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Ligand binding, allosteric modulation and constitutive activity of melanocortin-4 receptors

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

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Author's contribution:

I The author was involved in the optimization of the production of the receptor preparation by centrifugation and tangential flow filtration (TFF) for the ligand binding experiments as well as in the data analysis.

II The author was the principal investigator responsible for the planning and performing the experiments as well as the data analysis and writing of the manuscript.

III The author was the principal investigator responsible for the planning and performing the ligand binding and functional assays as well as the data analysis and writing of the manuscript.

ABBREVIATIONS

AC	adenylyl cyclase
ACTH	adrenocorticotropic hormone
ASIP	agouti signalling protein
ATP	adenosine triphosphate
BacMam	a recombinant baculovirus for delivering genes of interest into mammalian cells
BBV	budded baculoviruses
BRET	bioluminescence resonance energy transfer
cAMP	3',5'-cyclic adenosine monophosphate
CNS	central nervous system
CS	conformational selection
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
EC_{50}	concentration of the compound that produces 50% of the maximal possible effect
EDTA	ethylendiaminetetraacetic acid
FA	fluorescence anisotropy
FCS	fluorescence correlation spectroscopy
FDA	the United States Food and Drug Administration
FI	fluorescence intensity
FRET	Förster/fluorescence resonance energy transfer
GPCR	G protein-coupled receptor
G protein	guanine nucleotide-binding protein
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HTS	high-throughput screening
IB	incubation buffer
IC_{50}	concentration of the compound that produces 50% of the maximal inhibitory effect
IF	induced-fit
ivp	infectious viral particles
K_d	equilibrium dissociation constant of a ligand
k_{off}	dissociation rate constant of a ligand
k_{on}	association rate constant of a ligand
MC_1-MC_5	subtypes 1 to 5 of melanocortin receptors
MOI	multiplicity of infection
MPAK	mitogen-activated protein kinase
MSH	melanocyte-stimulating hormone
PKA	protein kinase A
PKC	protein kinase C
POMC	proopiomelanocortin

Sf9	*Spodoptera frugiperda* cell line
TFF	tangential flow filtration
TFI	total fluorescence intensity

1. LITERATURE OVERVIEW

1.1. G protein-coupled receptors

Various extracellular signals (chemical or physical) can initiate a chain of biochemical reactions inside the cell by interacting with membrane proteins called receptors. The largest family of membrane receptors are G protein-coupled receptors (GPCRs), which can transmit extracellular signals into cells mainly via G protein activation. Human GPCRs (about 800) are divided into five subfamilies based on their amino acid sequence and structure, among which the rhodopsin family is the largest (about 700) (Fredriksson *et al.* 2003; Wacker *et al.* 2017). These receptors consist of seven hydrophobic transmembrane α-helices, which are interconnected by intra- and extracellular loops. The receptors can have highly variable structures that allow the binding of specific ligands. Ligands are various extracellular signals, including hormones, neurotransmitters, toxins and drugs.

GPCRs regulate a wide spectrum of essential functions in the human body, and dysfunctions in their signalling are involved in a number of diseases. Thus, GPCRs are important drug targets for the pharmaceutical industry. Structural, ligand binding, and functional studies of GPCRs provide valuable information for understanding their signalling mechanisms and for the development of new drugs. The discovery of cellular signal transduction via G proteins and the studies of GPCRs have led to two Nobel Prize nominations, one in physiology or medicine to Alfred G. Gilman and Martin Rodbell (1994) and one in chemistry to Robert J. Lefkowitz and Brian K. Kobilka (2012). Although drug development has been successful in the case of many GPCRs, there are still a lot of receptors where the discovery of approved drugs has failed despite great efforts.

1.1.1. G protein-coupled receptors as signal transducers

Signal transduction is the process where a cell receives an extracellular signal and transmits it into the cell (Rodbell *et al.* 1971). A signal-stimulated membrane receptor activates a chain of biochemical reactions that produces a secondary signal or a change in the activity of a particular protein inside the cell.

The signal transduction process of GPCRs is usually regulated by the ligand binding to the receptor. The specificity of this signal transduction process is achieved by the complementarity between the ligand and the receptor. The signal transduction is mediated by the conformational changes of the receptor. For simplicity, the conformational changes of the receptor have been often described by conformational selection (CS) or induced-fit (IF) mechanism (Leff 1995; Castillo and Katz 1957). Based on the CS model, ligands bind preferentially (with higher affinity) to the receptor in a particular conformational state (active or inactive). Whereas, based on the IF model, ligand induces a particular conformational state of the receptor after the binding. Although, the ligand

binding process is likely much more complicated and consists of several steps of conformational adjustments (Meyer-Almes 2016). It has been shown that the receptor can undergo different conformational adjustments depending on the ligand (Nygaard *et al.* 2013).

Ligands are divided into two main categories based on the ligand binding site of the receptor – orthosteric ligands and allosteric ligands or modulators (Neubig *et al.* 2003). Orthosteric ligands bind to the active binding site of the receptor, and allosteric ligands bind to a topographically different, but conformationally linked recognition domain of the primary binding site. Allosteric ligands can usually modulate (activate or inhibit) the binding of an orthosteric ligand by altering the conformation of the orthosteric binding site of the receptor upon binding. Whereas in case receptor multimers are formed, cooperativity between the receptor binding sites can add another level of sophistication to the ligand binding process (Meyer-Almes 2016).

Ligands can also be divided based on the effect that ligand binding has on the receptor function. The three main categories are receptor activity enhancing agonists, non-altering antagonists, and reducing inverse agonists. The effect of ligand binding is determined by the level of constitutive/basal activity of receptors (Kenakin 2005; Bond and IJzerman 2006). The constitutive activity is the receptors' capacity to induce agonist-independent signalling. Elevated basal activity is required for antagonists to act as inverse agonists (Chai *et al.* 2003). Increasing number of GPCRs have shown the ability to exhibit increased basal activity in vitro (Costa and Cotecchia 2005; Coll 2013), but its mechanism is not yet fully understood.

GPCRs signalling is commonly mediated through the intracellular heterotrimeric G-proteins. Heterotrimeric G proteins consist of three subunits: α subunit, and tightly bound β and γ subunits (Hepler and Gilman 1992). In response to agonist binding or due to the constitutive activity, GPCRs associate with guanine nucleotide-binding proteins (G proteins) and acts as guanine nucleotide exchange factors. In the inactive state of the G protein trimer, guanosine diphosphate (GDP) is bound to the α-subunit. Upon activation, G proteins undergo conformational changes that lead to the release of GDP from the α-subunit and the subsequent binding of guanosine triphosphate (GTP). This results in further conformational changes that lead to the dissociation of the G protein from the receptor; and dissociation of the GTP-bound α-subunit from the βγ-complex. The released G protein components can interact with and regulate different effector systems, including ion channels and enzymes that generate second messengers. The end of the G protein activation cycle is marked by the GTP hydrolysis to GDP and subsequent reunification of the α-subunit and the βγ-complex (Milligan and Kostenis 2006).

The heterotrimeric G proteins are divided into four families based on the α-subunits: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ (Gilman 1987). Depending on the family, signalling is conveyed through different pathways (Kristiansen 2004; Landry *et al.* 2006; Birnbaumer 2007). The $G\alpha_s$ activates and $G\alpha_{i/o}$ inhibits the membrane-bound enzyme adenylyl cyclase (AC) that stimulates the production

of cAMP (3',5'-cyclic adenosine monophosphate) from ATP (adenosine triphosphate). Subsequently, the level of cAMP modulates the activity of protein kinase A (PKA) pathway. $G\alpha_{q/11}$ family activates the membrane-bound enzyme phospholipase Cβ (PLCβ), which can lead to the subsequent activation of protein kinase C (PKC) and Ca^{2+} release. $G\alpha_{12/13}$ family can modulate the activity of monomeric G proteins, PKA, or PKC pathways. Importantly, GPCRs can have preferential binding to some type of G proteins, but often they are able to couple to different types of G proteins.

1.1.2. Melanocortin-4 (MC_4) receptors

Five melanocortin receptor subtypes (MC_1-MC_5) that belong to the rhodopsin class of GPCRs were cloned in 1990s (Cone 2000). Melanocortin receptors are one of the smallest GPCRs in size consisting of about 300 amino acid residues. These receptors are attractive for drug development companies and researchers as they play a key role in the regulation of multiple important functions in the human body (Wikberg and Mutulis 2008). All melanocortin receptors, except the MC_2 receptors, have been shown to exhibit high constitutive activity in vitro (Coll 2013). It is unique, however, that their constitutive activity is regulated not only by endogenous peptide agonists but also by endogenous protein antagonists that can act as inverse agonists in case of elevated basal activity (Chai *et al.* 2003; Coll 2013).

The human MC_4 receptor was cloned in 1993 (Gantz *et al.* 1993), and its crystal structure has been recently solved (Yu *et al.* 2020). This 332 amino acid long receptor has the closest sequence homology to human MC_3 receptors (over 50%). The MC_4 receptors are mainly expressed in brain and spinal cord but have also been found in the periphery (Wikberg *et al.* 2000; Abdel-Malek 2001; Chaki and Okuyama 2005). MC_4 receptors are able to couple to different G protein families ($G\alpha_s$, $G\alpha_{i/o}$, and $G\alpha_{q/11}$), but they are also able to couple to the potassium channel Kir7.1 independently of G proteins (Tao 2010; Asai *et al.* 2013). Whereas, most of the earlier studies were directed at the $G\alpha_s$ stimulated cAMP production as the indicator for MC_4 receptor activation. In addition to the cAMP signalling pathway, the MC_4 receptors are also able to activate the mitogen-activated protein kinase (MPAK) signalling pathway and increase intracellular calcium concentration. The MC_4 receptors are involved in the regulation of several physiologically important functions, whereas their effects on eating behaviour and energy homeostasis have been most studied (Wikberg and Mutulis 2008). Activation of the MC_4 receptors has been shown to increase energy expenditure (accelerate metabolism) and reduce food intake. MC_4 receptor ligands are potential drugs for obesity, cachexia, sexual dysfunction, addiction, mood disorders, and neuropathic pain (Wikberg and Mutulis 2008; Tao 2010). Despite great efforts done so far, there is still a need for therapeutically approved drugs for MC_4 receptors (Ericson *et al.* 2017). Recently, bremelanotide (Vyleesi™) for MC_4 receptors was therapeutically approved for

the treatment of premenopausal women with hypoactive sexual desire disorder (HSDD) (Dhillon and Keam 2019). The United States Food and Drug Administration (FDA) has also just accepted (in May 2020) a new drug application of MC_4 receptor agonist setmelanotide for the treatment of monogenic and syndromic obesity (Kühnen *et al.* 2016; Clément *et al.* 2018).

1.1.3. MC_4 receptor ligands and modulators

The first experiments demonstrating the *in vivo* effect of melanocortin peptides were performed as early as the 1950s (Shizume *et al.* 1954), paving the way for the isolation and characterization of melanocyte-stimulating hormones (MSHs). Melanocortin peptides are among the first purified and sequenced biologically active peptides. The hypothesis that larger peptides may act as precursors to smaller forms led to the discovery of proopiomelanocortin (POMC), a precursor molecule of melanocortin peptides (Nakanishi *et al.* 1979). The melanocortin peptide agonists α-, β-, γ- and δ-MSH and adrenocorticotropic hormone (ACTH) are obtained by post-translational treatment of POMCs with prohormone convertases (Cone 2000). All endogenous melanocortin receptor agonists contain a conserved His-Phe-Arg-Trp sequence (also called the core sequence), which is necessary for the binding to and stimulation of melanocortin receptors. The melanocortins that bind to the MC_4 receptor are α-MSH, β-MSH, γ-MSH, and ACTH (Cone 2000). The melanocortin system is unique among GPCRs as it has two endogenous antagonists: agouti protein (also called the agouti signalling protein, ASIP) in the periphery and agouti-related protein (AgRP) in the central nervous system (Lu *et al.* 1994; Ollmann *et al.* 1997). Whereas AgRP is an MC_4 receptor antagonist. Both antagonists have shown to behave as inverse agonists for melanocortin receptors in case of elevated constitutive activity (Haskell-Luevano and Monck 2001; Nijenhuis *et al.* 2001; Chai *et al.* 2003).

Several active peptides and non-peptides have been synthesized in the search for ligands that are selective for melanocortin receptors. Peptide agonists, aliphatic NDP-α-MSH (also called melanotan I) and cyclic melanotan II (MTII), are derivatives of α-MSH, which bind with high affinity to melanocortin receptors and have increased stability (Sawyer *et al.* 1980; Al-Obeidi *et al.* 1989). For both of the synthetic agonists, L-phenylalanine amino acid residue in the core sequence was replaced with D-phenylalanine amino acid residue. Non-peptide agonist have also been found for MC_4 receptors, such as the small, potent, and selective tetrahydroquinoline derivative THIQ (Sebhat *et al.* 2002). Replacement of D-phenylalanine with a hydrophobic D-3-(2-naphthyl)alanine amino acid residue in the cyclic MTII led to the discovery of the high affinity MC_4 receptor antagonist SHU9119 (Hruby *et al.* 1995). Synthesis of several disulfide α-MSH analogues, such as HS024 (Kask *et al.* 1998), has led to the discovery of selective high affinity MC_4 receptor antagonists.

Different physiological metal ions have shown to act as modulators of the signal transduction of multiple GPCRs (May *et al.* 2007; Westhuizen *et al.*

2015). Ca^{2+} ions are critical cofactors for the high affinity ligand binding to MC_4 receptors and for the agonist-induced signalling in G_s protein and Kir7.1 potassium channel pathway (Kopanchuk *et al.* 2005; Veiksina *et al.* 2010; Yu *et al.* 2020). These ions have also found to be essential for the signalling of other melanocortin receptor subtypes (Salomon 1990; Kopanchuk *et al.* 2005; Mazina *et al.* 2012). Whereas Zn^{2+} and Cu^{2+} ions have been shown to inhibit the ligand binding to MC_1 and MC_4 receptors, while their role in the signalling of the receptors has remained inconclusive (Holst *et al.* 2002; Lagerström *et al.* 2003). In one case, it was reported that Zn^{2+} acts as an agonist and increases the peptide agonist-induced signalling of MC_4 receptors (Holst *et al.* 2002), but in another case, these ions exhibited no agonistic properties and reduced the peptide agonist-induced signalling in G_s protein pathway (Lagerström *et al.* 2003).

Ligand binding is also modulated by the oligomerization of MC_4 receptors (Kopanchuk *et al.* 2006; Lensing *et al.* 2019). There is growing number of studies showing that MC_4 receptors can form (homo- and hetero-) dimers or higher order oligomers (Kopanchuk *et al.* 2005; Elsner *et al.* 2006; Kopanchuk *et al.* 2006; Nickolls and Maki 2006; Rediger *et al.* 2009; Chapman and Findlay 2013; Müller *et al.* 2016). It has been demonstrated that ligand binding and signalling of MC_4 receptor homodimers is a complex dynamic process, which is governed by asymmetric regulation of co-operative binding sites (Kopanchuk *et al.* 2005; Kopanchuk *et al.* 2006; Lensing *et al.* 2019). In addition, ligand binding to MC_4 receptors can also be modulated by several accessory proteins (Cooray and Clark 2011; Asai *et al.* 2013).

1.2. Characterization of ligand binding to GPCRs

Binding to a target GPCR is essential for the pharmacological action of a drug molecule. Therefore, drug development has mainly focused on finding selective drug candidates with high affinity binding to the target receptors. Although the affinity is important, it does not reveal much about the kinetic properties of the ligands. There is a growing understanding that ligand's association rate and residence time (receptor occupation period by a ligand) can also be important indicators for discovering drug candidates with increased signalling efficacy, duration, and selectivity (Copeland *et al.* 2006; Hoffmann *et al.* 2015; Meyer-Almes 2016; Sykes *et al.* 2019). Therefore, ligand binding assays that enable to study the affinity, as well as the kinetic aspects of ligand-receptor interactions, are becoming increasingly appreciated.

1.2.1. Ligand binding assays

Majority of methods that are used to study GPCR and ligand interactions implement some type of labelling. After the introduction of radioisotope labelled

ligands at the end of 1960s (Paton and Rang 1965; Lefkowitz *et al.* 1970), the radioligand binding assay became widely used for the determination of affinity and kinetic properties of ligand binding to the receptors. Although the highly sensitive radioactive method is still often used today, it poses limitations for kinetic studies, due to the need to separate receptor-bound ligands from free ligands, which is usually achieved by filtration. Instead, a bead-based scintillation proximity method could be used for kinetic measurements of radioligand binding to receptors (Xia *et al.* 2016). Whereas the critical early time points could still be lost due to the settling of beads. In addition, the use of radioactive ligands poses restrictions due to the safety level, waste disposal, and high cost issues.

The availability of fluorophores with high brightness and photostability has enabled the development of fluorescence-based methods to measure ligand binding to GPCRs (Hertzberg and Pope 2000). Binding of labelled ligands to receptors may alter the properties of the fluorophores, such as fluorescence spectrum, intensity, lifetime, rotational and/or translational mobility. Most of the fluorescent methods are suitable for continuous online monitoring of receptor-ligand interactions, as there is no need for the separation of unbound ligands. High-sensitivity fluorescence measurements can be carried out in a population of molecules or in a single molecule level (Hern *et al.* 2010; Kasai *et al.* 2011). Ligand binding properties of GPCRs have been studied using fluorescence intensity (FI), fluorescence anisotropy (FA), fluorescence correlation spectroscopy (FCS) and (Förster/bioluminescence) resonance energy transfer (FRET/BRET) methods (Hoffmann *et al.* 2015; Sykes *et al.* 2019). Although these techniques have several advantages, they are also prone to some limitations, connected with autofluorescence, quenching and scattering of fluorescence emission, and inner-filter effects. Fluorescent methods are becoming increasingly popular, as high affinity fluorescent tracers are already available for numerous receptors (Ciruela *et al.* 2014; Vernall *et al.* 2014). Although, the fluorescent labelling can lead to substantial changes in the biochemical properties of ligands (especially for small-molecule ligands), which should, therefore, be treated as novel chemical entities (Vernall *et al.* 2014). However, the use of fluorophores is safer, and their waste disposal is cheaper compared to radioactive ligands.

1.2.1.1. Fluorescence anisotropy assay

Fluorescence anisotropy (FA) method is used to study various molecular interactions, including ligand binding to the receptor. FA method detects the change in rotational freedom of the fluorescent ligand upon the receptor binding process. This technique is based on the principle that receptor-bound fluorescent ligands that are excited by linearly polarized light emit polarized light, and fluorescent ligands that are freely rotating in solution emit depolarized light (Weber 1952; Owicki 2000). The values of fluorescence anisotropy (FA) are

obtained experimentally from the measurement results of fluorescence intensities parallel and perpendicular to the plane of excitation light, as shown in the following equation:

$$FA = \frac{I_{II} - I_{\perp}}{I_{II} + 2 \times I_{\perp}}, \quad (1)$$

where I_{II} and $I_{\perp}$ denote the fluorescence intensities parallel and perpendicular to the plane of excitation light and their sum ($I_{II} + 2 \times I_{\perp}$) corresponds to the total fluorescence intensity (TFI) of emitted light (Jablonski 1960). Instrumental differences in the sensitivities of parallel and perpendicular polarizers are calibrated by G-factor correction (Owicki 2000).

The FA ligand binding assay is easy-to-perform due to a simple principle and a low-demand apparatus that allows continuous online measurement of several samples simultaneously. Due to the high sensitivity, it is possible to perform experiments on small volumes of microtiter plates, which ensures economic high-throughput screening. Promising results have been obtained using this assay in kinetic studies of several GPCRs, such as endothelin A, muscarinic M1, A2A adenosine, melanocortin-4, serotonin 1A and dopamine 1 receptors (Junge *et al.* 2010; Huwiler *et al.* 2010; Kecskés *et al.* 2010; Veiksina *et al.* 2010; Tõntson *et al.* 2014; Allikalt *et al.* 2018). The high quality of FA data enables to determine the binding affinities and kinetic parameters of labelled and unlabelled ligands and mechanism of the binding process (Rinken *et al.* 2018). Although, the data analysis is more complex compared to radioligand binding assays, as the depletion of the fluorescent ligand in the binding process has to be taken into account in FA experiments (Roehrl *et al.* 2004; Veiksina *et al.* 2010). This is caused by the ratiometric nature of this method, which requires that the fluorescent ligand and the receptor to be used in comparable concentrations.

There is a variety of receptor sources that have been implemented for ligand binding assays starting from live organisms to solubilized receptors in liposome or high-density lipoprotein systems (Früh *et al.* 2011; Mitra *et al.* 2013). The use of native receptor sources for high-throughput assays is limited due to the low concentration GPCRs. Therefore, systems that enable overexpression of recombinant receptors are often used for these assays. Proper protein processing, including protein folding and post-translational modifications (glycosylation, phosphorylation, fatty acid acetylation) is necessary for recombinant GPCRs to retain properties close to its native receptors. Mammalian cells are most suitable for the processing of recombinant mammalian GPCRs, but the receptor expression levels remain relatively low. Whereas, insect cells enable mammalian-like protein processing but exhibit higher protein expression. Although, insect cells pose limitations for glycosylation (Katoh and Tiemeyer 2013), this can be solved by using an insect cell line that has been developed to allow higher eukaryotic like glycosylation (Aumiller *et al.* 2012).

The expression of recombinant GPCRs is achieved by the introduction of foreign genes into cells using chemical, physical or biological techniques. A widely used biological technique uses baculoviruses as foreign gene expression vectors for the production of large amounts of recombinant GPCRs. Recombinant baculoviruses are most commonly used in combination with the insect cell lines Sf9 or Sf21 (cells isolated from pupal ovarian tissue of fall armyworm, *Spodoptera frugiperda*). The baculovirus vector initiates early gene expression in the cell, leaving more time for post-translational modifications to occur (Massotte 2003). The combined insect cell-baculovirus expression system is a very efficient system for the large-scale production of recombinant receptors with the native protein-like properties.

It has been shown that a good signal output for FA ligand binding assay can be achieved with the implementation of membrane preparations of recombinant Sf9 cells (Veiksina *et al.* 2010). Although, the membrane preparations pose limitations due to the uncontrolled size of lipoparticles and orientation of the receptors. A novel approach to improve the experimental conditions for FA is the implementation of budded baculoviruses (BBVs), which display recombinant receptors on their surface (Veiksina *et al.* 2014). BBVs are surrounded by the plasma membrane that is derived from the infected Sf9 cells when the nucleocapsids bud out of the host cells (Braunagel and Summers 1994). In the BBVs (250–300 nm in length, 30–60 nm in diameter), membrane proteins are captured in the correct orientations forming a homogenous system for the FA ligand binding assays. Implementation of baculoviruses is a two in one method for gene delivery and receptor expression, which requires only Biosafety Level 1 conditions.

1.2.2. Functional assays

Functional assays are used to determine the biological activity and efficacies of ligands upon binding to the receptors. Many GPCRs mediate the biological effects of ligands through a cyclic adenosine monophosphate (cAMP) signalling pathway. Therefore, many functional assays are directed to detect changes in cAMP level in cells upon receptor activation. Commonly used methods implement labelled cAMP in competition assays or labelled cAMP-binding partners (PKA, Epac, cyclic nucleotide gated ion channels) in non-competition assays to determine the changes in the endogenous cAMP levels using radioactive or non-radioactive signals (Hill *et al.* 2010; Zhang and Xie 2012).

The FRET-based biosensors offer novel possibilities to monitor the activation of cAMP production in real-time in living cells. The biosensor consists of a cAMP recognition domain that is sandwiched between donor and acceptor fluorophores with overlapping emission and excitation spectra. The FRET-based technique is based on the principle that a radiationless energy transfer takes place if donor and acceptor fluorophores are close ($d < 10$ nm) (Klarenbeek *et al.* 2011). Whereas, the efficiency of FRET depends on the distance and orientation between the two fluorophores, which is altered upon cAMP binding

to the biosensor's recognition domain (Lohse *et al.* 2007; Okumoto *et al.* 2012). Therefore, the change in FRET signal depends on the change in cAMP concentration in the cell. In most cases, the values of FRET are obtained experimentally from the fluorescence intensity ratio of acceptor and donor fluorophores (or vice versa). Whereas, the issues of background fluorescence and spectral bleed-through can be compensated if the relative change of FRET (ΔFRET) is calculated as shown in the following equation:

$$\Delta\mathrm{FRET} = \frac{\left(I_0^{acceptor}/I_0^{donor}\right) - \left(I_t^{acceptor}/I_t^{donor}\right)}{\left(I_0^{acceptor}/I_0^{donor}\right)}, \qquad (2)$$

where $I_0^{acceptor}$, I_0^{donor} and $I_t^{acceptor}$, I_t^{donor} correspond to the fluorescence emission intensities of the acceptor and donor fluorophores before and after cell treatment, respectively.

First generation FRET-biosensors were designed based on cAMP-sensitive protein kinases or ion channels as the recognition domains (Adams *et al.* 1991; Fagan *et al.* 2001; Rich *et al.* 2001). These biosensors were soon substituted with cAMP sensitive Epac-based recognition elements, because of their uniform cellular expression, increased sensitivity, and higher signal-to-noise ratio. There are four generations of biosensors based on either Epac1 or Epac2 proteins that are tagged with various FRET-pairs (Nikolaev *et al.* 2004; Ponsioen *et al.* 2004; van der Krogt *et al.* 2008; Goedhart *et al.* 2010; Klarenbeek *et al.* 2015). Importantly, if native cAMP recognition elements are used for biosensors, they should be modified to become catalytically inactive to avoid disruption of cellular functions (Okumoto *et al.* 2012). The fourth-generation Epac-based FRET sensors have demonstrated higher dynamic range, brightness, and photostability (Klarenbeek *et al.* 2015). This was achieved by implementing modified structure of Epac1 and novel fluorophores. In the case of Epac-FRET sensors, the binding of cAMP induces a conformational change, which makes the conditions for FRET unfavourable. Therefore, FRET signal is high when there is low level of cAMP and low when there is high level of cAMP in the cell.

FRET-based biosensors were first microinjected into cells (Adams *et al.* 1991), but the development of genetically encoded sensors allowed non-invasive expression of biosensors, and monitoring of FRET change in living cells (Zaccolo and Pozzan 2002). Mammalian cells are most suitable to characterize the signal transduction of human GPCRs coupled to the cAMP pathway. Implementation of recombinant baculoviruses as foreign gene expression vectors in mammalian cells allow efficient transgene expression levels with low toxicity (Kost and Condreay 2002; Mazina *et al.* 2012; Mazina *et al.* 2013; Mazina *et al.* 2015a; Mazina *et al.* 2015b). This BacMam system is safe and convenient to use, as baculoviruses cannot replicate in mammalian cells and require only Biosafety Level 1 conditions.

2. AIMS OF THE STUDY

The general aim of the study was to gain additional information about ligand binding, allosteric modulation, and constitutive activity of melanocortin-4 (MC_4) receptors. To achieve this goal, several particular tasks were raised within this study:

- Optimization of the preparation method of budded baculoviruses and development of a fluorescence anisotropy (FA)-based assay for the characterization of ligand binding kinetics to MC_4 receptors.
- Design and implementation of novel fluorescent ligands with different kinetic properties for ligand binding studies of MC_4 receptors.
- Implementation of a cAMP biosensor assay for the characterization of signal transduction of MC_4 receptors in live cells.
- Characterization of the influence of different metal ions on the ligand binding to MC_4 receptors and on the activation of the receptor-dependent cAMP accumulation.

3. MATERIALS AND METHODS

3.1. Cell lines and Reagents

Spodoptera Frugiperda (Sf9) cells (Invitrogen Life Technologies) were grown as a suspension culture in antibiotic- and serum-free growth medium EX-CELL® 420 (Sigma-Aldrich) at 27 ºC in a non-humidified incubator. Chinese hamster ovary (CHO-K1) cells (ATCC®, LGC Standards) were modified to stably express human wild type MC_4 receptors (Link *et al.* 2020). The pcDNA3.1+ expression vectors (Invitrogen) encoding MC_4 receptors (Missouri S&T cDNA Resource Center) were transfected into CHO-K1 cells using ExGen 500 (Fermentas) according to the manufacturer's description and selected using 500 μg/ml geneticin (PAA Laboratories). Both, naive and modified CHO-K1 cells were grown as an adherent monolayer culture in high glycose Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/ml penicillin, 0.1 mg/ml streptomycin (PAA Laboratories) and in the latter case also with 400 μg/ml geneticin at 27 ºC in a humidified incubator with 5% CO_2.

Na-HEPES (Amresco), NaCl (AppliChem), KCl (AppliChem), Pluronic F-127 (Sigma-Aldrich), and Complete EDTA-Free Protease Inhibitor Cocktail (Roche Applied Science) were used to prepare the incubation buffer (IB). $CaCl_2$ (AppliChem), $MgCl_2$ (AppliChem), $MnCl_2$ (AppliChem), $ZnSO_4$ (ReaChim), $CuCl_2$ (Sigma-Aldrich), $BaCl_2$ (ReaChim) or $Sr(NO_3)_2$ (Acros Organics) were used to prepare salt stock solutions. Metal ion effects were studied using ethylendiaminetetraacetic acid (EDTA, Merck). Unlabelled ligands of MC_4 receptors were purchased from Tocris Bioscience (NDP-α-MSH, SHU9119, THIQ, HS024) and Bachem (JKC-363). Custom synthesis of MC_4 receptor fluorescent ligands was ordered from AnaSpec (Cy3B-NDP-α-MSH, Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys(Cy3B)-Pro-Val-NH_2; TAMRA-NDP-α-MSH, TAMRA-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) and CEPEP EESTI (UTBC101, Cy3B-Nle-c[Asp-His-DNal(2')-Arg-Trp-Lys]-NH_2; UTBC102, Ac-Lys(Cy3B)-c[Asp-His-DNal(2')-Arg-Trp-Lys]-NH_2). Forskolin (Tocris Bioscience) was used for a direct activation of adenylyl cyclase. The ligands were stored at –20 °C or lower in dimethyl sulfoxide (DMSO, AppliChem). Sodium butyrate used for the induction of gene expression was from Sigma-Aldrich. Expression vectors were purchased from Invitrogen Life Technologies (pcDNA3.1(+), pFastBac™1) or kindly provided by Dr. Kees Jalink from The Netherlands Cancer Institute (pcDNA3.1(+)-mTurq2Δ_Epac(CD,ΔDEP, Q270E)_td^{cp173}Ven, referred to as Epac^{H187}). Restriction enzymes (FastDigest RruI, FastDigest EcoRI, EcoRI, Eco105I) were from Thermo Fisher Scientific.

3.2. Recombinant expression systems in GPCR assays

3.2.1. Budded baculovirus preparation

Recombinant baculoviruses of human MC_4 receptors were generated via the transfection of Sf9 cells, as described by Veiksina *et al.* (Veiksina *et al.* 2010). The initial virus was amplified to achieve a high titer (10^8 ivp/ml), which was determined by a cell size-based assay (Veiksina *et al.* 2015; Laasfeld *et al.* 2017). The high-titer virus was then used for the production of budded baculoviruses by infecting Sf9 cells at a density of 2×10^6 cells/ml at the multiplicity of infection (MOI) of 3-30. The infection was carried out for 48-120 h at 27 °C. The cells were separated from budded baculoviruses by centrifugation at 1,000 for 10 min. The supernatant containing the virus was then concentrated 10–50 times, either by tangential flow filtration (TFF) or by high-speed centrifugation (Veiksina *et al.* 2015). For the TFF concentration, the supernatant was diafiltrated at 4 °C using polyethersulfone membranes with a cutoff of 100 kDa or 300 kDa (LV Centramate™ Lab TFF system with Omega™ Membrane LV Centramate™ Cassettes, Pall®). The insect cell growth medium was exchanged for the incubation buffer (IB), which was then reduced to the desired volume. For the centrifugal concentration, the supernatant was centrifuged at 48,000g for 40 min at 4 °C and the obtained pellet was washed and suspended in the desired IB volume. The IB (pH 7.4) contained 11 mM Na-HEPES, 135 mM NaCl, 5 mM KCl, 0.1% Pluronic F-127 (excl. for TFF), and Complete EDTA-Free Protease Inhibitor Cocktail (according to the manufacturer's description). The IB was also supplemented with 2 mM $CaCl_2$ and 1 mM $MgCl_2$ unless the budded baculoviruses was further used in the studies of divalent metal ion effects (Link *et al.* 2020). The budded baculovirus (BBV) preparations were aliquoted and stored at –90 °C or lower.

3.2.2. BacMam-Epac^{H187} virus

BacMam viruses of FRET-based cAMP biosensors were generated via transfection of Sf9 cells as previously described by Mazina *et al.* (Mazina *et al.* 2015a; Mazina *et al.* 2015b). In brief, the cAMP biosensor construct Epac^{H187} (Klarenbeek *et al.* 2015) was cloned into the pFastBac™1 vector using FastDigest RruI and FastDigest EcoRI restriction enzymes for Epac^{H187} and EcoRI and Eco105I for pFastBac™1. The resulting pFastBac-Epac^{H187} construct under the control of the cytomegalovirus promoter was transformed into DH10Bac™ competent cells (Invitrogen Life Technologies) to generate recombinant bacmids. The purified and verified bacmids were transfected into Sf9 cells with a transfection reagent ExGen 500 to generate BacMam viruses based on the Bac-to-Bac® expression system manual (Invitrogen Life Technologies). The initial virus was amplified to achieve a high titer, which was determined by a cell size-based assay (Laasfeld *et al.* 2017). The amplified BacMam virus was aliquoted and stored at –90 °C.

3.3. Fluorescence anisotropy assay

Fluorescence anisotropy (FA) measurements were performed with Cy3B-NDP-α-MSH and TAMRA-NDP-α-MSH (Veiksina *et al.* 2010) and with novel fluorescent ligands UTBC101 and UTBC102 (**Fig. 1**). The concentration of novel fluorescent ligands was determined by the absorbance of Cy3B (ε_{558} = 130,000 M^{-1} cm^{-1}). Budded baculoviruses of MC_4 receptors were used as a homogenous receptor source, as described by Veiksina *et al.* (Veiksina *et al.* 2014; Veiksina *et al.* 2015). In brief, assays were carried out in the incubation buffer in a total volume of 100 µl at 27 °C using 96-well half area, flat-bottom polystyrene NBS microtiter plates (Corning). Fluorescence intensities were measured on a PHERAstar (BMG Labtech, Germany) or Synergy™ NEO (BioTek, USA) microplate reader using optical modules with excitation and emission filters of 540 (slit 20 nm) and 590 (slit 20 nm) or 530 (slit 25 nm) and 590 (slit 35 nm), respectively. Parallel-polarized light was used to excite the samples, and the dual detection mode was used to register the emission. This allows to simultaneously record intensities that are parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the plane of excitation light. Erythrosine B was used as a standard to correct the sensitivities of both emission channels (G-factor) (Thompson *et al.* 2002). The fluorescence intensities ($I_{\parallel}$, $I_{\perp}$) were also background corrected (Owicki 2000). All measurements were carried out in kinetic mode after the addition of budded baculoviruses to the fluorescent ligand with or without a competing ligand corresponding to the nonspecific and total binding, respectively.

In saturation binding assays, serial dilutions of budded baculoviruses were added to two fixed concentrations of the fluorescent ligands (Cy3B-NDP-α-MSH, UTBC101, or UTBC102). Nonspecific binding was determined in the presence and total binding in the absence of 3 µM SHU9119 or NDP-α-MSH. Reactions were carried out in the IB with 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (Veiksina *et al.* 2015; Link *et al.* 2017) and with 1 mM EDTA for the removal of heavy metal ions from the BBV preparation (Link *et al.* 2020). The association kinetics of Cy3B-NDP-α-MSH and UTBC101 was measured for 3 h and UTBC102 for 0.5 h. After that, the dissociation was initiated with 3 µM SHU9119 or NDP-α-MSH.

In competition binding assays, fixed concentrations of MC_4 receptors and fluorescent ligand (UTBC101 or UTBC102) were incubated with serial dilutions of various unlabelled ligands (NDP-α-MSH, THIQ, SHU9119 or JKC-363). Reactions were carried out in the IB with 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (Link *et al.* 2017). The budded baculoviruses were pre-incubated for 0.5 h with either the unlabelled (pre) or the fluorescent ligand (post). After that, the competition reaction was initiated with either the fluorescent (pre) or the unlabelled ligand (post), and it was measured for 370 min. The time points of the competition assays were calculated from the addition of the fluorescent ligand to the medium containing MC_4 receptors.

In divalent metal ion modulated binding assays, a fixed concentration of MC_4 receptors were added to a fixed concentration of the fluorescent ligand (UTBC101, UTBC102 or TAMRA-NDP-α-MSH) in the presence (nonspecific binding) or absence (total binding) of 3 μM SHU9119. Reactions were carried out in the IB with 1 mM $CaCl_2$ in the presence or absence of EDTA (0.01-1 mM). Divalent metal ion salt solutions ($MgCl_2$, $CaCl_2$, $Sr(NO_3)_2$, $BaCl_2$, $MnCl_2$, $ZnSO_4$ or $CuCl_2$) were added to the binding reaction from the start or after the equilibrium had been achieved (after 3 h for UTBC101, 0.5 h for UTBC102 or 1.5 for TAMRA-NDP-α-MSH).

3.4. BacMam-Epac^{H187}-based FRET assay

BacMam-Epac^{H187}-based FRET assay was used to measure cAMP accumulation in CHO-K1 and CHO-K1-MC_4R cells, as described previously by Mazina *et al*. (Mazina *et al.* 2015a; Mazina *et al.* 2015b). The high-titer BacMam virus was used to transduce mammalian cells at the density of 10^5 cells/well at MOI of 9-25. The cells were seeded on a black clear-bottom 96-well cell culture plate (Corning Life Sciences) in growth medium containing the BacMam virus and 12 mM sodium butyrate in a 100 μl volume. The transduced cells were incubated for 30 h at 30 °C for the expression of cAMP biosensor. Before the experiment (0.5 h), the cell growth medium was replaced with the IB (excl. protease inhibitors) with 1 mM $CaCl_2$. The assays were performed on a Synergy™ NEO microplate reader (BioTek), with excitation at 420/50 nm and simultaneous dual emission at 485/20 nm and 540/25 nm. The fluorescence intensities of the FRET-based biosensors in the cells were measured before and after the addition of a ligand and/or a modulator. The change in FRET values was calculated, as shown in Equation 2.

3.5. Data analysis

Aparecium 2.0.20 software (http://gpcr.ut.ee/aparecium.html), which is developed in our laboratory, was used for the management of experimental data. The data was analysed using MATLAB 7.1 (MathWorks, Inc., Natick, MA, USA) or GraphPad Prism 5.04 (GraphPad Software, USA) with built-in or user-defined optimization (Veiksina *et al.* 2014) binding models. The data are presented as the mean ± standard deviation of at least three independent experiments unless stated otherwise. Statistically significant differences were determined by Student's t-test or nonparametric Mann-Whitney U test, where the significance level was set to 0.05.

4. RESULTS AND DISCUSSION

4.1. Development of fluorescence anisotropy assay implementing budded baculoviruses

Ligand binding to GPCRs, including to MC_4 receptors, is a complex dynamic process, which requires a measurement system that would allow real-time monitoring of the reaction to get a better insight (Kopanchuk *et al.* 2005; Kopanchuk *et al.* 2006; Hoffmann *et al.* 2015). Fluorescence anisotropy (FA) method used together with bright and photostable fluorescent ligands can be a powerful tool to conduct kinetic measurements of ligand binding. In the current work, the fluorescent ligand Cy3B-NDP-α-MSH was used to develop a specific step-by-step protocol for FA-based method to study the kinetics of ligand binding processes of MC_4 receptors and GPCRs in general (Veiksina *et al.* 2015).

An important aspect of the FA-based assay development is the source of receptors. Due to the ratiometric nature of FA, similar concentrations of receptors and ligands are required, which can be achieved by using receptor sources that express high levels of GPCRs. Good results for FA assay have been achieved with the implementation of Sf9 membrane preparations, which exhibit high expression levels of recombinant receptors (Veiksina *et al.* 2010). However, the preparation of budded baculoviruses (BBV) that display receptors of interest on their membranes is a uniform system that further improves the quality of FA assay results (Veiksina *et al.* 2014). As the implementation of BBVs in FA-based GPCR studies is a novel approach (**Fig. 1**), conditions for its production needed to be optimized.

The infection conditions of Sf9 cells with recombinant baculoviruses and the collection methods of budded baculovirus are important factors to achieve high expression levels of GPCRs. In case of MC_4 receptors, the multiplicity of infection (MOI) of Sf9 cells was varied from 3 to 30 using high-titer baculoviruses and the virus collection time was varied from 48 h to 120 h after the infection. After the infection period, Sf9 cells were separated from budded baculoviruses by low-speed centrifugation (1,000g). Subsequently, the obtained supernatant fraction, containing budded baculoviruses, was concentrated by using two methods – high-speed centrifugation and tangential flow filtration (TFF). In addition, sucrose gradient centrifugation (>80,000g) and ultrafiltration using protein concentrating cartridges were also studied for the preparation of virus-like particles. However, these methods were disregarded, as sucrose gradient centrifugation did not improve results, and ultrafiltration revealed considerably lower yields (Oliver Pulges unpublished data).

In the case of high-speed centrifugation, the supernatant containing BBVs was centrifuged at 48,000g for 40 min to collect the viruses as a pellet. The pellet was suspended in the IB to obtain the BBV preparation with the desired concentration. In the case of TFF concentration, the supernatant containing

BBVs was pumped in a tangential direction to the membrane, which helped to avoid build up and clogging of the membrane pores. The insect cell growth medium was passed through the membrane by applying pressure, whereas the budded baculovirus particles were largely retained in the system for reprocessing. Two different polyethersulfone membranes with a cutoff of 100 kDa and 300 kDa were tested, from which the latter proved to be more suitable for the BBVs. During the reprocessing with the TFF system, the BBV retentate was washed with the IB (without Pluronic F-127 to prevent foaming), after which the amount of the IB was reduced to the desired volume and was collected as the BBV preparation. A suitable amount of BBVs containing MC_4 receptors for FA assay was achieved by concentrating the supernatant in the range of 10–50 times, considering the initial volume of the Sf9 cell suspension.

The quality of BBV preparations containing MC_4 receptors was evaluated in FA-based ligand binding assay (**Fig. 1**). Optimal expression of the receptors was achieved at MOI of 3 and with the infection period of 96 h (Veiksina *et al.* 2015). The estimated concentration of MC_4 receptor binding sites was about 5 times lower in the BBV preparation obtained by TFF compared to centrifugation. The lower yield of TFF concentration compared to centrifugation may have been caused due to the loss of some BBVs in contact with the membrane. As the whole medium was not replaced, some proteases may have remained in the preparation. In principle, it was shown that TFF is applicable for the production of BBVs, but it is less effective than centrifugation in small-scale production, where the modest loss of receptors is more critical. In large-scale production, the use of TFF would be more reasonable as it is potentially less labour-intensive and less harmful to BBVs than centrifugation. Since studies within the current thesis did not require large-scale receptor production, it was decided hereafter to use the centrifugation method for the preparation of BBVs.

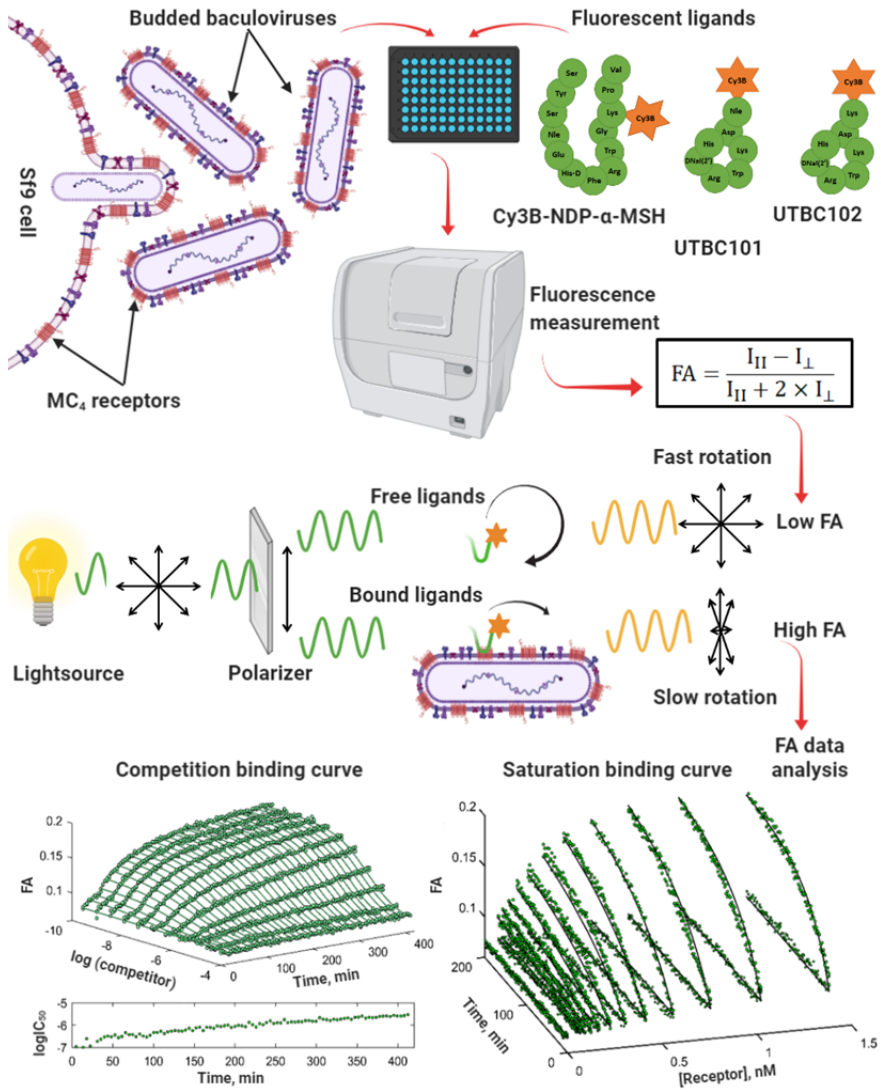


Figure 1. Fluorescence anisotropy (FA) based ligand binding assay with the implementation of budded baculoviruses as a receptor source. In the FA-based assay system, bright and photostable fluorescent ligands were used to characterize the ligand binding properties to GPCRs. Three custom synthesized peptides coupled with Cy3B-tag were used for MC_4 receptor studies – Cy3B-NDP-α-MSH, UTBC101, and UTBC102. Cy3B-NDP-α-MSH is the derivative of a high affinity agonist NDP-α-MSH; UTBC101 and UTBC102 are the derivatives of high affinity antagonist SHU9119 of MC_4 receptors. Budded baculoviruses (BBVs) are used as a melanocortin-4 (MC_4) receptor source that display the receptors on their surfaces. BBVs were obtained by the infection of Sf9 (*Spodoptera frugiperda*) cells with recombinant baculoviruses that encode the expression of MC_4 receptors. During the infection cycle, baculoviruses bud from the insect cell surface. After budding from the cell, viruses are surrounded by the host cell membranes that carry the recombinant receptors expressed by the host cell.

FA-based ligand binding assays were carried out on microplate readers for the fluorescence measurements. Fluorescent ligand binding to the receptors can be characterized by the change in FA values, which were calculated from the experimentally measured fluorescence intensities that are parallel (I_{II}) and perpendicular ($I_{\perp}$) to the plane of excitation light. FA values are low when fluorescent ligands are free in the solution and high when fluorescent ligands are bound to the receptor. Global data analysis (Veiksina *et al.* 2014) was used for the fitting of FA data to characterize the binding properties of labelled and unlabelled ligands.

4.2. Novel fluorescent ligands for MC_4 receptors

The fluorescent ligand Cy3B-NDP-α-MSH (**Fig. 1**), which was used to develop the FA-based assay system, enables to obtain high quality ligand binding data for MC_4 receptors. However, the slow dissociation kinetics of this reporter ligand limits reaching equilibrium in ligand binding studies. As there were no commercially available fluorescent ligands for MC_4 receptors, it was decided to synthesize two novel fluorescent derivatives of a high affinity antagonist SHU9119. Cy3B was coupled directly to SHU9119 at the first amino acid residue Nle resulting in a positively charged fluorescent ligand UTBC101 (**Fig. 1**). Whereas, UTBC102 was obtained by exchanging the amino acid that was coupled to Cy3B from Nle to Lys, which further increased the positive charge of the ligand (**Fig. 1**). The red-emitting Cy3B was chosen as it has proven to be a stable and high quantum yield fluorophore (Cooper *et al.* 2004; Turek-Etienne *et al.* 2004) suitable for FA measurements (Veiksina *et al.* 2010). The affinities of two novel fluorescent ligands were estimated from kinetic binding data as well as from a time snapshot of binding data corresponding to near equilibrium conditions.

The addition of BBV preparation containing MC_4 receptors to the fluorescent ligands caused a time-dependent increase in the FA values (**Fig. 2 A** and **B**, filled shapes). Whereas in the presence of excess NDP-α-MSH or SHU9119 the FA change was negligible (**Fig. 2 A** and **B**, open shapes). The difference between these FA values could be attributed to the specific ligand binding to MC_4 receptors. After the binding of UTBC101 (3 h) and UTBC102 (0.5 h) reached a plateau, the dissociation reaction was initiated by the addition of 3 μM NDP-α-MSH or SHU9119 (**Fig. 2 A** and **B**, filled shapes indicated by an arrow). This revealed that UTBC101 had a significantly slower dissociation compared to UTBC102 (half-lives of 160 ± 18 min and 7 ± 2 min, respectively). Whereas both ligands demonstrated faster dissociation kinetics compared to Cy3B-NDP-α-MSH having approximately 1.4 and 30 times shorter half-lives, respectively (Veiksina *et al.* 2014; Link *et al.* 2017). The apparent dissociation rate constants (k_{off}) were estimated by one-phase exponential decay for UTBC101 ($(1.1 \pm 0.3) \times 10^{-1}$ min^{-1}) and UTBC102 ($(4.4 \pm 0.5) \times 10^{-3}$ min^{-1}). These values were also used for the global fitting of the observed binding data, as described by Veiksina *et al*. (Veiksina *et al.* 2014). It was found that the apparent association rate constants (k_{on}) were quite similar, having values of

$(1.8 \pm 0.3) \times 10^7\ M^{-1}\ min^{-1}$ for UTBC101 and $(3.7 \pm 0.8) \times 10^7\ M^{-1}\ min^{-1}$ for UTBC102. The apparent dissociation constants (K_d) calculated from ratio of the kinetic rate constants (k_{off}/k_{on}) were 0.24 ± 0.04 nM for UTBC101 and 3.0 ± 0.6 nM for UTBC102. The concentration of MC_4 receptor binding sites in the BBV preparation was estimated to be twice as high for UTBC101 (48 ± 4 nM) compared to UTBC102 (21 ± 4 nM).

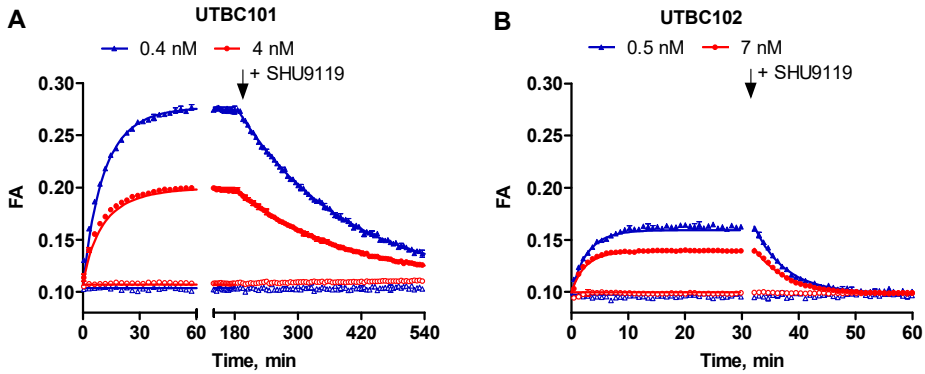


Figure 2. Kinetics of the fluorescent ligand binding to MC_4 receptors in budded baculoviruses. The binding reaction was initiated by addition of MC_4 receptors to UTBC101 (0.4 nM and 4 nM) (**A**) or UTBC102 (0.5 nM and 7 nM) (**B**) in the absence (filled shapes) or presence (open shapes) of 3 µM SHU9119. The concentration of MC_4 receptor binding sites for the fluorescent ligands was estimated to be 2.5 nM. After 3 h (**A**) or 0.5 h (**B**), the dissociation was initiated by the addition of 3 µM SHU9119 (shown with an arrow). The fluorescence intensities were measured, and corresponding changes in FA values were calculated. Data are from a representative experiment of at least three independent experiments performed in duplicates.

The addition of an increasing amount of BBVs containing MC_4 receptors to the fluorescent ligands caused an increase in the FA values corresponding to the total binding (**Fig. 3 A** and **B**, filled shapes). There was also an increase in the non-specific binding (**Fig. 3 A** and **B**, open shapes), but it was significantly lower than the total binding. These binding curves of the fluorescent ligands to MC_4 receptors correspond to the binding data obtained at near equilibrium conditions (**Fig. 3**). Near equilibrium conditions were achieved for UTBC101 after 3 h binding and for UTBC102 after 0.5 h binding to MC_4 receptors (Link *et al.* 2017). The binding parameters were obtained by simultaneous fitting of the total and non-specific signal, which takes into account the depletion of the fluorescent ligand during the reaction (Veiksina et al. 2014). The obtained K_d values were 0.21 ± 0.03 nM for UTBC101 and 3.7 ± 1.1 nM for UTBC102, which are in agreement with the K_d values that were calculated from the ratio of the apparent kinetic rate constants. The estimated concentration of MC_4 receptor binding sites in the BBV preparation were 40 ± 4 nM for UTBC101

and 18 ± 4 nM for UTBC102, which are in good agreement with the values obtained from the kinetic binding curves.

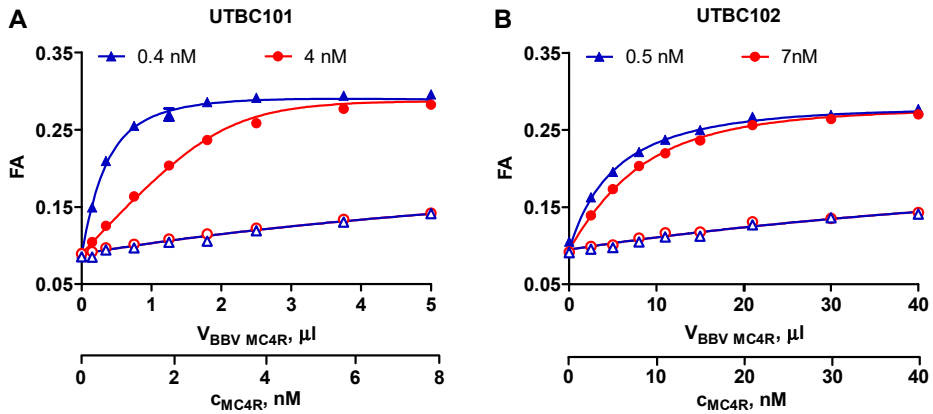
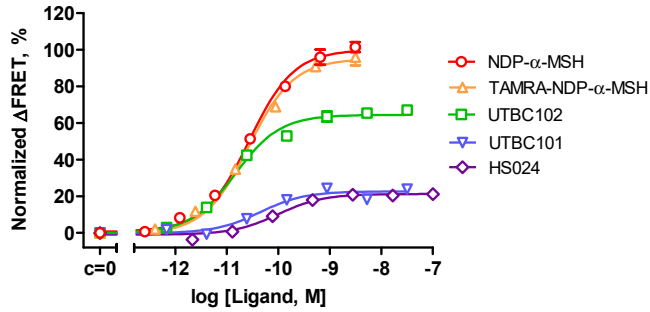


Figure 3. Time snapshots of the binding curves of the fluorescent ligands to MC_4 receptors in budded baculoviruses. Fixed concentrations of UTBC101 (0.4 nM and 4 nM) (**A**) or UTBC102 (0.5 nM and 7 nM) (**B**) were incubated with different amounts of MC_4 receptors in the absence (filled shapes) or presence (open shapes) of 3 µM SHU9119. The fluorescence intensities were measured after a 3 h (**A**) and 0.5 h (**B**) incubation period. Corresponding changes in FA values were calculated, and the global fitting of this data was used to characterize the binding properties and MC_4 receptor binding site concentrations (depicted on the lower x-axis) for these ligands. Data are from a representative experiment of at least three independent experiments performed in duplicates.

Although SHU9119 is mainly known as an MC_4 receptor antagonist (Hruby *et al.* 1995) the biological activity of its novel derivatives needed to be determined. The biological activity of the novel fluorescent ligands was studied in CHO-K1 cells stably expressing MC_4 receptors. To estimate the activation of MC_4 receptors coupled to G_s proteins, the accumulation of cAMP was detected by a FRET-based biosensor, which is expressed in the cells using the BacMam system (Mazina *et al.* 2015a; Mazina *et al.* 2015b). MC_4 receptor full agonist NDP-α-MSH caused a concentration-dependent increase in the ΔFRET values corresponding to cAMP accumulation (**Fig. 4**). The maximal level of cAMP accumulation was achieved within 30 minutes. Similar concentration dependent increase in cAMP accumulation was also observed for fluorescently labelled NDP-α-MSH (**Fig. 4**). Neither of the novel fluorescent ligands exhibited full agonistic properties – UTBC102 behaved as a classical partial agonist (68 ± 5%), and UTBC101 showed low partial agonistic activity (22 ± 2%) compared to NDP-α-MSH (**Fig. 4**). In our system, low partial agonistic activity was also found for HS024 (**Fig. 4**; 19 ± 3%), the previously known antagonist for MC_4 receptors (Kask *et al.* 1998).



Ligand	log [EC_{50}] ± SD		Efficacy ± SD, %	
NDP-α-MSH	-10.7	0.2	100	-
TAMRA-NDP-α-MSH	-10.9	0.3	96	2
UTBC102	-10.8	0.2	68	5
UTBC101	-10.7	0.3	22	2
HS024	-10.3	0.5	19	3

Figure 4. Ligand-dependent activation of cAMP production in CHO-K1 cells with MC_4 receptors. The ΔFRET values were measured 30 min after the addition of the indicated concentrations of ligands to CHO-K1-MC_4R cells expressing the cAMP biosensor BacMam-Epac^{H187}. After that, fluorescence intensities were measured, and corresponding changes in ΔFRET values were calculated and normalized to the maximal NDP-α-MSH signal. Data are from a representative experiment of at least three independent experiments performed in triplicates. A three-parameter logistic function was used to fit the data to characterize the ligand-specific effects shown in the table as the mean ± SD.

4.3. Fluorescent ligands as tracers to characterize unlabelled ligands

Fluorescent ligands can be used as tracers to determine the affinity and kinetic properties of unlabelled ligands binding to GPCRs using competition binding assays. Generally, it is assumed that competition binding assays are measured when equilibrium has been reached, but this is not always the case as equilibrium depends on the kinetic properties of fluorescent tracers as well as on the unlabelled competitors (Kopanchuk *et al.* 2006; Veiksina *et al.* 2010; Guo *et al.* 2013; Rinken *et al.* 2018). This has to be taken into account in the planning of the experiment and in the interpretation of results to avoid over- or underestimation of the unlabelled ligands' affinity.

It was found that the apparent pIC_{50} values also depend on the experimental design of the competition experiment (**Fig. 5**). The pIC_{50} values of unlabelled ligands could be underestimated when MC_4 receptors are pre-incubated with the fluorescent tracer (**Fig. 5**, red line) or overestimated when they are pre-incubated with the unlabelled ligand (**Fig. 5**, blue line) prior to the initiation of the competition reaction. In these assays, equilibrium was reached in less than an hour using UTBC102 (**Fig. 5**, solid line) or in more than 3 hours using

UTBC101 (**Fig. 5**, dashed line). Whereas in previous studies using the fluorescent tracer Cy3B-NDP-α-MSH, equilibrium was practically not reached in the competition binding assays (Veiksina *et al.* 2014). The time of equilibrium in these assays also depended on the unlabelled ligand. The rank order of which of the studied unlabelled ligand binds to MC_4 receptors the fastest and which one the slowest was estimated based on the time of equilibrium (THIQ > JKC-363 > SHU9119 > NDP-α-MSH) (Link *et al.* 2017). The kinetic rate constants of unlabelled ligands can be estimated by using multivariable global analysis of the competition binding data as described by Rinken *et al.* (Rinken *et al.* 2018), but this requires precise optimization of experimental conditions and collecting a substantial amount of high-quality experimental data. The apparent potencies of unlabelled ligands, which were determined with both novel tracers, were in good agreement with each other, except for some discrepancies between the apparent pIC_{50} values of SHU9119 (Link *et al.* 2017). It has been shown that unlabelled ligand's affinity can be underestimated in FA-based competition binding assays, if the unlabelled ligand has a substantially higher affinity than the fluorescent tracer does (Cha 1975; Huang 2003; Sinijarv *et al.* 2017; Rinken *et al.* 2018). This is likely the case for SHU9119 and UTBC102 (**Fig. 5**, solid line).

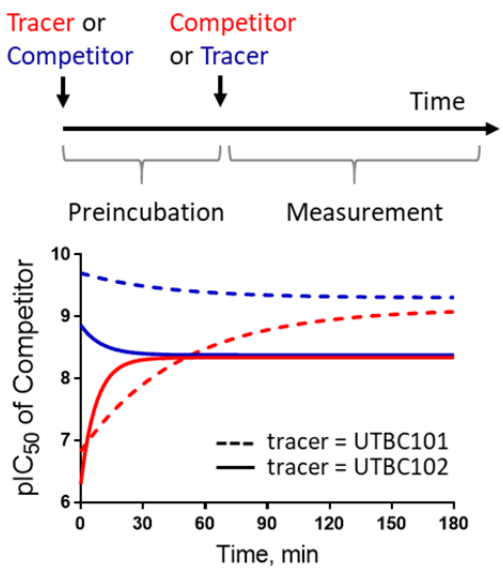


Figure 5. Time dependence of the apparent pIC_{50} values of unlabelled ligands in competition with fluorescent ligands binding to MC_4 receptors. MC_4 receptors in budded baculoviruses were preincubated for 0.5 h either with an unlabelled SHU9119 (blue) or with a fluorescent ligand (red) prior to the initiation of the competition reaction. The apparent pIC_{50} values of unlabelled SHU9119 at different time points were calculated from representative competition curves of UTBC101 (dashed line) and UTBC102 (solid line) of at least two independent experiments performed in duplicates.

4.4. Modulation of the ligand binding to MC_4 receptors by metal ions

Ca^{2+} is an essential cofactor for the high affinity ligand binding to MC_4 receptors, which has previously been shown with ligand binding experiments (Kopanchuk *et al.* 2005; Veiksina *et al.* 2010) and recently confirmed by solving the crystal structure of the receptor (Yu *et al.* 2020). At least 1 mM Ca^{2+} is required to achieve optimal ligand binding to MC_4 receptors (Kopanchuk *et al.* 2005; Link *et al.* 2020). Furthermore, we have found that the ligand binding level to MC_4 receptors in the presence of 1 mM Ca^{2+} could be further increased by the addition of a low amount of EDTA. In the case of UTBC101, the specific ligand binding level was increased more than twice (230 ± 24%), whereas, in the case of UTBC102, the increase was moderate (126 ± 15%). The specific ligand binding level to MC_4 receptors was considerably increased in these conditions without altering the affinity of the studied fluorescent ligands. Without calcium, EDTA did not induce ligand binding to MC_4 receptors.

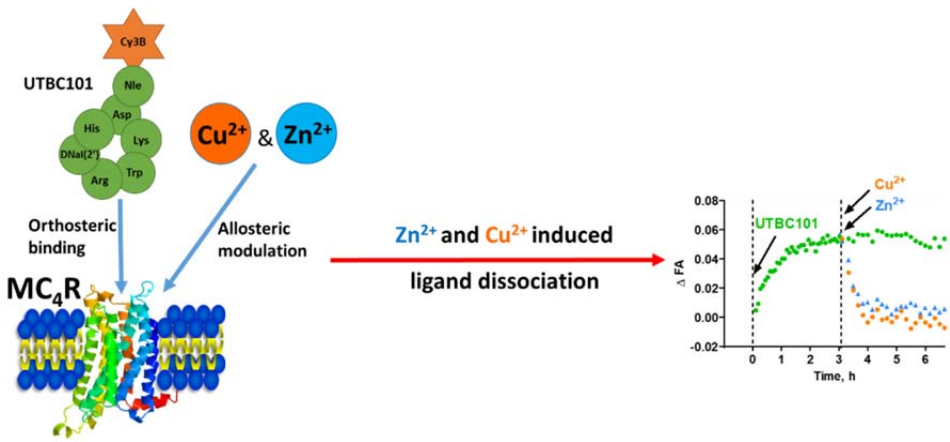


Figure 6. Influence of Zn^{2+} and Cu^{2+} on the binding of UTBC101 to MC_4 receptors in budded baculoviruses. The binding reaction was initiated by the addition of 0.8 nM MC_4 receptors to 0.4 nM UTBC101. After 3 h, $ZnSO_4$ (32 µM, blue triangles) or $CuCl_2$ (3 µM, orange circles) or the same amount of buffer (green circles) were added. The specific binding of ligands was characterized as the change in ΔFA values in time. Data are from a representative experiment of three independent experiments performed in duplicates.

The observed EDTA effect indicated that some heavy metals could be involved in the inhibition of ligand binding to MC_4 receptors. As a result of the determination of the metallic composition of the samples by ICP-MS, it was found that the receptor preparation contained in addition to buffer components (Na^+, K^+) also other metal ions in the micromolar range (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+})

(Link *et al.* 2020). By studying the effect of these ions for UTBC101 binding in the presence 1 mM Ca^{2+}, it was discovered that Mn^{2+} and Mg^{2+} had no significant effect on the level of specific ligand binding to MC_4 receptors. Whereas, already 10 μM Zn^{2+} and 0.3 μM Cu^{2+} caused a significant inhibition of the level of specific ligand binding to MC_4 receptors (Link *et al.* 2020). Zn^{2+} and Cu^{2+} caused a concentration-dependent inhibitory effect on the ligand binding to MC_4 receptors, but also caused the dissociation of already formed complexes (**Fig. 6**). The effective concentration of these ions, causing inhibition of ligand binding to MC_4 receptors was found to be in the physiologically relevant low micromolar range (Link *et al.* 2020). The effect of these metal ions was partially reversible upon the addition of excess EDTA.

The rate and level of fluorescent ligand dissociation from MC_4 receptors depended on the concentration of zinc and copper ions. The dissociation rate of UTBC101 was increased 20-fold by Zn^{2+} as the ion concentration was increased from 1 μM (k_{off} = 0.006 ± 0.002 min^{-1}; $\tau_{1/2}$ = 116 min) to 32 μM (k_{off} = 0.12 ± 0.06 min^{-1}; $\tau_{1/2}$ = 6 min). Cu^{2+} caused a similar trend in the dissociation of UTBC101 over the concentration range of 0.1 μM to 10 μM. Although, Cu^{2+} induced a more complex dissociation than Zn^{2+}, which induced one-phase exponential decay. Zinc and copper ions also induced the dissociation of UTBC102 and TAMRA-NDP-α-MSH from the complex with MC_4 receptors at a similar concentration range (Link *et al.* 2020). The ligands' dissociation rate dependence on ion concentration indicates that zinc and copper ions do not compete with the ligands for the orthosteric-binding site, but act as negative allosteric modulators.

4.5. Modulation of the signal transduction of MC_4 receptors by metal ions

The effect of Zn^{2+} and Cu^{2+} on the biological activity of MC_4 receptors was estimated by the change in cAMP accumulation in CHO-K1-MC_4R cells using a FRET-based biosensor. Zn^{2+} caused a concentration-dependent increase in the accumulation of cAMP achieving almost the level of full agonists (93 ± 6%), whereas half of the maximal response was reached already at a 1.1 μM concentration (pEC_{50} = 5.95 ± 0.12) (**Fig. 7**, triangles). While Cu^{2+} induced a concentration-dependent biphasic change in cAMP accumulation, causing a marginal increase in cAMP levels at concentrations up to 1.5 μM (33 ± 9%) and a decrease in cAMP levels even below baseline at higher concentrations (**Fig. 7**, circles). A slight increase of cAMP accumulation was also seen in the naive CHO-K1 cells without MC_4 receptors, but there was no decrease phase observed. This indicated that only the inverse agonistic effect of Cu^{2+} could be mediated through MC_4 receptors. As Zn^{2+} and Cu^{2+} can modulate the signal transduction of MC_4 receptors at low micromolar levels, these ions may play a physiologically important role for these central nervous system receptors as endogenous agonists and inverse agonists, respectively. Upon neuronal stimula-

tion, the free zinc concentration has been found to reach tens of micromolar in brain slices (and even higher at synapses), while free copper concentration is more tightly controlled (Thompson *et al.* 2000; Li *et al.* 2001b; Li *et al.* 2001a; Ueno *et al.* 2002; D'Ambrosi and Rossi 2015).

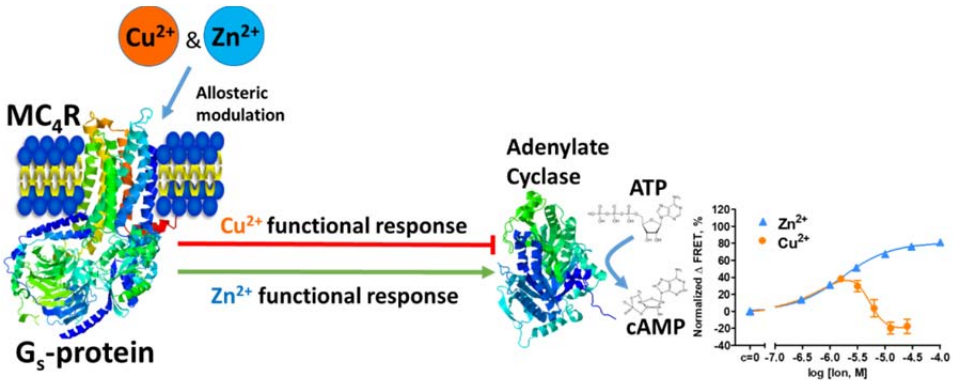


Figure 7. Influence of Zn^{2+} and Cu^{2+} on the cAMP production in CHO-K1 cells expressing MC_4 receptors. CHO-K1-MC_4R cells expressing cAMP biosensor BacMam-Epac^{H187} were treated with increasing concentrations of $ZnSO_4$ (blue triangles) and $CuCl_2$ (orange circles) for 0.5 h in the presence of 1 mM $CaCl_2$. After that, fluorescence intensities were measured, and corresponding changes in ΔFRET values were calculated and normalized to the maximal NDP-α-MSH signal. Data are from a representative experiment of three independent experiments performed in triplicates.

In vitro studies have shown that MC_4 receptors exhibit a relatively high level of constitutive activity without agonistic ligands (Haskell-Luevano and Monck 2001; Nijenhuis *et al.* 2001; Coll 2013). The reason for this constitutive activity may be Zn^{2+} ions, as zinc is often an ingredient of cell growth media (Bozym *et al.* 2008). Whereas, the presence of Cu^{2+} could influence the constitutive activity of MC_4 receptors in an opposite manner when cell growth media are supplemented with serum or copper salts are added (Chaderjian *et al.* 2005; Qian *et al.* 2011). The level of constitutive activity of receptors is critical in functional studies, as it determines the behaviour of ligands as activators or inhibitors (Kenakin 2005; Bond and IJzerman 2006). It has been shown that an endogenous antagonist AgRP of MC_4 receptors can behave as an inverse agonist for the cAMP pathway in case of elevated basal activity (Haskell-Luevano and Monck 2001; Nijenhuis *et al.* 2001). Differences in its functionality have also been observed for the tetrapeptide His-DNal(2′)-Arg-Trp, which has behaved as an antagonist, but also as a partial agonist (Holder *et al.* 2002; Chen *et al.* 2006; Lensing *et al.* 2019). This was also observed in the current work, where previously described antagonist HS024 (Kask *et al.* 1998) behaved as a partial agonist (**Fig. 4**, diamonds).

The effect of Zn^{2+} and Cu^{2+} was studied on the signalling of the MC_4 receptor agonist NDP-α-MSH. Both ions influenced the level of basal and maximal cAMP accumulation but did not change the potency of NDP-α-MSH. This indicates that these ions function as allosteric modulators of MC_4 receptors and not as orthosteric competitors. To estimate the allosteric nature of Zn^{2+}, a Schild analysis was performed by studying the inhibition of the agonistic signal of Zn^{2+} by an MC_4 receptor ligand HS024. HS024 inhibited the agonist signal of Zn^{2+} in a concentration-dependent manner, but the slope of the Schild plot was significantly below unity (Link *et al.* 2020), which indicates again that zinc acts as an allosteric modulator of MC_4 receptors. As a control, inhibition of the agonistic signal of NDP-α-MSH by HS024 was also studied, resulting in a Schild plot with a slope close to unity, as it should be for an orthosteric ligand (Link *et al.* 2020). As Cu^{2+} exhibits a biphasic signal, it was not possible to perform a classical Schild assay. However, it was shown that only the inverse agonistic signal of Cu^{2+} was blocked by HS024 in a concentration-dependent manner (Link *et al.* 2020), which confirms once again that these ions modulate the signal of MC_4 receptors by suppressing its activity.

5. CONCLUSIONS

The general aim of the study was to gain additional information about the ligand binding, allosteric modulation, and constitutive activity of melanocortin-4 (MC_4) receptors. The results that were acquired during this study enabled to make the following conclusions:

- The developed budded baculovirus preparation of MC_4 receptors and various fluorescent ligands enable high quality characterization of ligand binding to MC_4 receptors using a homogeneous FA assay. The developed step-by-step FA method could be applicable to other GPCRs.
- The slow dissociation of Cy3B-NDP-α-MSH ($\tau_{1/2}$ = 224 min) imposes limitations for reaching reaction equilibrium. Therefore, novel fluorescent peptides (UTBC101 and UTBC102) were synthesized. These Cy3B-labelled derivatives of SHU9119 exhibited high binding affinities towards MC_4 receptors, but rather different binding kinetics. The dissociation half-lives of UTBC101 and UTBC102 were 1.4 and 30 times shorter compared to Cy3B-NDP-α-MSH, respectively.
- It was shown that the equilibrium of competition reactions depends on the kinetic properties of labelled and unlabelled ligands and on the design of competition assay. Both UTBC101 and UTBC102 are suitable for the characterization of unlabelled ligand binding to MC_4 receptors using FA assay. They complement each other in these studies, as UTBC102 enables to reach reaction equilibrium faster, and UTBC101 enables to cover a wider range of competitor potencies.
- The CHO-K1 cell line stably expressing MC_4 receptors was developed to assess the level of receptor activation via the detection of cAMP accumulation using a FRET-based biosensor. It was shown that UTBC102 is a partial agonist in MC_4 receptor-dependent cAMP accumulation, while UTBC101 exhibits only low partial agonistic properties. This offers new possibilities for the signal transduction studies of MC_4 receptors, as there are no other previously reported fluorescent ligands with such functional properties.
- It was shown that Ca^{2+} ions are essential for the high affinity ligand binding to MC_4 receptors at millimolar concentrations, while Mg^{2+} and Mn^{2+} had an only negligible effect. At the same time, Zn^{2+} and Cu^{2+} ions inhibited the ligand binding already at physiologically relevant low micromolar concentrations in the presence of Ca^{2+}.
- Zn^{2+} and Cu^{2+} ions inhibited the formation of MC_4 receptor-ligand complexes as well as induced dissociation of already formed complexes. As the dissociation rate of ligands depended on the concentration of Zn^{2+} and Cu^{2+}, it was proposed that these ions act as negative allosteric modulators of ligand binding to MC_4 receptors.

- Zn^{2+} and Cu^{2+} have different effects on the constitutive activity of MC_4 receptors in the cAMP pathway. Low micromolar concentrations of Zn^{2+} cause activation of MC_4 receptors up to full agonistic levels, whereas Cu^{2+} ions suppress the activity even below baseline levels. This indicates that these ions could play a physiologically relevant role as endogenous agonists or inverse agonists of MC_4 receptors, respectively. In addition, in cellular studies, it should be taken into account that Zn^{2+} and Cu^{2+} ions are common ingredients of the cell growth media.
- The FA-based ligand binding and FRET-based functional assays that were further developed in this study have already been successfully applied for the characterization of several other GPCRs. As both zinc and copper are endogenous ions in the body, their involvement in the regulation of signal transduction with other GPCRs requires further studies.

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SUMMARY IN ESTONIAN

Melanokortiin-4 retseptorite ligandiga seostumine, allosteeriline modulatsioon ja konstitutiivne aktiivsus

Melanokortiin-4 (MC_4) retseptorid on peamiselt kesknärvisüsteemis leiduvad G-valguga seotud retseptorid, mis reguleerivad kehas mitmeid olulisi funktsioone, nagu energia homeostaas, toitumine ja seksuaalsed funktsioonid. Seetõttu pakuvad need retseptorid ravimiarendusfirmadele suurt huvi. Nende plasmamembraanis asuvate retseptorite poolt vahendatav signaaliülekanne rakku on keeruline dünaamiline protsess, mille kõiki iseärasusi senini veel täpselt ei mõisteta.

Käesoleva doktoritöö üldiseks eesmärgiks oli saada lisateavet MC_4 retseptorite ligandi seostumise, allosteerilise modulatsiooni ja konstitutiivse aktiivsuse kohta. Töö üldise eesmärgi saavutamiseks püstitati mitu konkreetset ülesannet:

- Fluorestsentsanisotroopia (FA) baasil põhineva katsesüsteemi arendamine, kasutades pungunud bakuloviiruste preparaati uuritavate retseptorite allikana, et iseloomustada ligandi seostumiskineetikat MC_4 retseptoritele.
- Uudsete fluorestsentsligandide arendamine ja rakendamine ligandi seostumise iseloomustamiseks MC_4 retseptoritele.
- MC_4 retseptorite signaaliülekande uurimine FRET-il põhineva biosensori katsesüsteemi abil, mis tuvastab sekundaarse virgatsaine cAMP kontsentratsiooni rakus. Antud retseptorite konstitutiivse aktiivsuse ja selle inhibeerimise määramine.
- Erinevate metalliioonide mõju iseloomustamine MC_4 retseptorite ligandi seostumisele ja cAMP tootmise aktiveerimisele.

Kokkuvõtvalt võimaldasid antud töö tulemused jõuda järgmiste järeldusteni:

- MC_4 retseporeid ekspresseerivad pungunud bakuloviirused võimaldavad kõrgkvaliteedilist ligandi seostumise määramist nendele retseporitele FA katsesüsteemis.
- Cy3B-NDP-α-MSH aeglane dissotsiatsioon ($\tau_{1/2}$ = 224 min) seab piirangud MC_4 retseptoritega seostumisreaktsiooni tasakaalu saavutamisele. Seetõttu sünteesiti uued Cy3B-märgistatud fluorestsentspeptiidid (UTBC101 ja UTBC102), mis on SHU9119 derivaadid. Mõlematel on kõrge seostumisafiinsus MC_4 retseptoritele, kuid üsna erinev seostumiskineetika. UTBC101-l on 1,4 korda ja UTBC102-l on 30 korda lühem dissotsiatsiooni pooleluiga võrreldes Cy3B-NDP-α-MSH-ga.
- Konkureerimisreaktsioonide tasakaal sõltub märgistatud ja märgistamata ligandide kineetilistest omadustest ja katse ülesehitusest. Mõlemad, nii UTBC101 kui ka UTBC102, sobivad märgistamata ligandide seostumise iseloomistamiseks MC_4 retseptoritele kasutades FA katset. Seejuures nad täiendavad üksteist, kuna UTBC102-ga saavutatakse

tasakaal kiiremini, aga UTBC101 võimaldab iseloomustada kõrgema afiinsusega märgistamata ligande.

- MC_4 retseptoreid stabiilselt ekspresseeriv CHO-K1 rakuliin arendati välja selleks, et hinnata retseptorite aktivatsioonitaset cAMP tootmise kaudu, kasutades FRET-il põhinevat biosensorit. Selle katsesüsteemi abil näidati, et UTBC102 käitub kui osaline agonist, samas kui UTBC101 omab ainult väga madalat osalist agonistlikku aktiivsust. See pakub uusi võimalusi MC_4 retseptorite signaaliülekande uurimiseks, kuna teisi selliste funktsionaalsete omadustega fluorestsentsligande teadaolevalt pole.
- Millimolaarses kontsentratsioonis Ca^{2+} ioonid on hädavajalikud suure afiinsusega ligandi seostumiseks MC_4 retseptoritega, samas kui Mg^{2+} ja Mn^{2+} olulist mõju ei oma. Seevastu Zn^{2+} ja Cu^{2+} ioonid pärssisid ligandi seostumist juba füsioloogiliselt olulistel mikromolaarsetel kontsentratsioonidel Ca^{2+} ioonide juureolekul.
- Zn^{2+} ja Cu^{2+} ioonid pidurdasid MC_4 retseptor-ligand komplekside moodustumist, samuti algatasid juba moodustunud komplekside dissotsiatsiooni. Nende ioonide kontsentratsioonist sõltuv retseptor-ligand komplekside dissotsiatsioonikiirus viitab sellele, et Zn^{2+} ja Cu^{2+} ioonid on MC_4 retseptorite negatiivsed allosteerilised modulaatorid.
- Zn^{2+} ja Cu^{2+} avaldavad erinevat mõju MC_4 retseptorite konstitutiivsele aktiivsusele cAMP signaalrajal. Madal mikromolaarne Zn^{2+} aktiveerib MC_4 retseptoreid pea täisagonistilikul tasemele, samas kui Cu^{2+} vähendab retseptorite aktviisust isegi alla baastaset. Seega võivad ioonid toimida MC_4 retseptorite endogeensete agonistide või pöördagonistidena. Seda tuleks arvestada ka rutiinsete rakukatsete läbiviimisel, kuna need ioonid on sageli rakusöötmete komponendite hulgas.

Antud töö tulemused on rakendatavad ka teiste retseptorsüsteemide uurimiseks, sest järjest enam kasutatakse FA-l ja FRET-biosensoril põhinevaid katsesüsteeme. Lisaks on selles töös näidatud endogeensete metalliioonide mõju uurimine oluline ka teiste GPCR-ide signaaliülekannete puhul, kuna need ioonid esinevad nii kudedes kui ka rakukultuuri kasvulahustes.

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