hidden" efficacies in addition to considered "primary" activity (discussed in Hynes et al., 2013). Low expression level of GPCRs in primary cells (human or from transgenic animals with disease phenotype) are no longer an obstacle for highly sensitive, single cell, real-time, label-free detection systems that would provide clinically/therapeutically more relevant functional results. However, a considerable increase in the complexity of "resolving" these signals precludes these technologies from becoming trivial and commonly available to a wide range of scientists (Kenakin, 2009; Lundstrom, 2013).

Assay costs, simple performance, quickly obtainable results and highly qualified personnel are only a few of the aspects addressed when an assay system and receptor sources are chosen for solving certain scientific questions. Thus, any tools that allow for solving the enormous complexity of GPCRs' machinery (even in extremely small steps) have their own right to existence.

3. AIMS/OBJECTIVES OF THE STUDY

Since melanocortin receptors are involved in the regulation of a wide variety of physiological functions, melanocortin ligands have remarkable therapeutic potential for the treatment of several human disorders. Thus, it is very important to understand the mechanisms of receptor-ligand interactions and use this for the design of novel, receptor subtype selective, potent and metabolically suitable drugs.

The general aim of the current thesis was development and validation of novel assay systems for studies of the binding properties of novel melanocortin ligands and for characterisation of receptor-ligand interaction mechanisms that would enhance our general understanding of the melanocortin system.

To address this general aim, several particular tasks were proposed (with special attention paid to the MC_4 subtype):

- Comparison of ligand binding and secondary messenger activation properties of new melanocortin receptor active compounds
- Implication of novel radioligand for characterisation of the MC_4 receptor ligand binding mechanism
- Implication of a fluorescence anisotropy-based assay for characterisation of the MC_4 receptor ligand binding properties
- Implication of budded baculoviruses as a receptor source for characterisation of the MC_4 receptor ligand binding properties in a fluorescence anisotropy-based assay

4. MATERIALS AND METHODS

4.1. Cell cultures

- B16-F1 murine melanoma cells were cultured as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with a 10% heat-inactivated foetal calf serum (FBS) at 37 °C in a humidified 5% CO_2 cell incubator. Cells from frozen stocks were used in MC_1 receptor radioligand binding assays (**Paper I**), whereas freshly cultured cells were used for assessment of ligand functional effects on MC_1 receptor in cAMP assay (**Paper I**).
- COS-1 cells were grown as monolayer culture in DMEM supplemented with 10% heat-inactivated FBS at 37 °C in humidified 5% CO_2 cell incubator. 50% confluent COS-1 cells were transiently expressed with either one of the recombinant human MC_3, MC_4 and MC_5 receptor DNA (described earlier (Schiöth et al., 1997)) by using Lipofectine reagent (Gibco-BRL) according to the manufacturer's instructions. After 48 h transfected COS-1 cells were used for determination of ligands' functional effects in a cAMP assay (**Paper I**) or used for membrane preparation (**Paper II**).
- Sf9 cells were grown:
  - in 50–100 ml Sf-900 II medium at 27 °C in small spinner bottles (250 ml) as described earlier (O'Reilly et al., 1992). Recombinant human MC_3, MC_4 and MC_5 baculoviruses (prepared as described in (Kopanchuk et al., 2005)) were added to the cell culture ($2–3 \times 10^6$ cells/ml) and the incubation continued for additional 72 h before harvest, centrifugation and membrane preparation or freezing of concentrated cell suspension. Cells from frozen stocks were used for assessment of tertiary amides' binding properties in radioligand binding assays (**Paper I**). Sf9 cell membrane preparations were used in MC_4 receptor kinetic radioligand binding experiments (**Paper II**).
  - in serum-free insect cell medium (BD BaculoGold Max-XP) with supplementation of 25 U/ml penicillin and 25 μg/ml streptomycin. For production of cells expressing MC_4 receptors, 150 ml of Sf9 cell suspension in a 500-ml Erlenmeyer flask at a density 2×10^6 cells/ml was infected with a high-titre supernatant of baculovirus encoding the human MC_4 receptor and grown for 4 to 5 days with shaking at 120 rpm on an orbital shaker at 27 °C in a humidified atmosphere. Then cells were harvested, centrifuged and cell membrane preparation prepared for usage in FA-based ligand binding assays (**Paper III**).
  - in serum-free insect cell growth medium EX-CELL 420 in Celstir spinner flasks (stirring rate 115 rpm) at 27 °C in a nonhumidified environment. For the production of budded baculoviruses that display MC_4 receptors on cell membrane envelope, 500 ml of Sf9 cell suspension at a density of

2×10^6 cells/ml was infected with a high-titre supernatant of recombinant baculovirus encoding the human MC_4 receptor at a multiplicity of infection MOI = 3 and was grown for ≈ 96 h. Then supernatant fraction of cell suspension was used for preparation of the MC_4 receptor budded baculoviruses for FA-based ligand binding assays (**Paper IV**).

4.2. Receptor preparations

4.2.1. Cell membrane preparations

Membrane preparations of the MC_4 receptors from Sf9 and COS-1 cells for kinetic radioligand binding experiments were prepared as following (**Paper II**):

COS-1 cells (48 h post transfection) and Sf9 cells (72 h after infection with recombinant MC_4 receptor baculovirus) were collected by centrifugation at 800 × g for 5 min and homogenised with a glass–Teflon Dounce homogeniser (five times by 10 strokes with 30 s intervals) in an ice-cold buffer solution (B1) containing 20 mM Na-HEPES, 1 mM $CaCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 0.25 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml soybean trypsin inhibitor, pH 7.4. The homogenate was centrifuged at 700 × g for 5 min at 4 °C, the pellet was then homogenised once more in B1 solution and centrifuged. The supernatants were combined; membranes were collected by centrifugation at 70,000 × g for 60 min at 4 °C, washed once with a B1 buffer, resuspended in new B1 and recentrifuged. The final pellet was resuspended in a B1 buffer at a concentration of 1–3 mg of protein/ml and aliquots were stored at −80 °C. Protein was determined using the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as the standard.

MC_4 receptor Sf9 cell membrane preparations for FA-based ligand binding assays were prepared as following (**Paper III**):

4 to 5 days after infection of cells with a high-titre recombinant baculovirus encoding the MC_4 receptor, Sf9 cells were collected by centrifugation at 1000 × g for 10 min and homogenised using a Bandelin Sonopuls sonicator (70 W, 70%, three passes by 10 s each) in ice-cold buffer (B2), which contained 20 mM Na–Hepes, 1 mM $CaCl_2$, and a Complete EDTA-Free Protease Inhibitor Cocktail (Roche Applied Science) according to the manufacturer's description, pH 7.4. The homogenate was centrifuged at 30,000 × g for 40 min at 4 °C, and the pellet was homogenised and centrifuged once more as described above. The final pellet containing Sf9 cell membranes was resuspended in a B2 solution in a ratio of 1:15 in regard to the initial cell suspension volume, corresponding to a concentration of approximately 1 mg of protein/ml, and aliquots were stored at −80 °C until used. Protein content was determined using the Bradford method with BSA as the standard.

4.2.2. Budded baculovirus preparation

For the production of budded baculoviruses (BBV) that display MC_4 receptors on their membrane envelope (**Paper IV**), 500 ml of Sf9 cell suspension at a density of 2×10^6 cells/ml was infected with a high-titre supernatant of corresponding baculovirus at a multiplicity of infection MOI = 3 and was grown in a spinner flask for ≈ 96 h with an agitation of 115 rpm at 27 °C in a nonhumidified environment. The supernatant fraction that contained BBVs was then collected after the centrifugation of cell suspension at 1000 × g for 10 min. Following centrifugation of the collected supernatant at 48,000 × g for 40 min revealed pellet that contained BBVs. This was carefully washed with sterile incubation buffer (IB), which contained 20 mM Na-HEPES, 1 mM $CaCl_2$, Complete EDTA-Free Protease Inhibitor Cocktail (Roche Applied Science) according to the manufacturer's description, and 0.1% Pluronic F-127, pH 7.4. Then, the obtained pellet was resuspended in sterile IB in a ratio of 1:20 in regard to the initial cell suspension volume. The obtained baculovirus preparations were aliquoted and aliquots stored at –90 °C until used for analysis.

The same scheme was used for production of BBVs displaying human NPY1 receptor (neuropeptide Y receptor Y1)) on their membrane envelope with the exception that the supernatant fraction was collected 48 h post infection.

4.3. Recombinant baculovirus construction

Cloning of baculoviruses containing human MC_3 (Gantz et al., 1993a), MC_4 (Gantz et al., 1993b) and MC_5 (Chhajlani et al., 1993) receptor genes was performed as described in (Kopanchuk et al., 2005). Obtained baculovirus vectors were used for expression of these receptors into Sf9 cells, which were further used in radioligand binding experiments (as whole cells (**Paper I**) or membrane preparation (**Paper II)**). Shortly, cDNA (in pcDNA3.1) of three human MC receptors were subcloned into the HinDIII–XbaI site of the pFdHPhMC2R vector and transformed into DH10Bac-competent cells for transposition into baculovirus DNA. All vector inserts were sequenced. Recombinant baculovirus DNAs was transfected into Sf9 cells using cellfectin. Viruses were amplified about 2−3 times and stocks were prepared. Then baculovirus single clone purification procedures were performed and the clonal baculoviruses (encoding Flag-MC receptors) yielding the highest receptor expressions were selected and used further.

To obtain recombinant MC_4 receptor baculoviruses further used in FA-based ligand binding assays (**Paper III, IV)**, human MC_4 receptor cDNA in pcDNA3.1 was subcloned into the EcoRI–XhoI site of the pFastBac1 vector and transformed into DH10Bac-competent cells. All plasmid constructs were sequenced. Recombinant baculovirus was generated by homologous recombination via transfection of 7×10^5 insect cells with 5 μg of purified recombinant

bacmid DNA in the presence of 16 µl of ExGen500 in 35-mm tissue culture plates. Viruses were harvested after 72 h of incubation at 27 °C and amplified at a multiplicity of infection of 0.1 in suspension culture until high-titre virus supernatants reached. Large-scale virus stocks were harvested 96 h post infection by centrifugation at 1000 × g for 10 min, and supernatants were stored at 4 °C. Virus titres were estimated by standard plaque assay or by titration assay based on viable and infected cell size differences (Janakiraman et al., 2006); for more details see (Reinart-Okugbeni, 2012).

The same scheme was utilised for generation of NPY1 receptor recombinant baculovirus constructs, with the exception that cDNA (in pcDNA3.1, obtained from the University of Missouri-Rolla cDNA Resource Center) was subcloned into the BamHI-XbaI site of the pFastBac1 vector.

4.4. Radioligand binding assays

Screening of binding of tertiary amides to human MC receptors (**Paper I**) were performed using [^{125}I]-NDP-α-MSH radioligand binding as described earlier (Schiöth et al., 1995). Briefly, each of the radioligand binding assays comprised a dilution series of 12 concentrations in duplicates, and each compound was analysed with at least two repeats. B16-F1 murine melanoma cells (naturally expressing MC_1 receptors) or Sf9 cells expressing either one of the recombinant human MC_3, MC_4 or MC_5 receptors were taken from frozen stocks and washed with binding buffer B3 (minimum essential medium with Earle's salts, pH 7.0, 0.2% BSA, 1 mM 1,10- phenanthroline, 0.5 mg/l leupeptin and 200 mg/l bacitracin). The assays were performed in 96-well binding plates. To each well approximately 50,000 cells, 0.5 nM [^{125}I]-NDP-α-MSH (giving about 50,000 cpm) and appropriate concentrations of tertiary amide in binding buffer were dispensed. The plates were then incubated for 1 h at 37 °C, centrifuged (3500 × rpm for 5 min at 5 °C) and supernatant aspirated in order to separate the free radioligand from the cell bound radioligand. The cells were washed then with an ice-cold B3 buffer, detached from the plates with 0.1 N NaOH and the radioactivity was counted in a Wallac, Wizard automatic gamma counter.

Radioligand binding assays on Sf9 cell membranes or COS-1 cell membranes expressing MC_4 receptors (**Paper II**) were performed by incubation of membranes (5 µg protein/100 µl in equilibrium binding or 40 µg protein/ 700 µl in kinetic experiments) in the incubation buffer containing 20 mM K-HEPES, 5 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mg/ml BSA and 0.5 mM Na-acetate (pH 7.4). Binding of [^{125}I]-NDP-α-MSH or [^{125}I]THIQ was determined after incubation of membranes with different concentrations of radioligand (1 pM–6 nM), or in the case of displacements with a fixed concentration of radioligand (0.6 nM) and different concentrations of competing ligands. Incubations with [^{125}I]-NDP-α-MSH were carried out for 3 h at 25 °C and terminated by rapid filtration through 0.3% polyethyleneimine and 1 mg/ml

BSA pretreated GF/B glass–fibre filters using a Brandel cell harvester, followed by three washings with 5 ml of ice-cold 50 mM Tris–HCl buffer (pH 7.4). Incubations with [^{125}I]THIQ were carried out for 1 h at 25 °C and terminated by filtration through non-pretreated filters. Ice-cold 10 mM Na-acetate buffer (pH 4.5) used for the washing of filters was found to be optimal to minimise the amount of non-specifically bound [^{125}I]THIQ. Non-specific binding was determined in the presence of 3 μM I-NDP-α-MSH or I-THIQ.

Radioligand association kinetics was determined after addition of the radioligand to the membrane suspension in the incubation buffer. At timed intervals aliquots (200 or 500 μl) were filtered on GF/B filters and washed with the corresponding buffer. Non-specific binding was determined by incubation of the membranes with the radioligand and 1000-fold excess of non-labelled ligand.

Dissociation experiments were performed after preincubating the membrane suspension with [^{125}I]-NDP-α-MSH (0.6 nM) or [^{125}I]THIQ (0.2 and 0.6 nM) for appropriate times at 25 °C. Dissociation was then initiated by adding non-labelled NDP-α-MSH (final concentration 3 or 100 μM) or I-THIQ (final concentration 3 μM), or hAGRP(83-132) (final concentration 0.8 or 10 μM), or by 100-fold dilution of the reaction's medium. At timed intervals aliquots were filtered on GF/B filters and washed with the corresponding buffer.

4.5. cAMP assay

The ability of compounds of interest to influence the levels of cAMP (**Paper I**) was, for the MC_1 receptor, assessed in B16-F1 murine melanoma cells that express the MC_1 receptor naturally, or in transiently transfected COS-1 cells with MC_3, MC_4 and MC_5 receptors. In brief, cells were harvested and incubated for 20 min at 37 °C in 50 μl FBS-free DMEM containing 0.5 mM 3-isobutyl-1-methylxanthine and appropriate concentrations of substances of interest. cAMP was then extracted from cells by adding 4.4 M perchloric acid to achieve a final concentration of 1.25 M. After 5 min the samples were neutralised with 5 M KOH/1 M Tris and centrifuged at 3000 × rpm for 5 min at 20 °C. After centrifugation 50 μl of supernatants or cAMP standards ranging from 0.2 to 250 nM were added to 96-well microtitre plates. The cAMP concentration was measured by a bovine adrenal cAMP binding protein competitive binding assay using [^{3}H]-cAMP (specific activity 24 Ci/mmol) as labelled ligand (Nordstedt and Fredholm, 1990). In brief, [^{3}H]-cAMP (0.14 pmol, giving approximately 11,000 cpm), the sample or cAMP standard and the binding protein were incubated at 4 °C for 150 min after which the incubates were harvested by filtration on Whatman GF/B filters using a semiautomatic Brandel cell harvester. Each filter was rinsed with 10 ml ice-cold 50 mM Tris·HCl (pH 7.4), punched out and placed into scintillation vials with scintillation fluid, and the radioactivity of bound [^{3}H]-cAMP was counted and the concentration of cAMP in samples was calculated.

4.6. FA-based ligand binding assays

FA measurements with Cy3B-NDP-α-MSH (**Paper III, IV**) (GE Healthcare Life Sciences, Sweden) were performed in the simplest buffer solution required for ligand binding to MC_4 receptor (20 mM Na-HEPES, 1 mM $CaCl_2$, Complete EDTA-Free Protease Inhibitor Cocktail (according to the manufacturer's description, Roche Applied Science) and 0.1% Pluronic F-127 (Invitrogen), pH 7.4). The detergent has been found to be essential for stabilising the signal during anisotropy measurements and has no significant influence on the properties of NDP-α-MSH binding to the MC_4 receptor (Do et al., 2006). Whereas lower fluorescence intensity and anisotropy signal stability were found for TAMRA-NDP-α-MSH (AnaSpec, USA) in this buffer solution (**Paper III**). Increasing the ionic strength of the buffer (addition of NaCl and/or KCl) significantly improved fluorescence signal characteristics of TAMRA-NDP-α-MSH, and modified Krebs–Ringer buffer (135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mg/ml.BSA, and 11 mM Na-Hepes, pH 7.4) supplemented with Complete EDTA-Free Protease Inhibitor Cocktail and 0.1% Pluronic F-127 was found to be optimal for assays with this ligand.

Stocks of the fluorescent ligands in dimethyl sulphoxide (DMSO) were stored at −20 °C and diluted with appropriate assay buffers on the day of the experiment. The concentration of fluorescent ligands was confirmed by absorbance readings of Cy3B (ε_{558} = 130,000 M^{-1} cm^{-1}) and TAMRA (ε_{547} = 80,000 M^{-1} cm^{-1}). Black 96-well half area, black flat bottom polystyrene NBS microplates (Corning, Product No. 3993) were found to give optimal results for our assays (low background fluorescence and low adsorption of ligands onto the plastic surface) and were used in all experiments.

Assays were performed in a total volume of 100 µl at 27 °C (optimal temperature for growing of Sf9 cells) on a PHERAstar (BMG Labtech, Germany) microplate reader using an optical module with excitation and emission filters of 540 nm (slit 20 nm) and 590 nm (slit 20 nm), respectively. The dual emission detection mode allows the simultaneous recording of intensities that are parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the plane of excitation light. Sensitivities of channels (G factor) were corrected with a gain adjustment of the photomultiplier tubes (PMTs) using erythrosine B as a standard (Thompson et al., 2002).

All experiments were carried out in the kinetic mode and reactions were started by the addition of receptor preparation (Sf9 cell membranes or BBVs) to the microplate wells that contained the fluorescent ligand with or without competing ligands, and fluorescence intensities were registered at the appropriate time points. Unless otherwise stated, approximate MC_4 receptor concentration used per well was 1.5 nM in case of membrane preparation used and 0.5 nM when BBVs were used as a receptor source.

The ligand-specific effects were measured in the presence (nonspecific binding) or absence (total binding) of an excess of NDP-α-MSH (3 µM), and

specific binding was defined as the difference between these values. In addition, the background fluorescence of the assay, which was caused by membranes, buffers, competing ligands etc., was measured in the absence of the fluorescent ligand and was subsequently subtracted separately from all channels of all the total and nonspecific binding data, resulting in background-corrected values. Steady-state FA signals at time t after the initiation of the binding reaction was calculated as parameter $r(t)$ from the Eq. 1.

Concentration dependent binding of both, Cy3B-NDP-α-MSH and TAMRA-NDP-α-MSH, to MC_4 receptors was determined by varying the concentration of receptor and keeping the ligands' concentration constant (**Paper III**) or, in case of Cy3B-NDP-α-MSH used in tandem with BBVs (**Paper IV**), also by varying the concentration of Cy3B-NDP-α-MSH and keeping the receptor's concentration constant.

In the case of competitive binding experiments, fixed concentrations of fluorescent ligands (1 nM) and receptor preparations were incubated with increasing concentrations of the MC_4 receptor agonists (NDP-α-MSH and β-MSH (AnaSpec, USA), α-MSH, Ro27-3225, MT II, HP-228 and H-6268 (Bachem AG, Switzerland), and I-THIQ (kindly provided by Dr. Felikss Mutulis (Mutulis et al., 2003))) and antagonists (HS-024 (Tocris Bioscience, UK), SHU9119, JKC-363 and H-2716 (BachemAG, Switzerland)), and reactions were followed in the kinetic mode as described above. The experiments for all studied MC_4 receptor ligands were performed in duplicates using a 10-point dilution series.

The dissociation kinetics were measured after the preincubation of the receptor preparation with 1 nM Cy3B-NDP-α-MSH or TAMRA-NDP-α-MSH for certain time period. Subsequently, the dissociation was initiated by the addition of 3 μM NDP-α-MSH, 3 μM SHU9119, 3 μM I-THIQ or 10 mM EDTA (final concentrations), and the reactions were followed in the kinetic mode as described above.

4.7. Data analysis

For the analysis of experimental data the BindAid software (**Paper I**) (Wan System AB, Umeå, Sweden), GraphPad Prism™ 4.03 (**Paper II**) or 5.00 (**Paper III, IV**) (GraphPad Software Inc., San Diego, CA, USA) or MATLAB 7.1 programme (**Paper IV**) (MathWorks, Inc., Natick, MA, USA) was used (see original publications for more details).

5. RESULTS AND DISCUSSION

As MC receptors participate in the regulation of a wide variety of different physiological functions, selective and high-affinity MC receptor ligands would be perspective drug candidates for the treatment of diseases regulated by these receptors. In order to obtain non-peptide, MC receptor active compounds suitable for medical use, the library of 210 tertiary amides (compounds that have centrally located amide moiety with three different substituents) was designed and synthesised in Uppsala University in the laboratory of Prof. Jarl Wikberg (**Paper I**). We tested these compounds on their ability to bind to the four melanocortin receptor subtypes MC_1, MC_3, MC_4 and MC_5. Almost all synthesised compounds inhibited [^{125}I]-NDP-α-MSH binding on recombinant human MC receptors with affinities in the micromolar range. Despite considerable structural differences, most of the studied tertiary amides showed quite similar binding affinities for all the MC receptors tested. Only few of them were selective for one of the receptor subtypes having selectivity pattern $MC_1 > MC_4$ and $MC_5 > MC_3$. In order to determine whether synthesised tertiary amides are MC receptor activators or inhibitors, some of the title compounds were tested for their ability to promote or block the accumulation of cAMP in cells expressing different MC receptor subtypes. In these functional assays all the studied compounds demonstrated the ability to inhibit the increase of cAMP accumulation caused by full agonist α-MSH. In some cases the inhibition was incomplete as the remained level of cAMP was higher than the control level of cAMP. These observations led to the notion that tested compounds act as MC receptor antagonists and/or partial agonists, but the data available did not allow for clearly distinguishing these two options.

In the course of our studies aiming at the development of MC receptor subtype selective organic agonists and antagonists (Mutulis et al., 2002a, 2002b, 2005), radioligand binding assays have had an essential role. Historically, MC receptor radioligand binding assays have been developed for intact cells, which have relatively high nonspecific binding and require substantial amounts of living cells. As we were interested in improving the accuracy and sensitivity of our radioligand binding assays, we proposed that using a simpler system, where the receptors are confined in a membrane fraction rather than in whole cells, would be beneficial as less confounding effects should become introduced by ligand diffusion into the cells, cell metabolism, etc. We introduced membrane preparations from recombinantly transfected Sf9 cells as a suitable, adequate and simpler model system for studies of the MC receptor ligand binding in radioligand binding assays (Kopanchuk et al., 2005, Mutulis et al., 2005). During the characterisation and evaluation of these membrane preparations we found that the presence of Ca^{2+} ions is mandatory for achieving specific [^{125}I]-NDP-α-MSH binding to the melanocortin receptors (Kopanchuk et al., 2005). Moreover, [^{125}I]-NDP-α-MSH demonstrated heterogeneous and incomplete dissociation kinetics from the binding with MC receptor complexes (in different

extent for different receptor subtypes). Besides that, [^{125}I]-NDP-α-MSH in competition with 11 MC receptor peptides gave grossly different shapes of competition curves ranging from shallow to super-steep. As the "extent" of heterogeneity varied for different peptides and receptor subtypes, we proposed that ligand binding to the MC receptors is governed by a complex dynamic homotropic cooperative regulation. These findings indicated that more attention has to be paid to the kinetic characteristics of ligand binding to the MC receptors as well. Despite the fact that [^{125}I]-NDP-α-MSH has been widely used for studies of the MC receptors and their active compounds, we realised that very little was known however about its own kinetic properties. Our recently synthesised radioiodinated low molecular weight high-affinity MC_4 receptor selective ligand [^{125}I]THIQ (Mutulis et al., 2003) gave us the chance to compare the properties of two radioligands in MC_4 receptor-directed studies. We used these two radioligands, [^{125}I]-NDP-α-MSH and [^{125}I]THIQ, together in one study to characterise ligand binding mechanism to MC_4 receptor (**Paper II**). Whereas the kinetic characteristics of used ligands were different, they both demonstrated heterogeneity in their binding patterns to MC_4 receptors. The association kinetics of [^{125}I]-NDP-α-MSH was slow and heterogeneous, where the rate constant for slower component did not depend on the radioligand concentration, whereas the rate constant for the faster component depended linearly on the [^{125}I]-NDP-α-MSH concentration. Dissociation of [^{125}I]-NDP-α-MSH from the complex with the MC_4 receptor was biphasic and incomplete as it reached the plateau – the apparent irreversibility of bound ligand can be considered as a practically non-dissociable fraction; whereas the rate of dissociable fraction was independent from the preincubation time (an average $k_{off} = 0.006 \pm 0.001\, min^{-1}$). On the contrary, [^{125}I]THIQ demonstrated considerably faster association kinetics, which proportionally accelerated within the increase of [^{125}I]THIQ concentration. Due to the practical limitations of the radioligand binding method for the determination of very fast kinetics, no heterogeneity in [^{125}I]THIQ association kinetics was observed in the concentration range used. However, dissociation of this ligand from the complex with the MC_4 receptor was complete, fast and biphasic when initiated by non-radioactive I-THIQ. The analysis revealed that part of the [^{125}I]THIQ dissociates very fast with $\tau_{1/2} < 1\, min$, while another part released with a $\tau_{1/2} = 20\, min$. But more intriguingly, when dissociation of [^{125}I]THIQ was initiated by an excess of non-labelled peptide ligand I-NDP-α-MSH, a heterogeneous dissociation pattern was also observed, but the dissociation rate of the second phase became considerably slower, having a $\tau_{1/2} = 115\, min$. When human AGRP(83-132) was used as a displacer, the dissociation rate of the second phase became even slower with the half-life being more than 5 h. Moreover, when displacement of [^{125}I]-NDP-α-MSH and [^{125}I]THIQ was studied, the peptide and low molecular weight non-peptide ligands demonstrated different abilities to compete with either [^{125}I]-NDP-α-MSH or [^{125}I]THIQ for binding to MC_4 receptors (Fig. 5).

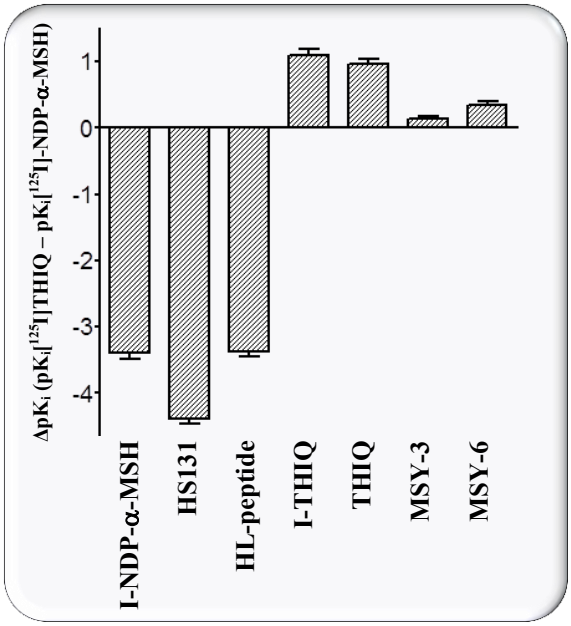


Figure 5. Differences in the apparent affinities of peptide ligands (I-NDP-α-MSH, HS131, HL-peptide) and low molecular weight non-peptide ligands (I-THIQ, THIQ, MSY-3, MSY-6) for the MC_4 receptor measured in competition with [^{125}I]-NDP-α-MSH and [^{125}I]THIQ. Data are presented as differences of negative logarithms of means of apparent ligand binding constants to MC_4 receptors measured in competition with [^{125}I]-NDP-α-MSH (pK$_i$[^{125}I]-NDP-α-MSH) and [^{125}I]THIQ (pK$_i$[^{125}I]THIQ), respectively.

In competition with [^{125}I]-NDP-α-MSH the peptide ligands had up to 20,000-fold higher apparent affinities than in competition with [^{125}I]THIQ. However, the low molecular weight ligands demonstrated almost similar abilities to compete with both radioligands, only with a slight, 1.5–12-fold preference for inhibiting the binding of [^{125}I]THIQ (Fig. 5).

The nature of the obtained results indicated that MC_4 receptor-ligand interactions are complex and that at least two ligand-binding sites are inter-connecting. Our attempts to explain the obtained results directed us to the development of a minimal model of the MC_4 receptor-ligand interactions that can account for all experimental data. It was proposed that interconnecting ligand binding sites are located on individual/separate receptor subunits within the dimeric/oligomeric complex of the MC_4 receptor. According to this model (Fig. 6), MC_4 receptors exist as preformed dimers RR and the binding of a ligand may occur only on one of the receptor subunits at a time. After conformational changes caused by the ligand binding to either one of the receptor units, the ligand molecule becomes "locked" into the complex and herewith it opens the possibility for the second ligand molecule to bind to the other receptor subunit. When both receptor units have bound [^{125}I]-NDP-α-

MSH, the radioligand can be released only from the non-isomerised unit, while it remains practically irreversibly bound to the other, isomerised unit due to the very slow de-isomerisation rate of the complex $R^*LR \rightarrow LRR$. This was also supported by results from the experiment where dissociation of [^{125}I]-NDP-α-MSH from the complex with the MC_4 receptor was initiated by dilution – similar, biphasic and incomplete, dissociation patterns were observed and the off rate of the dissociable fraction was 0.005 ± 0.001 min^{-1}. Whereas, [^{125}I]THIQ dissociation studies revealed that the release of the slower component of [^{125}I]THIQ was remarkably retarded when dissociation was initiated by I-NDP-α-MSH or hAGRP(83-132) – in these cases the rate limiting step of [^{125}I]THIQ dissociation is reaction $R^*LRL \rightarrow R^*LR$ (i.e. release of I-NDP-α-MSH from the non-isomerised site). This was supported by the fact that the rate constant of the slower component for the [^{125}I]THIQ dissociation in the presence of I-NDP-α-MSH ($k_{off} = 0.005 \pm 0.001\ min^{-1}$) coincided with the faster component's rate constant of [^{125}I]-NDP-α-MSH dissociation.

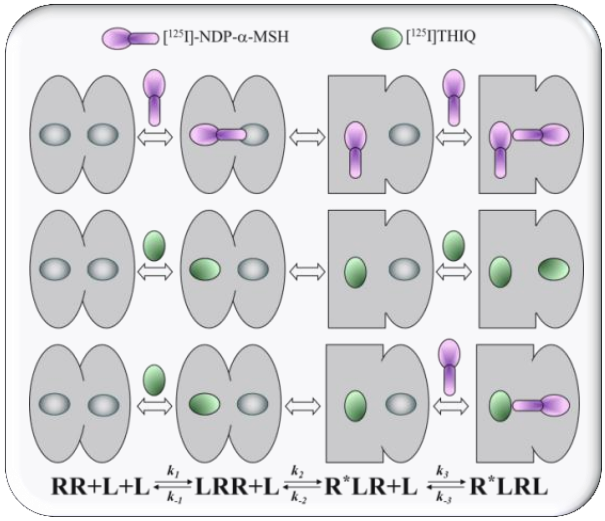


Figure 6. Schematic illustration of the model of ligand binding to tandemly arranged ligand binding sites on the MC_4 receptor dimer and the conformational transformations associated therewith.

Thus, the slow binding kinetics of [^{125}I]-NDP-α-MSH and the presence of interconnecting MC_4 receptor binding sites becomes critical when [^{125}I]-NDP-α-MSH is used for the determination of binding potencies of new MC_4 receptor active ligands. Gross differences in the apparent potencies of the same ligand were observed depending on the radioligand used in competition assays (Fig. 5). This probably would lead to the situation that some of the potentially perspective compounds are "lost" in radioligand HTS assays. That is why the kinetic properties of the competitor should also be taken into account in the

interpretation of data from competition binding experiments, but the filtration step-implicating radioligand binding assays are limited for the possibility of measuring the kinetics of the competing ligand. Developments in fluorescence-based ligand binding methods allow for achievement of even higher sensitivity, throughput, experimental "flexibility", etc., in comparison with radioactive methods. We implicated the FA-based assay system for studies of the MC receptors (**Paper III, IV**). This method allowed us online monitoring of ligand binding reactions to the MC_4 receptor and characterisation of binding dynamics to the receptor of both labelled and non-labelled ligands. Obtained kinetic information allows for estimation of more correct binding parameters essential for understanding ligand binding and its regulation mechanisms and design of new drugs with desirable properties. The binding properties of two different red-shifted fluorophore-labelled peptide ligands, Cy3B-NDP-α-MSH and TAMRA-NDP-α-MSH, to MC_4 receptors in membranes of Sf9 insect cells were compared and characterised (**Paper III**). The Cy3B-NDP-α-MSH demonstrated improved assay performance in comparison with TAMRA-labelled NDP-α-MSH having higher photostability, insensitivity to buffer properties, and better signal-to-noise ratio. Even though the FA signal of TAMRA-NDP-α-MSH was not stable, the binding of both ligands to MC_4 receptors in Sf9 cell membranes was saturable and had high affinity. In competition binding assays all studied MC_4 receptor-specific non-labelled ligands displaced fluoroligands' binding in a concentration-dependent manner with potencies in agreement with the order of their pharmacological activities. However, due to the slow kinetic characteristics of labelled NDP-α-MSH (fluorescent as well as radioactive) it is practically impossible to reveal equilibrium binding constants from competition binding experiments. Nevertheless, the FA assay system allows for monitoring of the receptor-ligand interaction process in real time thus giving the possibility to obtain at least rough estimates concerning the competitive ligand's affinity, but also binding kinetics. For example, on-line monitoring of Cy3B-NDP-α-MSH competition binding dynamics revealed that even 3 h of incubation for some of the ligands is not enough to reach the reaction's/interaction's equilibrium as apparent affinities (expressed as pIC_{50} values in Fig. 7) were still changing in time. Ignoring this kinetic factor may lead to over- or underestimation of the compound's apparent affinities.

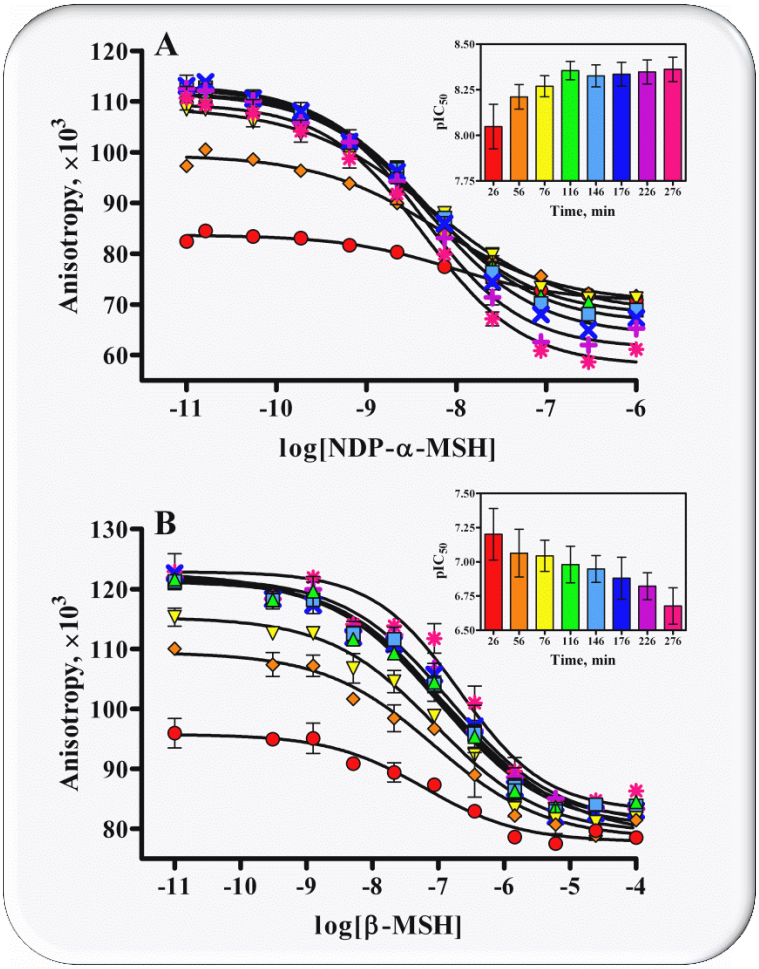
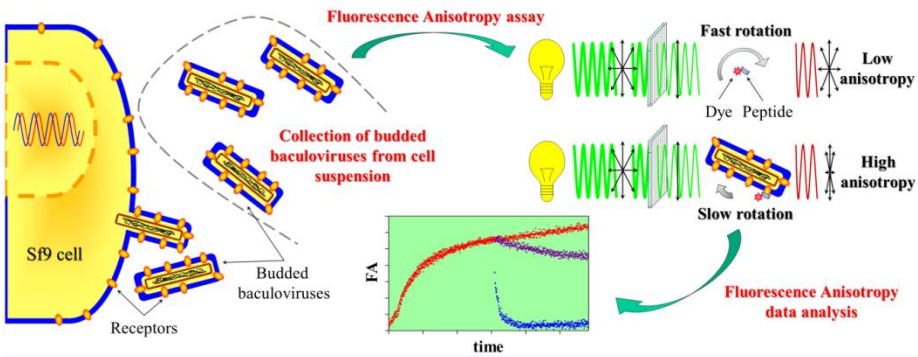


Figure 7. Dynamics of competition binding of Cy3B-NDP-α-MSH with NDP-α-MSH (A) and β-MSH (B) to the MC_4 receptor in Sf9 cell membranes. Presented data correspond to FA values determined 26 min (○), 56 min (◇), 76 min (▽), 116 min (△), 146 min (□), 176 min (×), 226 min (+) and 276 min (✱) after the initiation of competition reaction. Calculated pIC_{50} values (negative logarithm of the competitive ligand concentration causing 50% inhibition of anisotropy change caused by Cy3B-NDP-α-MSH binding to the receptor) are indicated in insets.

Although different GPCR preparations have been employed for FA assays, including whole cells (Wilkinson et al., 2001), membranes from overexpressed cells (Huwiler et al., 2010; Harris et al., 2003; Cornelius et al., 2009; Do et al., 2006), solubilised receptors (Rossi and Taylor, 2011) and GPCRs inserted into VLPs (Jones et al., 2008), implementation of Sf9 cell membranes with MC_4 receptors allowed us to achieve high levels of receptor protein expression (33 ± 3 pmol/mg protein). Thus, we were able to overcome one of the main

obstacles hindering the wider use of FA method for general studies of GPCRs as well as for ligand screening purposes, namely low receptor concentration in receptor preparations. Such a high receptor expression level provided a good assay window and led the influence of light scattering and autofluorescence from cellular components to be minimal because small amounts of membranes were used in assays. However, although good experimental conditions can be achieved in FA-based assays with Sf9 cell membrane preparations, homogenised membranes cannot be considered a homogeneous assay system – rather a mixture of lipoparticles of different shapes and sizes that range from a few tens of nanometres to more than a micrometre in diameter (Bailey et al., 2009). Additionally, the orientation of receptor proteins within such a lipoparticles is a controversial issue with limited control as well. In contrast to soluble receptor/protein studies, the generation of isotropic assay systems, where all receptor spatial orientations are evenly distributed and present in reaction's mediums, is more challenging when membrane receptors are studied. However, in the case of BBVs that we introduced as MC_4 receptor carriers in FA-based ligand binding assays (**Paper IV**), the initial isotropic conditions are primarily fulfilled (see Box 1). Cy3B-NDP-α-MSH on obtained BBV preparation demonstrated MC_4 receptor specific binding with improved FA assay performance and stability – the autofluorescence and light-scattering from the BBV preparation was more than five times lower than that observed from homogenised Sf9 cell membrane preparation with MC_4 receptors. Moreover, BBV preparation was highly stable in solutions and demonstrated long-lasting FA signal stability – the signal remained stable for at least 12 h after the initiation of the binding reaction, which is advantageous for assays where kinetically slow ligands are studied or when large numbers of samples are being screened at the same time. For the comparison, when membrane preparations from the same Sf9 cell system were used as receptor sources, the FA signal started to decrease after only 3 h of incubation (**Paper III**).

Box 1| Budded baculoviruses as a tool for homogeneous FA-based ligand binding assays



Baculovirus Surface Display (BVSD) technology is based on the ability of baculoviruses to express foreign proteins on both the surface of insect cells and their envelope. Budded baculoviruses (BBV) are produced during the insect cell infection cycle as nucleocapsids that bud from the insect cell surface. Baculoviruses are rod-shaped viruses (approximately 40–50 nm in diameter and 200–400 nm in length) that are surrounded by a double lipid bilayer envelope, which is derived directly from the host cell surface and carries membrane proteins from the host cell surface. Thus, BBVs can be considered as an essentially soluble cell-free system in which membrane proteins, including G protein-coupled receptors, are displayed on the surface of BBVs in their native conformation and environment. Moreover, BVSD represents more or less a "one size fits all" solution – viruses that are used for the delivery of genetic information into the cells and for the expression of receptors are also used for the exposure of those receptors on their own surfaces. Furthermore, manipulations with baculoviruses could be conducted in Biosafety Level 1 conditions (they are neither hazardous for the environment nor dangerous for humans), which considerably simplifies the handling and maintenance of baculovirus/insect cell system. Cost-effective, high expression level, large scale production possibilities make this system a highly attractive tool for the assessment of receptor-ligand binding interactions in fluorescence-based assays. We have shown that BBV fraction from Sf9 cells can be successfully used as a source of GPCRs in steady state FA-based assays and that this preparation has several advantages over other conventional receptor preparations (**Paper IV**).

To achieve sufficient changes of signal, FA assay demands that reactions are performed under second-order conditions where ligand and receptor concentrations are comparable ([L] ≈ [R]). This causes the excessive fluorescent ligand depletion during the course of the reaction which has to be taken into the account in the mathematical description of the interaction's processes. Here usual approximations like application of pseudo-first order reaction conditions, often used in radioligand binding assays, cannot be used anymore and this considerably complicates the data analysis. Besides specific binding, non-

specific binding sites may also contribute to the depletion of fluorescent ligand thus producing significant biases in results, if not taken into account. We proposed sets of equations for global analysis of direct and competitive equilibrium binding experiments as well as for binding kinetics (**Paper IV**). For analysis of binding kinetics we adopted analytically solved closed form equations for the simplest case of second-order reversible reactions (Malatesta, 2005).

The global data analysis from multiple variable experiments (Fig. 8) can significantly increase the content of accessible information if comparing with that from single variable experiments. However, the global data analysis approach also demands very good assay quality with initial isotropy conditions. Implementation of BBVs largely fulfilled these requirements. In the current study we performed multivariable global data analysis based on the simple second-order kinetic mechanistic model that assumes maximally three fluorescent ligand states (n = 3): free ligand, receptor-bound ligand and non-specifically bound ligand. The FA signal can be defined as the weighted sum of the anisotropy values r_i for these different fluorescent ligand states i:

$$r(t) = \sum_{i=1}^{n} f(t)_i \cdot r_i \quad (2)$$

The sum of fractions is 1 and the weighting parameter $f(t)_i$ describes the fractional proportion of the each fluorescent ligand's state in the corresponding time point. Mathematical description of each particular fraction's behaviour in the course of reaction could be found in the Supplementary materials of **Paper IV**. The applied mathematical approach (simultaneous fitting of two data surfaces (overall/total and nonspecific binding signals) as a function of both, time and receptor concentration; Fig. 8) allowed for determination of the receptor concentration, affinity and kinetic parameters of fluorescent ligand binding and state anisotropy values for different fluorescent ligand populations.

We have performed two types of receptor-ligand titration experiments: by keeping fluorescent ligand concentration constant (1 or 0.5 nM) and varying the concentration of receptor, or by keeping the amount of receptors constant and varying the concentration of Cy3B-NDP-α-MSH. It was revealed that at low Cy3B-NDP-α-MSH concentrations the interactions between the fluorescent ligand and the MC_4 receptor could be described as a simple one-site binding process, whereas at higher Cy3B-NDP-α-MSH concentrations the appearance of additional low affinity receptor binding site, that was "invisible" at lower fluorescent ligand concentrations, becomes evident. These observations are consistent with previously obtained results from MC_4 receptor studies with radiolabelled [^{125}I]-NDP-α-MSH. The emergence of an additional lower affinity MC_4 receptor-ligand interaction site with slow association kinetics observed in present study supports the previously developed model where ligand binding to MC_4 receptor is regulated by tandemly arranged ligand binding sites on the MC_4 receptor dimer.

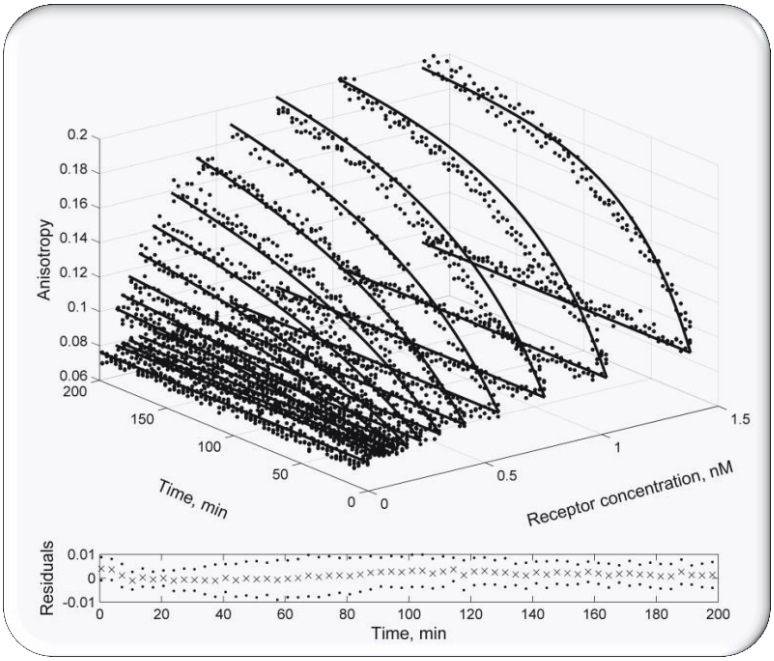


Figure 8. Association kinetics of 0.5 nM Cy3B-NDP-α-MSH binding to different concentrations of the MC_4 receptors in the BBV preparation. Two experimental data surfaces, one in the presence and one in the absence of excess of non-labelled NDP-α-MSH, were globally fitted and the lines represent the results of the non-linear optimisation procedure. The final concentrations of the MC_4 receptor binding sites in the wells were posteriorly calculated after global fitting and were presented on the receptor concentration axis (1.26, 0.93, 0.69, 0.51, 0.38, 0.28, 0.21, 0.153, 0.113, 0.083, 0.062 and 0 nM). The distribution of residuals between the experimental data and the global fit is presented on the lower panel as the mean of all time courses (×) ± the standard deviation (dashed lines).

From the displacement curves obtained in competition binding experiments with MC_4 receptor specific agonists and antagonists we were able to estimate apparent binding affinities K_i^{app}, which are in agreement with the affinities found from literature and determined with other methods. However, instead of the heterogeneous displacement curves we obtained from radioligand competition experiments (Kopanchuk et al., 2005), relatively homogeneous competition patterns have been observed in the majority of FA-based displacement experiments with BBVs. It could be explained by different assay performance conditions used. In the FA assays we used a comparable receptor and labelled ligand concentrations, and in these conditions ligands compete primarily for the high affinity sites; whereas in radioligand competition experiments the concentration of radioligand was over 10 times higher than the receptor concentration, and ligands are competing for both high and low affinity MC_4 receptor sites.

6. CONCLUSIONS

In summary, the results from biochemical studies of melanocortin receptors allowed us to make the following conclusions:

- Most of the compounds from the library of 210 designed and newly synthesised tertiary amides were able to compete with [^{125}I]-NDP-α-MSH for the binding to melanocortin receptors MC_1, MC_3, MC_4 and MC_5 with micromolar affinities (**Paper I**).
- Seven of the title compounds tested for their ability to promote or block the accumulation of cAMP, demonstrated the ability to inhibit the cAMP accumulation caused by α-MSH on all studied MC receptor subtypes (**Paper I**). In some cases the inhibition was incomplete as the remained level of cAMP was higher than the control level of cAMP. This indicates that tested compounds act as MC receptor antagonists and/or partial agonists (no more additional studies were performed to clearly distinguish between these two, competitive antagonistic and partial agonistic, activities).
- Membrane preparations from transfected Sf9 cells recombinantly expressing corresponding MC receptors are suitable targets for radioligand binding studies, improving the quality of the assay (**Paper II**).
- Comparative studies of the MC_4 receptor with two different radioligands, [^{125}I]THIQ and [^{125}I]-NDP-α-MSH, revealed that ligand binding to this receptor is regulated by complex mechanism (**Paper II**). Differences in binding kinetics of these two radioligands were the base for the development of model according to which the ligand binding occurs to the tandemly arranged interconnecting ligand binding sites on the MC_4 receptor dimer. It was demonstrated that the complex mechanism of the ligand binding to the receptor and different kinetic characteristics of radiolabelled ligands and non-labelled competitive ligands may cause the situations where apparent binding potency of competitor differs up to four orders of magnitude, depending on the radioligand used in the competition binding assay. This in turn would lead to the "loss" of potentially perspective compounds in radioligand HTS assays.
- We have implemented the FA method for the comparative characterisation of two fluorescent ligands', Cy3B-NDP-α-MSH and TAMRA-NDP-α-MSH, binding dynamics to the MC_4 receptors expressed on the membranes from homogenised Sf9 cells (**Paper III**). The Cy3B-NDP-α-MSH has been found to be more suitable for continuous monitoring of the binding reactions, thus enabling characterisation of both labelled and non-labelled ligand binding dynamics to the receptor.
- BBVs that display MC_4 receptors on their surfaces were for the first time implemented in an FA assay system as a source of GPCRs and found to be suitable for investigation of the receptor-ligand interactions (**Paper IV**).

- The inherent properties of the FA method and high assay quality provided by BBVs leads the experimental data to a level when the application of global data analysis is feasible and reasonable. Global analysis strategy worked out for a mathematical description of FA kinetic data with analytical expressions allows for the estimation of binding parameters for labelled as well as for unlabelled ligands (**Paper IV**).

All findings pointed out here can be considered as single steps in our progress of studies aiming at development of assay systems that would facilitate seeking novel MC receptor specific ligands as well as allow for characterisation of receptor-ligand interaction mechanisms that would enhance our general understanding of the melanocortin system. We believe that our findings and developments would also be applicable for other GPCR systems and would become a valuable tool for pharmaceutical and academic studies of pharmacologically active compounds and GPCRs in general.

7. SUMMARY IN ESTONIAN

Uudsete meetodite arendamine ligandide sidumisomaduste uurimiseks melanokortiini 4 retseptorile

Käesolev doktoritöö käsitleb uuringuid, mille põhieesmärgiks oli uute katsesüsteemide väljatöötamine, mida saaks kasutada nii melanokortiini retseptorite spetsiifiliste ligandide avastamiseks kui ka üldiselt retseptor-ligand vastasmõju mehhanismide iseloomustamiseks. Viis melanokortiini (MC) retseptorite alatüüpi ($MC_{1–5}$) kuuluvad suure raku membraanil asuvate G valguga-seotud retseptorite (GPCR) perekonda ning osalevad mitmete oluliste füsioloogiliste funktsioonide regulatsioonil nagu pigmentatsioon, seksuaal- ja toitumiskäitumine, energiatasakaalu reguleerimine, valu ja keha temperatuuri reguleerimine, immuunsed ja põletikuvastased reaktsioonid, jne. Seega on MC retseptoritele spetsiifilised ligandid perspektiivsed ravimikandidaadid selliste haiguste ravimiseks nagu rasvumine ja anoreksia, melanoom, erektsiooni ja seksuaalsuse häired, aga ka ärevushäired ning depressioon. Uute tõhusate ravimite leidmine sõltub suurel määral ka meie teadmistest retseptor-ligand vastasmõju mehhanismide kohta ning oskusest kasutada neid teadmisi uudsete efektiivsete raviainete disainimiseks. Lisaks sellele, oleneb tihti ka meetodist, mis on antud juhul meie „silmadeks" selles katsesüsteemis, millist toimeaine mõju me üldse näeme ja kui hästi me seda detekteerida ning iseloomustada suudame. Siin töös arendatud uudsed fluorestsentsanisotroopial (FA) põhinevad kastesüsteemid võimaldavad loobuda radioaktiivsete ühendite kasutamisest ning jälgida retseptor-ligand vastasmõjusid reaalajas. See võimaldab saada täiendavat informatsiooni organismide melanokortiinse süsteemi funktsioneerimise kohta, aga ka luua automatiseeritud katsesüsteem uute aktiivsete ühendite leidmiseks.

Töö üldise eesmärgi saavutamiseks püstitati mitu konkreetset tööülesannet:

- Uute MC retseptoritele aktiivsete ainete sidumis- ja funktsionaalomaduste karakteriseerimine ja võrdlemine
- Uue radioaktiivse ligandi kasutamine MC_4 retseptor-ligand vastasmõju mehhanismi karakteriseerimiseks
- FA meetodil põhineva katsesüsteemi kasutamine MC_4 retseptor-ligand vastasmõju omaduste karakteriseerimiseks
- Rakkudest pungunud bakuloviiruste (BBV) kasutamine retseptor allikatena FA põhinevas katsesüsteemis MC_4 retseptor-ligand vastasmõju omaduste karakteriseerimiseks

Kokkuvõtvalt võimaldasid biokeemiliste uuringute tulemused jõuda järgmiste järeldusteni:

- Enamik testitud ühenditest (ühendite raamatukogu koosnes 210 uudselt disainitud ja sünteesitud tertsiaarsest amiidist) omasid sidumisaktiivsust MC_1, MC_3, MC_4 ja MC_5 melanokortiini retseptori alatüüpidele, konkureerides [^{125}I]-NDP-α-MSH seostumisega mikromolaarse afiinsusega (**Publikatsioon I**).

- Seitsmest ühendist koosnevat valimit testiti cAMP akumulatsiooni katses ja kõik seitse ühendit demonstreerisid võimet inhibeerida α-MSH poolt stimuleeritud cAMP akumulatsiooni kõigi uuritud MC retseptori alatüüpide korral (**Publikatsioon I**). Mõnel juhul oli inhibeerimine mittetäielik, kuna lõplik cAMP tase jäi kõrgemale kontrolltasemest. Sellest tulenevalt võis järeldada, et testitud ühendid käituvad retseptori antagonistidena ja/või osaliste agonistidena.
- *In vitro* katsetes osutusid membraanpreparaadid, mis olid valmistatud rekombinantset retseptorvalku ekspresseerivatest Sf9 rakkudest, oluliselt parematekskui tervete rakkude preparaadid, mistõttu kasutati neid edasistes radioligandi sidumise uuringutes (**Publikatsioon II**).
- Uurides kahe erineva radioligandi, [^{125}I]THIQ and [^{125}I]-NDP-α-MSH, sidumisomadusi MC_4 retseptorile, täheldati et nende sidumiskineetika on üksteisest väga erinev, aga kummagi ligandi sidumine pole kirjeldatav lihtsa biomolekulaarse reaktsioonmudeliga (**Publikatsioon II**). Mõlemate radioligandi sidumiskineetika komplekssus ja erinevused viisid mudelile, mille järgi ligandid saavad seostuda kindlas järjekorras MC_4 retseptori dimeeri tandemis asetsevatele sidumiskohtadele. Ligandide järjestikune seostumine tekitab osaliselt mittekonkurentse olukorra, kus võib juhtuda, et uuritava ühendi näiv afiinsus resteptorile võib erineda kuni neli suurusjärku, olenevalt väljatõrjumiskatses kasutatava radioligandi omadustest. See võib omakorda viia oluliste ravimkandidaatide „kaotuseni“ laiaulatuslikes kiirsõelumis katsesüsteemides, kus ligandide kineetikale erilist tähelepanu ei pöörata.
- Võtsime kasutusse ja arendasime edasi FA põhinevat meetodit ligandide sidumise uurimiseks MC_4 retseptorile. Leidsime kaks fluorestsentsligandi, Cy3B-NDP-α-MSH ja TAMRA-NDP-α-MSH, mis võimaldasid jälgida ligandi sidumise dünaamikat MC_4 retseptorile homogeniseeritud Sf9 rakkude membraanides (**Publikatsioon III**). Seejuures osutus Cy3B-NDP-α-MSH olulisemalt stabiilsemaks, võimaldades uurida nii märgistatud kui märgistamata ligandide sidumisdünaamikat mitmete tundide jooksul.
- Näitasime esmakordselt, et rakkudest pungunud bakuloviirused koos nende pinnal olevate MC_4 retseptoritega on väga sobiv materjal retseptor-ligand vastasmõjude uuringuteks FA katsesüsteemis (**Publikatsioon IV**). Viirusosakesed tagavad retseptorpreparaadi homogeensuse ja ka stabiilsuse lahuses väga pika katseaja jooksul. Antud lähenemine ja metoodika on perspektiivne ka teiste GPCRide uurimisel ja automatiseeritud kiirsõelumissüsteemide arendamisel.
- FA meetodile iseloomulikud omadused ja BBV poolt tagatud katsesüsteemi kõrge kvaliteet on viinud eksperimentaalsed andmed uuele tasemele, kus globaalse andmeanalüüsi rakendamine on võimalik ja põhjendatud. Töötati välja globaalanalüüsi strateegia FA kineetiliste andmete matemaatiliseks kirjeldamiseks, mis võimaldab arvutada kineetilisi sidumisparameetreid nii märgistatud kui märgistamata ligandide jaoks (**Publikatsioon IV**).

Käesolevas töös arendatud uudsed fluorestsentsanisotroopial põhinevad kaste-süsteemid võimaldavad loobuda radioaktiivsete ühendite kasutamisest ning jälgida retseptor-ligand vastasmõjusid reaalajas. See võimaldab saada täien-davat informatsiooni organismide melanokortiinse süsteemi funktsioneerimise kohta ning luua automatiseeritud katsesüsteem uute aktiivsete ühendite testimiseks. Seejuures usume, et meie arendused on rakendatavad ka teiste retseptorsüsteemide uurimiseks ning neist saab oluline vahend nii biokeemiliste mehhanismide uuringuteks, kui ka uute farmakoloogiliselt aktiivsete ühendite leidmiseks.

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1. **Veiksina, S.**, Kopanchuk, S., Rinken, A. (2014) Budded baculoviruses as a tool for a homogeneous fluorescence anisotropy-based assay of ligand binding to G protein-coupled receptors: the case of melanocortin 4 receptors. *Biochim. Biophys. Acta (BBA) – Biomembranes*, 1838(1B):372–381.
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