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REGULATION OF LIGAND BINDING TO MELANOCORTIN RECEPTOR SUBTYPES

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Author's contribution

Paper **I**: Responsible for melanocortin receptor binding assay design; performance of these experiments and analysis of binding data together with S. Veiksina, participation in writing of biochemical part of the manuscript.

Paper **II**: Responsible for performing of part of radioligand binding experiments, data analysis and writing of corresponding part of the manuscript.

Paper **III:** Main person responsible for the paper, including planning and data analysis, performing experimental part (together with S. Veiksina and excluding cell and molecular biology work) and active participation in writing of the manuscript.

Paper **IV**: Main person responsible for the paper, including planning and data analysis, performing experimental part (together with S. Veiksina and excluding cell, molecular biology work and BRET analysis), design of mathematical models and active participation in writing of the manuscript.

LIST OF ABBREVIATIONS

7-TM	seven transmembrane (receptor)
AC	adenylyl cyclase
ACTH	adrenocorticotropic hormone; corticotropine
ADP	adenosine diphosphate
AGRP	agouti-related protein
ATP	adenosine triphosphate
ASIP	agouti protein or agouti signaling protein
BRET	Bioluminescence Resonance Energy Transfer
BSA	bovine serum albumine
cAMP	cyclic adenosine-3',5'-monophosphate
CRE	cAMP responsive element
CREB	cAMP responsive element binding protein
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine -3',5'-monophosphate
CTC	Cubic Ternary Complex
CTX	*Vibrio cholerae* toxin
DAG	diacylglycerol
EDTA	ethylenediaminetetraacetic acid
ETC	Extended Ternary Complex
FRET	Fluorescence Resonance Energy Transfer
GAP	GTPase-activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
G protein	guanine nucleotide-binding protein
GPCR	G protein-coupled receptor
GRKs	G protein-coupled receptor kinases
GTP	guanosine triphosphate
HEPES	4-(2-hydroxyethyl)- 1-piperazineethane sulfonic acid
Jak	Janus kinase
IP_3	inositol-triphosphate
KNF	Koshland, Némethy and Filmer
$MC_{1–5}$	melanocortin receptor subtypes 1–5
MSH	melanocyte stimulating hormone
MWC	Monod, Wyman and Changeux
NC-IUPHAR	International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification
NMDA	*N*-methyl-D-aspartate
QCM	Quaternary Complex Model
PC	prohormone convertase
PKA	protein kinase A

PKC	protein kinase C
POMC	pro-opio-melanocortin
PSD-95	postsynaptic density protein 95
PTX	*Bordetella pertussis* toxin
RGS	regulator of G protein signaling
SANS	Small-Angle Neutron Scattering
SPA	scintillation proximity assay
STAT	signal transducers and activators of transcription
TCM	Ternary Complex Mechanism
VND	vomeronasal duct

1. GENERAL INTRODUCTION TO GPCR SIGNALING

1.1. Transmembrane Signaling

Cells are highly responsive to specific signals from its environment and this ability to react to external signals is crucial for their survival. Specific signal proteins "receptors" are responsible for transmitting information initiated by very diverse signals like photons, odorants, tastants, hormones, neurotransmitters *etc*. Most of receptors are located in the plasma membrane (cell surface receptors), but several of them are found also inside the cell (intracellular receptors). The three largest classes of signal receptors are all cell-surface receptors: enzyme-linked receptors, ligand-gated ion channels, and G-protein-coupled receptors (GPCR), while forth class, steroid receptors, are mainly intracellular receptors. GPCR are among the largest and most diverse protein families in mammalian genomes. On the basis of homology with rhodopsin, they are predicted to contain seven membrane-spanning helixes, an extracellular N-terminus and an intracellular C-terminus. This gives rise to their other names, the 7-transmembrane (7-TM) receptors or the heptahelical receptors.

1.2. Concept of GPCRs and Heterotrimeric G Proteins

1.2.1. GPCR Classification

Several classification systems have been proposed that divide the GPCRs based on their ligand binding properties and/or amino acid sequences. In one of the frequently used systems, GPCRs were classified into six families, termed as the "A-F" classification system of Kolakowski (Kolakowski, 1994). "Family A" contained the large rhodopsin-related members, which included the biogenic amine receptors; "Family B" consisted of the glucagon, parathyroid hormone, and calcitonin-related receptors; "Family C" was the metabotropic glutamate receptors; "Family D" was the STE2 yeast pheromone receptors; "Family E" was the STE3 yeast pheromone receptors; and "Family F" was the slime mold cAMP receptors.

Bockaert and Pin introduced their classification of GPCR in 1999, based on receptor size and the interaction points of the ligand (Bockaert and Pin, 1999). Their classification system resembles the system of Kolakowski. "Family 1" included receptors that are activated by ligands binding within the transmembrane regions. "Family 2" included receptors that bind large peptide-like ligands and have long N-termini with hormone binding domains. "Family 3"

contains receptors with very large N-termini. ”Family 4” and “Family 5” contained VND pheromone receptors and frizzled/smoothened receptors.

After the human genome became available in 2001 (Venter *et al*., 2001) appear new classifications that were based on phylogenetic criteria. Phylogenetic studies of Fredriksson *et a*l. (Fredriksson *et al*., 2003) indicates that most human GPCRs can be grouped into five main families named; Glutamate (G), Rhodopsin (R), Adhesion (A), Frizzled/Taste2 (F), and Secretin (S), forming the GRAFS classification system. Within each family receptors share a common evolutionary origin. Also new effort in GPCR classification development continued by International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) (Foord *et al*., 2005)

In human, the number of different GPCRs is proposed to be 948 (Takeda *et al.,* 2002), corresponding to about 5% of the total number of human genes (Collins, 2004). Venter *et al.* reported the presence of 616 GPCR genes that belong to the rhodopsin class, the secretin class and the metabotropic glutamate class in the human genome (Venter *et al*., 2001). The rhodopsin class is the largest and contains four main groups termed alpha, beta, gamma, and delta with 13 distinct branches (Fredriksson *et al.*, 2003).

1.2.2. Structure of GPCRs

The secondary structure of the GPCR was first identified in the early 1980s (Hargrave *et al*., 1983). This was bovine rhodopsine – membrane spanning protein that transforms energy from light into cascades of intracellular reactions. After that, based on the analogy to bacteriorhodopsin (which structure had been determined by electron (Henderson and Unwin, 1975) and X ray-based crystallography (Henderson, 1975) the structural models for GPCRs were proposed. According to that, all GPCRs are composed of a single protein characterized by the presence of highly conserved molecular architecture encoding seven transmembrane hydrophobic regions, each of about 20–28 amino acids long and separated from each other by hydrophilic segments, which form three extracellular and three intracellular loops (Muller, 2000). The extracellular parts are often glycosylated and intracellular C-terminus may be palmitoylated. Usually extracellular loops also contains two highly conserved cysteine residues which form disulfide bond to stabilize the receptor structure. The overall sequence analysis has demonstrated that GPCRs have poor sequence homology (≈ 25%), except within the transmembrane domains (20–60%).

The first crystal structure of the “real” GPCR appeared in 2000, when Palczewski *et al*. reported the high-resolution structure for the bovine rhodopsin receptor (Palczewski *et al*., 2000). With 2.3 Å resolution, it was confirmed that the α-helical transmembrane domains are arranged in a closely packed bundle

forming the transmembrane receptor core. In addition, an extracellular ligand binding domain of the metabotrobic glutamate receptor and an extracellular follicle-stimulating hormone binding domain have been solved (Kunishima *et al*., 2000; Fan and Hendrickson, 2005). However there is very little direct information about structures of other GPCR.

1.2.3. Structure and Classification of Heterotrimeric G Proteins

A great deal of information concerning GPCR structure and function has been acquired from studies on β-adrenergic receptors. In the late 1950s, it was already known that these receptors are able to stimulate the adenylate cyclase – enzyme in cell plasma membranes. Initially, it was speculated that the receptor and the enzyme were parts of a single molecule and it took two more decades to realize that both functions are carried out by different membrane proteins (Vauquelin and Van Liefde, 2005). First of all, Martin Rodbell postulated in early 70s that the process which initiate signalling cascades within the cell require three parts: a discriminator (receptor in the cell wall), a transducer (what it was he didn't know, but it required guanosine triphosphate (GTP)), and an amplifier (which synthesize the cyclic adenosine monophosphate (cAMP)) (Rodbell *et al.,* 1971). And finally, in 1977, Alfred Gilman identified and purified that transducer to which GTP binds and named it "G protein" (Ross and Gilman, 1977). The full name of "G protein" is GTP-binding regulatory protein because in the active state it binds GTP.

There are two types of G proteins: heterotrimeric G proteins which participate in signal transduction into cell and monomeric G proteins (or small G proteins), which mediate intracellular signaling. The heterotrimeric G proteins consist of three subunits: α, β and γ (last two forms a tight complex which do not dissociate under non-denaturing conditions). The diversity of heterotrimeric G proteins has been demonstrated already in the beginning of 1980-s, with the purification of G_s (s = stimulatory for adenylyl cyclase), G_i (i = inhibitory for adenylyl cyclase), and G_t (t = transducine which activate cGMP phosphodiesterase in retinal cells) proteins (Landry and Gies, 2002). G protein subunits are highly homologuous in both primary sequence and tertiary structure. Based on the differences in their genes, there has been found at least 27 different α subunits (including splice variants), 5 β subunits and 14 γ subunits (Downes and Gautam, 1999; Landry *et al*., 2006). Their molecular weights are in the ranges:

- α subunit: 39–52 kDa
- β subunit: 35–39 kDa
- γ subunit: ~ 8 kDa

The α subunit consists of two domains: the GTPase domain and the α-helical domain. The GTPase domain contains the GDP/GTP binding site. The helical domain comprises 6 helices and participates in effector recognition. Certain α subunits and βγ complex are post-translationally modyfied by lipids and thus tethered to the plasma membrane.

The usual classification of G proteins based on the similarity of their α subunit structure and activating response, which divide them into four subfamilies: G_s including α_s and α_{olf} (olf = olfactive); G_i including $\alpha_{i1–i3}$, $\alpha_{o1–o2}$, $\alpha_{t1–t2}$, α_z and α_{gust} (gust = gustducin); G_q including α_q, α_{11}, $\alpha_{14–16}$ and G_{12} including α_{12} and α_{13} (Landry and Gies, 2002). A second classification of G proteins can be made based on their sensitivity to *Vibrio cholerae* toxin (CTX) or *Bordetella pertussis* toxin (PTX). CTX can ADP-ribosylate all α_s subunits which lead to constitutive activation of G proteins. But ADP-ribosylation of α_i, α_o and α_t by PTX lead to deactivation of corresponding G proteins. Certain α subunit can be ADP-ribosylated by both toxins (α_t and α_{gust}) and other subunits are insensitive to them.

1.3. Principles of GPCR Signal Transduction

"Signal transduction" describes how individual cells receive, process, and ultimately transmits information derived from external "signals", such as hormones, drugs, or even light" – Martin Rodbell (1969).

Most endogenous signals are agonists (substances which binding to the receptor triggers a response into the cell) and very few are antagonists (which binding blocks receptor, but fails to activate the signal transmission). Molecular pharmacology divides agonists also into full (evoke maximal observed response), partial (evoke a partial response) and inverse agonists (counteract with existing basal activity).

The classic paradigm of the GPCR signal transduction is a simple linear model: upon agonist binding, conformational changes in the receptor protein activates a heterotrimeric G protein, which can activate or inhibit a variety of downstream effector molecules (Neer, 1995). Here GPCRs act as guanine-nucleotide exchange factors for the heterotrimeric G proteins, where activated receptor induces a conformational change in the associated G protein α subunit leading to release of bound GDP and followed by binding of GTP (Iiri *et al*., 1998). GTP binding to the α subunit induces a conformational changes, which cause dissociation of the α subunit and the βγ complex from each other and from the GPCR. The GTP bound α subunit as well as the βγ dimer are now able to interact with effector proteins. The slow inherent GTPase activity of the α subunit hydrolyzes the bound GTP to GDP, leading to the inactive α-GDP subunit. The resultant α-GDP complex reassociates with the βγ heterodimer and so inactivates also this. Efficient coupling of the heterotrimeric G protein to

receptor requires all three subunits to be present and initiates conformational changes in the receptor with increase of affinity for agonists (Yasuda *et al*., 1996). Also the paradigm of G protein subunit dissociation has been challenged (Bunemann *et al.*, 2003).

The intrinsic GTPase activity of purified α subunits measured *in vitro* is much slower than the rate of termination of many cellular G protein responses. This suggested that cells containe factors that regulate the GTPase activity of α subunit. Subsequently, a new gene family called "regulators of G protein signaling" (RGS) was identified (Dohlman and Thorner, 1997). RGS proteins may have several functions; one function of RGS proteins is to serve as "GTPase-activating proteins" (GAPs) for α subunit, hence to accelerate GTP hydrolysis and so the signal termination. Besides G-protein inhibition, some of them can enhance G-protein activation, serve as effectors, and act as scaffold proteins to gather receptors, G-proteins, effectors, and other regulatory proteins together (Landry and Gies, 2002).

Another adaptive response used by cells to terminate GPCR signaling is the receptor desensitization (deactivation). Desensitization is the consequence of receptor phosphorylation, arrestin binding, sequestration and down-regulation. Some of the effector proteins that are activated by many GPCRs, including GPCR kinases (GRKs) and second messenger-activated protein kinases, mediate receptor phosphorylation. In turn, the phosphorylated receptors recruit β-arrestins, which uncouple receptors from G proteins and facilitate receptor internalization, especially via clathrin-coated pits. Receptor internalization precedes receptor degradation, which can occur via both lysosomes and proteasomes. Some receptors can be also dephosphorylated and recycled to the plasma membrane (Ferguson, 2001).

The complexity of GPCR signaling has been further underlined by data indicating that GPCRs may not solely act via heterotrimeric G proteins but a number of other proteins, apart from G proteins, interact directly with GPCRs and modulate their signal transduction (Hall *et al.*, 1999).

1.4. Signal Transduction Pathways

Agonist binding to a GPCR leads to activation of a heterotrimeric G protein, which in turn is linked to either activating or inhibiting different signaling pathways. Several parallel pathways may be activated in response to agonist stimulation of a receptor, from α subunits and βγ complexes, or from the activation of two different G proteins. Signals arising from GPCR are rarely unique. The regulation of cellular events occurs by the integration of several highly complex signaling networks rather than by isolated pathways (Lowes *et al.*, 2002).

1.4.1. The cAMP Pathway

For many transmembrane signaling systems adenylyl cyclases (ACs) has been taken as the final effector enzymes which integrate and interpret signals from different pathways. Molecular cloning studies have identified nine mammalian genes that encode membrane-bound ACs, and one gene encoding a soluble isoform that is insensitive to G proteins. The regulation of membrane-bound AC isoforms is very diverse, depending on particular protein composition in the cell. The α subunits of G_s activate all membrane-bound AC subtypes, but α_i inhibit only couple of isoforms. The βγ complexes may be as activator or inhibitor, depending on AC isoform involved. Some membrane bound AC isoforms may be regulated by phosphorylation, but also calcium ions and calmodulin are very strong modulators for some isoforms of the enzyme (Patel *et al*., 2001; Sunahara and Taussig, 2002).

AC use ATP to generate adenosine-3',5'-monophosphate (cAMP). The cAMP molecule is a "second messenger," one of a family of small diffusible substances that powerfully induce cytoplasmic responses. The primary effector of cAMP is the cAMP-dependent protein kinase (PKA). PKA is a tetrameric complex of two catalytic subunits and two inhibitory (regulatory) subunits. cAMP activates PKA by binding to the regulatory subunits and causing them to release active catalytic subunits. Catalytic subunits modify the activities of target enzymes by phosphorylation of specific serine and threonine residues. Catalytic subunit can translocates even to the nucleus where it phosphorylates the CREB (cAMP responsive element binding protein) family of transcription factors. Once phosphorylated, CREB proteins activate the expression of specific genes containing consensus CRE (cAMP responsive element) sequences in their promoters (5-TGACCTCA-3'). This signal transduction way is used by melanocortin receptors for melanogenesis (skin pigmentation) initiation (Buska and Ballotti, 2000).

1.4.2. Other Pathways

The α subunits as well as the βγ complex can modulate different effectors and activate different signaling pathways. Therefore the main GPCR signaling pathways described should be named as conventional. Most of the pathways are initiated by the activation of effector enzymes that produce intracellular second messengers. Effector enzymes include adenylyl cyclases (discussed above), cGMP-specific phosphodiesterase, phospholipase C, and others. For example, some G_q family α subunits activate the plasma membrane bound enzyme phospholipase C-β (PLCβ), which cleaves the lipid phosphatidyl-inositol-4,5-bisphosphate into diacylglycerol (DAG) and the cyclic alcohol inositol-triphosphate (IP_3). Both, DAG and IP_3, are second messengers. DAG activates protein

kinase C, which by phosphorylation regulates catalytic activity of following enzymes of the pathway. Second, IP_3, acts directly on specific calcium channel receptors in the endoplasmatic reticulum and so increases the cytosolic Ca^{2+} concentration, which leads to activation of other protein kinases and activation of following steps of the pathway. α subunits from another family ($G_{12/13}$) can regulate the small G-protein RhoA via specific of Rho-family guanine nucleotide exchange factors (RhoGEF). An unique signaling cascade is connected with α_t subunit, which regulates a cyclic GMP-gated Na^+/Ca^{2+} channel through its effector cGMP phosphodiesterase. There are also data that some G-proteins subunits bind to and activate directly some ion channels. Thus, some K^+ channel proteins (GIRKs – G protein activated inward rectifier potassium channels) in the heart and some neuronal Ca^{2+} channels are opened by direct binding of βγ complex. However, considerably higher concentrations of βγ complexes in comparison with α-GTP are required to modulate the activities of effector proteins. The role of βγ complexes in regulation of some kinases (for example phosphoinositide-3' kinase-γ) and small G proteins has been also found (McCudden *et al*., 2005).

Recent studies have identified also several unconventional GPCR signaling pathways. For example, some GPCRs may interact with different cytoplasmic scaffold proteins, which can link the receptors to various signaling intermediates and intracellular effectors (Hall and Lefkowitz, 2002). Scaffold proteins are defined as proteins that associate with two or more partners to enhance the efficiency and/or specificity of cellular signaling pathways. There are evidences that the association of the β_1-adrenoreceptors with postsynaptic density protein 95 (PSD-95) link the receptors to effectors such as the *N*-methyl-D-aspartate (NMDA) class of glutamate receptor channels, which are known to be regulated by β_1-adrenoreceptor stimulation in neurons (Hu *et al*., 2000). Another example: stimulation of the angiotensin 1 receptor has been found to activate in addition to traditional G-protein pathways also the Janus kinase, (Jak)-signal transducers and activators of transcription (STAT) signaling pathway, which is usually activated by cytokine or growth factor receptors. Jak-s are tyrosine kinases and STATs are transcription factors that can shuttle between the cytoplasm and the nucleus to regulate the expression of various genes. The ability of the angiotensin 1 receptor to regulate Jak/STAT signaling has been found to be dependent on a direct interaction between the receptor and Jak2 (Ali *et al*., 1997). There is reported that also melanocortin 5 receptors induce the tyrosine phosphorylation and activation of the Jak2 signalling pathway (Buggy, 1998), possibly by the same mechanism.

1.5. Oligomerization of GPCRs

During the last decade, there has been accumulated substantial amount of evidence to indicate that many GPCRs function as associates (for review: Rios *et al.*, 2001; Bouvier, 2001; Milligan, 2001; Terrillon and Bouvier, 2004; Park *et al.*, 2004). These associates could be between two receptor monomers to form dimers or between multiple receptor monomers to form oligomers (present experimental techniques rarely distinct between dimers and oligomers). Association can occur among the same receptor subtypes (homooligomerization) or even between different receptor subtypes (heterooligomerization). As there can be also constitutive oligomerization and ligand-dependent oligomerization, the description of GPCR systems become increasingly complex. Here the product of a single gene is no longer as determined functional unit as it may exist as a monomer, dimer, or larger oligomer and all of these may have unique signaling properties or roles in the signaling process (Rios *et al*., 2001). From the other side, the diversity created by GPCR oligomerization provides the opportunity to design novel pharmaceutical agents, such as bivalent ligands specific only for heterodimers.

The existence of higher order structures has been confirmed by different biochemical and biophysical studies. Earliest evidences were from binding experiments and by manipulations with chimeric receptor constructs. For example, the kinetics of [^{3}H]oxotremorine-M binding to M_2 muscarinic receptors had two kinetic phases for its association (Hirschberg and Schimerlik, 1994). For these receptors also the biphasic inhibitory effects on antagonist binding have been found (Wreggett and Wells, 1995). The complex binding curves of agonists and antagonists to GPCRs were interpreted as evidence for negative or positive cooperativity (cooperativity discussion in next section) that could be explained by site-site interactions within receptor molecule or between receptors of dimeric complexes. Additionally, it has been demonstrated that functional receptor dimers can be formed by co-expressing two reciprocal nonfunctional chimeras (Maggio *et al*., 1993).

Next evidences about receptor associations have been obtained by using selective co-immunoprecipitation. A weak side of this technique is risk of artifactual aggregations (due to the inherent hydrophobic nature of GPCRs) during the solubilization/immunoprecipitation processes (Rios *et al.*, 2001).

The relative localization of receptor monomers in formation of associates has been visualized by Fluorescence- (FRET) and Bioluminescence Resonance Energy Transfer (BRET) methods. These techniques measure the energy transfer from the light emitter-donor (which in case of FRET is excited by an external light source) to the energy acceptor. The most often used donors are bioluminescent protein *Renilla Luciferase* or some variants of green fluorescent protein (GFP). Other variants of GFP are used also as acceptors of the emitted light. The energy transfer between donor and acceptor is measurable only when

they are located within 50 Å (in case of BRET) or within 100 Å (in case of FRET) distance. The appearance of energy transfer has been taken as indication of formation of associates. However, recent data about the rhodopsin structure indicated that the monomeric unit has a diameter of 43 Å (Palczewski *et al*., 2000). This indicates that BRET can occur between receptors that are over one and FRET can occur between receptors that are more than two receptor diameters apart from each other. In addition, the resonance energy transfer techniques do not distinguish effects caused by agonist-mediated formation of dimers and conformational changes in the receptor molecules affecting the level of the energy transfer signal (Rios *et al*., 2001).

Recently, using atomic force microscopy, it has shown that rhodopsin in native disk membranes of rod outer segments are arranged in large para-crystalline arrays (Liang *et al*., 2003).

The acceptance of the new concept has picked up a new question about the stoichiometry between receptor(s) and G protein(s). There is already a finding that one chemoattractant GPCR forms in detergent solution of the pentameric assembly ($receptor_2$*α*β*γ), where exact mass of the conglomerate has been determined by Small-Angle Neutron Scattering (SANS) (Baneres and Parello, 2003). However, while the existence of GPCR dimers/oligomers is largely accepted, their functional role and mechanism has remained more enigmatic and in some cases even controversial.

1.6. Mathematical Models

Paul Ehrlich (1854–1915) and John Newport Langley (1854–1936) are generally credited with the introduction of the concept of receptors or receptive substances to describe the interactions of drugs with cells. A few years later, Alfred J. Clarke (1885–1941) started mathematical modeling of ligand-receptor interactions, becoming so a father of modern receptor theory (Bond and Lefkowitz, 2005; Kenakin, 2004; Colquhoun, 2006). Clark was the first who attempted to quantify the action of ligands (drugs) by looking at their dose-response relationships. His Occupancy Theory models were based on the assumption that the law of mass action dictates the binding (B) of ligand (L) to the receptor (R), according to the equilibrium dissociation constant, K_d, and then subsequently resulted a response. Dissociation constant was used to characterize the strength of ligand-receptor binding (affinity) (Eq. 1).

$$B = \frac{B_{\max} \cdot L}{K_d + L} \qquad (1),$$

where B_{max} is a maximal ligand binding.

Ariëns and Stephenson, independently from each other modified Clark's Occupancy Theory in the 1950s by introducing another term – efficacy (i.e. the ability of the ligand-receptor complex to elicit a physiological response, or "intrinsic activity") that describes the level of response, which agonists produce when they occupy the same number of receptors (full and partial agonism concept) (Ariëns, 1954; Stephenson, 1956). Stephenson used term 'affinity' for the binding step and 'efficacy' for the production of response by the ligand binding. In general terms, for drug development these two parameters have to be measured separately and considered together (Colquhoun, 2006).

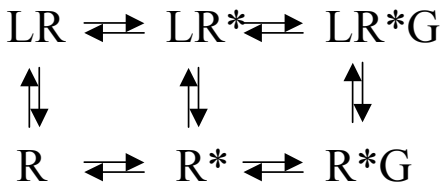
Del Castillo and Katz (1957) assumed that ligands bind to the receptor in the resting state R and cause isomerization it to active ligand bound LR* state (Scheme 2.):

$$R + L \rightleftharpoons RL \rightleftharpoons RL^* \qquad (2)$$

The isomerization is a conformation change by which the information about agonist binding is transferred from the binding site to other sites of the protein (Del Castillo and Katz, 1957). Shortly after, in 1961, the general term "allostery" was introduced by Monod and Jacob to describe the ability of enzymes to have their biological activity modified by the binding of ligands to sites that were topographically distinct from the substrate-binding site. This term "allostery" is nowadays used widely to indicate a remote modulation between different parts of a molecular complex. An allosteric modulator of ligand binding to GPCRs is also the G protein itself. To account that allosteric modulation De Lean in 1980 proposed a new model with ternary complex mechanism (TCM) (Scheme 3.) (De Lean *et al.,* 1980). TCM allowed a ligand-bound activated receptor to form a G protein complex resulting in activation.

$$\begin{array}{ccc} LR & \rightleftharpoons & LRG \\ \uparrow\downarrow & & \uparrow\downarrow \\ R & \rightleftharpoons & RG \end{array} \qquad (3)$$

In 1993, Samama *et al*. introduced ETC (Extended Ternary Complex) model that combines early two-state model, which assumes that receptor can independently to be in inactive form R or in active state R* and only active conformation of the receptor R* can interact with G protein (Samama *et al*., 1993) (Scheme 4.).



(4)

The cubic ternary complex model (CTC) added also possibility that receptors in inactive state R can interact with G proteins (Weiss *et al*., 1996).

Formally CTC model is identical with the allosteric two-state model of Hall, which describes the interaction of an allosteric modulator and ligand on a receptor that can adopt active and inactive conformations (Hall, 2000). On the next level new possibilities have opened with the combination of CTC and Hall models where receptor may have multiple binding sites and to bind to G protein, which are presented as quite sophisticated quaternary complex model (QCM) (for review Christopoulos and Kenakin, 2002).

For earlier models, there was common assumption that they have a single ligand binding site in the receptor. Nowedays the growing number of studies have identified the existence of GPCR dimmers/oligomers, that logically presume at least one additional ligand binding site on the complex. The presence of two ligand binding sites generates the possibility of cooperative interaction between the sites during the binding process. Cooperativity is usually regarded as a special case of allosteric interaction between different binding sites. Allostery and cooperativity are often found from the dose-response or binding curves, i.e. from the behavior of a large population of individual receptors. However, the quantification of allostery and cooperativity strongly depends on the knowledge of detailed molecular mechanisms of the system (Krusek, 2004).

Cooperative interactions may be positive or negative. Positive cooperation occurs when the binding of one ligand molecule increases the affinity of the receptor for the second ligand. The second ligand can either be the same ligand (homotropic cooperation), or a chemically different ligand (heterotropic cooperation). Negative cooperation occurs when the binding of one ligand decreases the affinity for the binding of second ligand.

The simplest mechanism to describe cooperative influence is to induce conformational changes in the protein subunits by ligand binding. It is assumed that each subunit can exist in two states, one conformation, which predominates in the nonliganded state (termed as “T-state”) and a conformation which predominates in the liganded state (the “R-state”). Several models have been proposed to describe possible molecular mechanisms of cooperativity. Two classical and most famous models are the concerted model of Monod, Wyman and Changeux (symmetry model of allosterism, frequently regarded as the MWC model) (Fig. 1B) (Monod *et al*., 1965) and the sequential model of

Koshland, Némethy and Filmer (frequntly regarded as the KNF model or the induced fit model) (Fig. 1C) (Koshland *et al*., 1966). The concerted and sequential models are specific cases of a more general model, proposed by M. Eigen where ligand can bind and conformation changes can occur independently (Fig. 1A) (Eigen 1968). In the reaction schemes in Fig. 1 the ligand-preferred R-states are presented as circles, nonliganded T-state as squares and ligand as L.

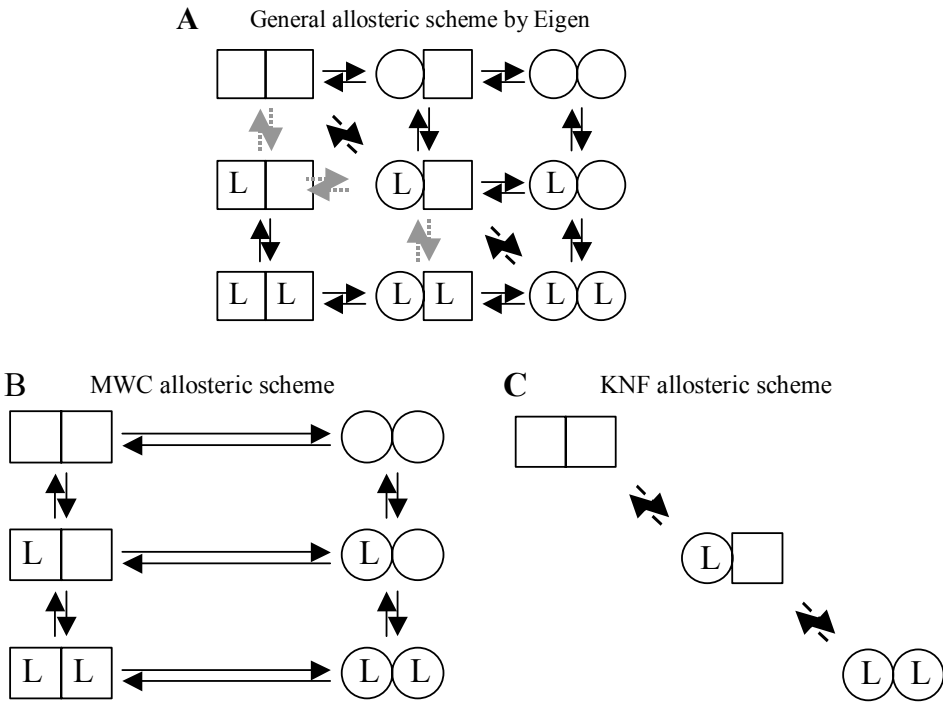


Fig. 1: Schemes of allosteric models of ligand binding.

According to the MWC model the subunits change their shape together to preserve the symmetry of the entire molecule (Fig. 1B). The alternative KNF model postulates that each subunit changes its shape by ligand binding and that changes in one subunit influence the shape of other subunits of the protein (Fig. 1C). The advantage of the KNF model is that it explains also negative cooperativity, whereas the MWC model describes only positive cooperativity. The disadvantage of the KNF model for allosteric interactions is that the changes described mathematically have to take place too quickly. We have also met this problem in interpreting data of our IV paper and found solution in combination of former models (dotted lines on Fig. 1A).

Once the existence of cooperativity has been found, very often it has been quantitatively characterized with the Hill number (h). It estimates the minimal number of interacting binding sites in positively cooperating systems involved. But the Hill equation is an empirical description that does not give insight into the real physical mechanism. Acerenza and Mizraji (1997) introduced another constant instead of the Hill coefficient. They use phenomenological "global dissociation quotient" which is analogous to the dissociation constant, but depend on ligand concentration (Eq. 5):

$$B = \frac{B_{\max} \cdot L}{K(L) + L} \tag{5}$$

In the absence of cooperativity, the $K(L)$ is constant and cooperative behavior can be measured as dependence of $K(L)$ on ligand concentration. This approach was useful also in our proposed scheme (IV paper, equation A28) where dissociation "constant" was dependent on ligand concentration. But it has to be noticed that David Colquhoun resumed his paper with the words: "Binding experiments do not measure affinity for any ligand that causes a conformation change" and "These problems do not mean, of course, that all the inferences that have been made about the binding site are wrong. But it does mean that we are not sure which of them are right" (Colquhoun, 1998).

In summary Eaton *et al*. (1999) have concluded about modeling of biological systems: "There are many levels at which a scientific question can be answered. An answer that is quite satisfying to a scientist from one discipline may be totally unsatisfactory to a scientist from another" and during the preparation of these theses we understood it well.

2. THE MELANOCORTIN SYSTEM

Observation of Fuchs in 1912 that pituitary extract caused darkening of the skin of frogs (Eberle, 1988) was beginning of melanocortin receptors fascinating history. The subsequent studies heralded the beginning of modern pituitary endocrinology and made contribution in the discovery of melanocortin hormones and their receptors. The melanocyte stimulating hormones (MSHs) were also among the first biologically peptides to be purified and sequenced in the 1950s (Cone, 2000). In the subsequent years, extensive studies showed that these peptides were derived from a common larger precursor protein named pro-opio-melanocortin (POMC). The full amino acid sequence of this protein was not known until the cloning of its cDNA in 1979, when it became as the first example of a prohormone precursor encoding a variety of different neuropeptides and peptide hormones.

The true breakthrough came in 1992 with the cloning of the first two melanocortin receptors (MC_1 and MC_2) by two independent groups: Mountjoy *et al.* (1992) and Chhajlani and Wikberg (1992). Subsequent cloning experiments added additional three more subtypes – MC_3, MC_4 and MC_5 receptors (Gantz *et al.,* 1993a, Gantz *et al.* 1993b; Roselli-Rehfuss *et al*., 1993; Chhajlani *et al*., 1993). It was discovered that the melanocortins possessed a surprisingly large number of different physiological effects. The most well-known of them are skin pigmentation changes, anti-inflammatory and antipyretic actions, influence on sexual behaviour, food intake regulation, influence on learning, attention and memory. Accordingly, the melanocortin system has become important target for drug developments. This system consists of:

- the melanocortin peptides α-, β-, and γ-melanocyte-stimulating hormones (α-, β-, γ-MSH) and adrenocorticotropic hormone (ACTH; corticotropin).
- a family of five seven-transmembrane G protein-coupled melanocortin receptors ($MC_{1–5}$ subtypes).
- the endogenous melanocortin antagonists agouti (named also agouti signaling protein, ASP; ASIP) and agouti-related protein (AGRP).
- in addition, two ancillary proteins – mahogany and syndecan-3 have been found that modulate the activity of the melanocortin peptides (Gantz *et al*., 2003).

2.1. The Melanocortins

All melanocyte stimulating hormones together with adrenocortropic hormone and several other peptides are produced by proteolytic cleavage of the common large precursor protein named pro-opio-melanocortin (POMC). Cleavage is performed by the prohormone convertases PC1 and PC2, which belong to a

family of serine proteinases (Seidah and Chretien, 1994). PC1 generates the ACTH, whereas PC2 leads to cleavage of first 13 amino acids of ACTH, yielding α-MSH. The term opiomelanocortins is generally used for the most of the POMC peptides, the term melanocortins is mainly used for ACTH/MSH-derived peptides (Cone, 2000).

Melanocortins and their precursor POMC have been identified in pituitary gland, in brain and in various peripheral tissues of all classes of vertebrates. The structure of MSH peptides of different vertebrates is more or less variable, but the identifying feature of structure of all MSH sequences and ACTH is the core tetrapeptide **His-Phe-Arg-Trp**, which is crucial for their biologic activity.

The sequence of ACTH for mammalian and nonmammalian vertebrates contained 39 amino acid residues (Fig. 2.). In most cases the sequences of mammalian ACTH in the 1–24 region are identical (except for the guinea pig), but have several differences in the 25–39 region. In nonmammalian vertebrates ACTH contains some modifications also in the 1–24 region. ACTH is selective activator for MC_2 receptor.

Usually, α-MSH contains the first 13 N-terminal amino acid residues of ACTH and this sequence is almost identical for 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 ensure stability of the α-MSH molecule against exopeptidases and increase the potency of the peptide (Abbott *et al*., 2000). Mammalian α-MSH is a basic peptide with a pI ranged from 10.5–11.0 (Cone, 2000). In addition to the above mentioned core tetrapeptide **His-Phe-Arg-Trp** also **Tyr^2** and **Pro^{12}** residues are common for all α-MSH peptides (Fig. 2.).

The structure of the β-MSH peptides of the different species is more variable, but it contains six conserved residues: **Tyr^5, His^9-Phe^{10}-Arg^{11}-Trp^{12}** and **Pro^{15}**. The β-MSHs contains usually 18 amino acid residues and its pI ranges from 5.2 to 5.8 (Fig. 2.).

ACTH	H-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	H-Asp-Glu-Gly-Pro-**Tyr**-Arg-Met-Glu-**His-Phe-Arg-Trp**-Gly-Ser-**Pro**-Pro-Lys-Asp-OH
γ-MSH	H-Lys-**Tyr**-Val-Met-Gly-**His-Phe-Arg-Trp**-Asp-Arg-**Phe**-NH_2
δ-MSH	H-Asp-Gly-Lys-Ile-Tyr-Lys-Met-Thr-**His-Phe-Arg-Trp**-NH_2

Fig. 2. Structure of the natural melanocortin peptides. The unique feature for melanocortins is core tetrapeptide His-Phe-Arg-Trp.

The mammalian γ-MSH exists as dodecapeptide (named also as [Lys]-γ^1-MSH), which is cleaved from longer peptide of 22 to 31 amino acids residues (named γ^3-MSH) and contain also the six conserved amino acid residues: **Tyr^2, His^6-**

Phe^{7}-Arg^{8}-Trp^{9} and **Phe^{12}** (Fig. 2.). The γ-MSH from different species contains carboxamide group and in most cases also N-terminal Lys. However the γ-MSH sequence has not been found in the structure of POMC precursor of some vertebrate species (Cone, 2000).

The δ-MSH is the most recent discovery of among the MSH peptides. It has been found only in the structure of POMC precursor of the some of cartilaginous fishes like the dogfish, the stingray, sharks and rays (Dores *et al*., 2003) (Fig. 2.).

2.2. The Melanocortin Receptors

The melanocortin receptors belong to the rhodopsin family of GPCRs. There are five known melanocortin receptors $MC_{1–5.}$ The subtypes MC_1, $MC_{3–5}$ are specific for melanocyte stimulating hormones and the MC_2 receptor for adrenocorticotropic hormone. The MC receptors have high sequence homologies, ranging from 60% identity between MC_4 and MC_5 receptors, to 38% homology between MC_2 and MC_4 receptors. The MC receptors are one of the smallest G protein-coupled receptors known, having short amino- and carboxyl-terminal ends and a very small second extracellular loop. All MC receptors are functionally coupled to activate adenylyl cyclase (AC) and so modulate a cAMP-dependent signaling pathway (Catania *et al*., 2004). There are also reports about involvement of other signal pathways, including phosphoinositol pathway for the MC_3 receptor (Konda *et al*., 1994) and the Jak/STAT pathway for MC_5 receptor (Buggy *et al*., 1998). There have been found consensus recognition sites for protein kinase C (PKC) in the structure of MC receptors, and in some cases also for protein kinase A (PKA), which may be subject of regulation by phosphorylation (Shinyama *et al.,* 2003; Gao *et al.,* 2003). All the MC receptors have several potential N-glycosylation sites in their N-terminal domains; they also have conserved cysteines in their C-terminus, which may serve as sites for fatty acid acylation for anchoring of the C-terminus to the plasma membrane (Wikberg *et al*., 2000).

MC_1 receptor (previously called as MSH-receptor) was the first member of the MC receptors family, cloned in 1992 (Mountjoy *et al.,* 1992; Chhajlani and Wikberg, 1992). It is a 317-amino acid protein. The MC_1 receptor was first detected in melanoma cells, but later it has been found also in several other tissues, like melanocytes, immune/inflammatory cells (e.g., neutrophils, monocytes), human dermal microvascular endothelial cells, Sertoli cells (Abdel-Malek, 2001). Melanin pigment formation is under the control of MC_1 receptors, where the receptor activation 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 (Wikberg *et al*., 2000). Furthermore the recent studies have shown that MC_1 receptors could

serve as a novel target for anti-inflammatory therapies (Catania *et al*., 2004). The affinities of the ligands (both natural and synthetic) for the human MC_1 receptor decrease in row: NDP-α-MSH > α-MSH ≥ ACTH > β-MSH >> γ-MSH (Cone, 2000; Chhajlani and Wikberg 1992).

The gene of MC_2 receptor (originally called as ACTH-receptor) was first time isolated in 1992 (Mountjoy *et al.,* 1992). This encodes a 297-amino acid protein, which is expressed in the adrenal cortex *zona reticularis* and *zona fasciculate*, where it regulates steroid secretion. This receptor is unique within MC receptor family by having no significant response to any of the MSH peptides (Cone, 2000).

In 1993, two independent groups reported the cloning and characterization of the rat (Roselli-Rehfuss *et al*., 1993) and human (Gantz *et al*., 1993) MC_3 receptors. The human MC_3 receptor gene encodes for a protein of 360 amino acids. The greatest expression of MC_3 receptor mRNA is in the brain, but expression occurs also in peripheral nervous system, in placenta, in several human gut tissues including the stomach, pancreas and duodenum, in the heart, in human monocytes, testis, ovary (Cone, 2000; Catania *et al*., 2004; Abdel-Malek, 2001). In contrast to the other MC receptors, MC_3 receptor is functionally coupled not only to activation of AC through G_s protein, but is also coupled to G_q protein, which causes the activation of inositol 1,3,4-triphosphate turnover and modulation of intracellular calcium $[Ca^{2+}]_i$ (Konda *et al*., 1994). MC_3 receptor appears to be involved in modulation of autonomic functions, energy homeostasis, feeding and inflammation, and recent data suggest that the MC_3 receptor could serve as novel target for treatment of sexual dysfunctions (Wikberg *et al*., 2000; Catania *et al*., 2004; Gantz and Fong, 2003; Martin and MacIntyre, 2004). The MC_3 receptor is the only MC receptor having higher affinity for γ-MSH than other subtypes (NDP-α-MSH > γ-MSH = ACTH ≥ α-MSH = β-MSH) (Cone, 2000; Catania *et al*., 2004; Abdel-Malek, 2001).

The MC_4 receptor was also cloned in 1993 (Gantz *et al*., 1993b) and it is a 332-amino acid long 7-membrane spanning protein. It is widely expressed in the central nervous system, including the cortex, the thalamus, the hypothalamus, the brainstem, and the spinal cord, but it has not been found in the periphery (Catania *et al*., 2004; Wikberg *et al*., 2000; Abdel-Malek, 2001). Distribution of MC_4 receptor is consistent with its involvement in autonomic and neuroendocrine functions. It is involved in the central regulation of feeding behaviour and regulation of bodyweight. There are also evidences that MC_4 receptor modulates erectile function and sexual behaviour (Catania *et al*., 2004; Wikberg *et al*., 2000; Martin and MacIntyre, 2004), which have made it very atractive for many pharmaceutical companies. The order of potencies of melanocortin peptides for activation of MC_4 receptor is NDP-α-MSH >> α-MSH = ACTH > β-MSH >> γ-MSH (Catania *et al*., 2004; Wikberg *et al*., 2000).

The last known receptor of the MC receptors family was MC_5 receptor, which was cloned in 1993 (Chhajlani *et al*., 1993). The human gene of MC_5 receptor encodes of protein of 325 amino acids. This receptor is widely expressed in peripheral tissues – adrenal glands, fat cells, kidney, liver, lung, lymph nodes, thymus, mammary glands, testis, ovary, stomach, skin, skeletal muscle and exocrine glands, such as lacrimal, preputial, sebaceous, prostate, seminal, pancreatic *etc*., and plays an important role in production and/or secretion of the major products in these glands (Cone, 2000; Catania *et al*., 2004; Wikberg *et al*., 2000). Expressions of MC_5 receptor have been detected also in the brain and in B- and T- lymphocytes (Cone, 2000; Catania *et al*., 2004; Wikberg *et al*., 2000; Abdel-Malek, 2001). MC_5 receptor seems to be involved in systems connected with stress. The best-described functions of MC_5 receptor are regulation of hair lipid production, water repulsion and thermal regulation (Abdel-Malek, 2001). The potencies of MC peptides to activate MC_5 receptor are in order: NDP-α-MSH >> α-MSH ≥ ACTH = β-MSH >> γ-MSH (Catania *et al*., 2004; Wikberg *et al*., 2000; Abdel-Malek, 2001).

2.3. Ancillary Proteins in Melanocortin System

In contrast to other seven-transmembrane receptor systems there has been found also endogenous antagonists for MC receptors: ASIP and AGRP. ASIP was cloned from the mouse in the early 1990s (Bultman *et al.,* 1992; Miller *at al.,* 1993). Then it was found that this protein promotes biosynthesis of red/yellow pheomelanin and inhibits biosynthesis of black/brown eumelanin affecting the MC_1 receptor. Expression of ASIP takes place normally 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 also in a small amount in the lung and kidney (Gantz and Fong, 2003). In the hypothalamus AGRP acts as a potent orexigenic (appetite-stimulating) factor due to its ability to antagonise melanocortins at MC_3 and MC_4 receptors (Gantz and Fong, 2003). ASIP and AGRP both are relatively small, easily diffusible 132 residue glycoproteins with cysteine-rich C-terminal domains. Within 40 amino acid residues of C terminal, there are 10 cysteines, which form a network of five disulfide bonds. It is proposed that this region of the proteins determines their subtype selectivity (McNulty *et al*., 2005; Chai *et al*., 2005). ASIP has a nanomolar affinity for MC_1, MC_2 and MC_4 receptors, a lower affinity for MC_3 receptor, and no detectable affinity for MC_5 receptor; AGRP has nanomolar affinity for MC_3 and MC_4 receptors and very little affinity for MC_1, MC_2 and MC_5 receptors (MacNeil *et al*., 2002). However, there is very little known about the mechanism of molecular interactions between MC receptors and their antagonists and it would require additional investigations.

There has been also published data about other specific proteins that interact with MC receptors. One of these, mahogany (its human ortholog is attractin), was identified as the product of the murine *Mahogany* gene. Unlike to agouti proteins, mahogany is not cytosolic, but a type 1 transmembrane protein of 1428 amino acids with a large extracellular domain and a relatively short cytosolic tail of 128 amino acids. It appears to function as a co-receptor for the endogenous ASIP (but not AGRP) (He *et al.,* 2004). It is clear that mahogany is involved in mammalian coat coloration; but, there is also evidence that mahogany is involved in obesity suppressing (Gantz and Fong, 2003;Yeo and Siddle, 2003; Yang and Harmon, 2003). However, the role of mahogany in the melanocortin pathway is not fully understood.

Syndecan-3 is a heparan sulphate proteoglycan, a class of single-pass transmembrane molecules whose ectodomain is shed from the cell surface in response to defined stimuli. It is proposed that syndecan-3 might act as an AGRP co-receptor. Syndecan-3 has been shown to augment AGRP antagonism of α-MSH at MC_4 receptor, but the relative contribution of syndecan-3 to AGRP function is at present unknown (Gantz and Fong, 2003; He *et al.,* 2004; Yang and Harmon, 2003).

2.4. Melanocortin receptor active synthetic compounds

The development of novel and selective peptic agonists and antagonists for MC receptors has closely followed to the identification of new MC receptor subtypes. Due to the involvement in a vast array of physiological functions, including, energy balance, pigmentation, sexual function and inflammation, these receptors and their specific ligands have become the centre of interest for many researches from both academic and industrial laboratories.

The first modifications have been made in the "core" sequences of natural MSH peptides mentioned above. Enzymatic stability could be increased by introduction of unnatural structures, such as $_D$-Phe group for α-MSH, forming NDP-α-MSH ([Nle^4, $_D$-Phe^7]-α-MSH named also as melanotan-I (MT-I)). It was the first synthetic superpotent agonistic peptide for all the MSH-binding MC receptors (Sawyer *et al.,* 1980). NDP-α-MSH has similar affinities for $MC_{1,3–5}$ receptors and its radio-iodinated derivative has become as a valuable tool for studies on MC receptors *in vitro* and *in vivo* (Fig. 3.).

Subsequent modifications in the melanocortin "advanced" core tetrapeptide sequence His-$_D$-Phe-Arg-Trp have led to discovery of melanotan-II (MT-II; Ac-Nle^4, c[Asp^5, $_D$-Phe^7, Lys^{10}]-α-MSH[4-10]-NH_2). MT-II is a cyclic lactam analogue of α-MSH with even higher agonistic potency on all MC receptors and with prolonged activity (Hadley *et al*., 1989) (Fig. 3.).

MS05 (Fig. 3.) was one of the first subtype selective agonists found and it had more than 1200-fold higher affinity for MC_1 receptor over all other MC

receptor subtypes (Szardenings *et al.,* 2000). It has been proposed to use as an anti-inflammatory agent.

Using the MT-II cyclic template as a starting point, subsequent studies led to the discovery of first potent and selective antagonist SHU9119 ([$_D$-Nal(2')^7]-MT-II) for MC_3 and MC_4 receptors, which has been found to be also as partial agonist at the MC_1 and MC_5 receptors (Schiöth *et al*., 1997) (Fig. 3.).

There has been found also MC_4 receptor-specific antagonist named HS131 (Schiöth *et al*., 2003), which has 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 (Fig. 3.).

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

Fig. 3. Structures of the synthetic melanocortin peptides.

Peptides, in general, are regarded to be unsuitable for drug development due to their low enzymatic stability, poor oral bioavailability and high cost of production. Therefore, intensive search for highly selective non-peptic ligands for MC receptors is going on. Up to now the majority of new non-peptic agonists are derivates of piperidines or piperazines (Amgen, Bristol-Myers Squibb, Eli Lilly, Merck, Neurocrine, Taisho, Procter and Gamble) or substituted guanidines (Chiron, Melacure) (Bednarek and Fong, 2004) (Fig. 4.). The 4,4-disubstituted N-acylpiperidine derivate (**1** in Fig. 4.) discovered by Merck research group (Sebhat *et al.,* 2002) is a potent MC_4 –selective compound, which potency (EC_{50}) is 2.1nM at hMC_4 receptor, 2850nM at hMC_1 receptor, 2487nM at hMC_3 and 737nM at hMC_5 receptor (Sebhat *et al.,* 2002). The Taisho company has introduced low molecular weight MC_4 receptor antagonist, dipiperazine derivate (**2** in Fig. 4.), for the treatment of anxiety and depression. This compound has been shown to elicit anxiolytic and antidepressant effects when administered subcutaneously or orally, and binds to MC_4 receptor with IC_{50} value of 7.9 nM and displaying no apparent affinity for MC_1 and MC_3 receptors, even at 1 μM concentration (Chaki *et al.,* 2003). The guanidine-based MC receptor ligand (**3** in Fig. 4.) has been discovered and patented by Melacure AB. This compound appeared to be very selective for MC_1 and MC_4 receptors: K_i values are 0.5 μM at MC_1 receptor, 5.8 μM at MC_3 receptor, 0.01 μM at MC_4 and 4.9 μM at MC_5 receptors (Bednarek and Fong, 2004). All these compounds have been proposed for the treatment of obesity and/or erectile dysfunction and appear to reflect an unwavering commitment of the pharmaceutical research community towards the development of drugs for these two therapeutic areas.

Although, much has been learned from various structure-activity relationships of MC ligands since of the first studies about the MC system, but still it is difficult to design ligands *de novo* with specific activities for specific MC receptors. However, even there has not been discovered new subtype-selective ligands for MC receptors, the understanding about the functioning of the MC receptor system has considerably grown (Irani, 2004; Holder and Haskell-Luevano, 2004).

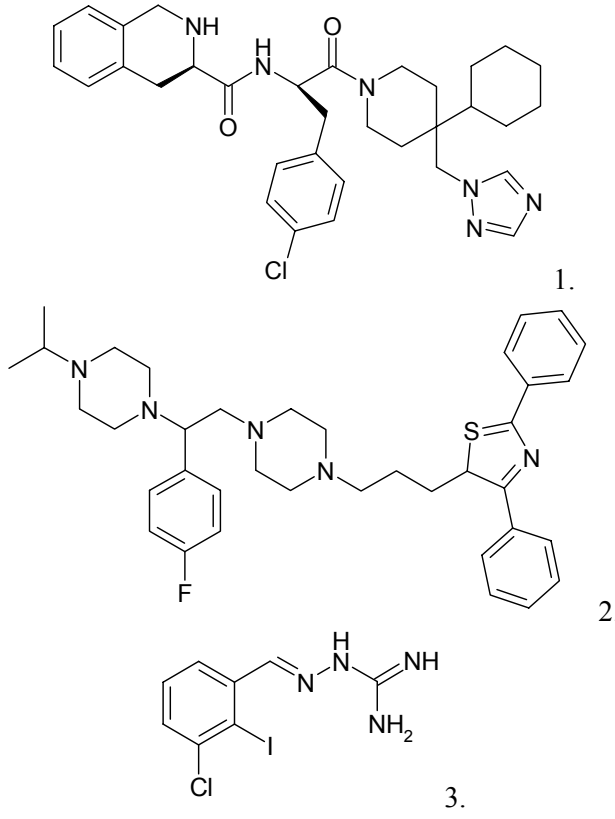


Fig. 4. Non-peptic specific MC receptor compounds from Merck (**1**), Taisho (**2**) and Melacure AB (**3**).

3. EXPEREIMENTAL TECHNIQUES AND PROCEDURES

3.1. Materials

[^{125}I][Nle^4, $_D$-Phe^7]α-MSH ([^{125}I]NDP-α-MSH) was prepared by iodination using chloramine T and purified to be radiochemically pure (2190 Ci/mmol) by High Performance Liquid Chromatography (HPLC). [Nle^4, $_D$-Phe7]α-MSH (NDP-α-MSH), α-MSH, β-MSH, γ^1-MSH, γ^2-MSH, Lys-γ^1-MSH, [Nle^4]-γ^2-MSH, Ser-Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2 (MS05) (Szardenings *et al*., 2000), cyclic [Ac-Cys^{11}, $_D$-Nal^{14}, Cys^{18}, Asp-NH_2^{22}]-β-MSH(11–22) (HS014) (Schiöth *et al*., 1998), cyclic [Ac-Cys^3, Nle^4, Arg^5, $_D$-Nal^7, Cys-NH_2^{11}]-α-MSH(3–11) (HS024) (Kask *et al.,* 1998), cyclic [Ac-Cys-Gly-d-Nal-Arg-Trp-Cys-NH_2] (HS131) (Schiöth *et al*., 2003), Ac-His-DPhe-Arg-Trp-NH_2 (HL-peptide) (Haskell-Luevano *et al*., 2001) were synthesised on solid phase, purified by HPLC and checked by Liquid Chromatography/Mass Spectrometry (LC/MS) by Dr. Ilze Mutule (Uppsala University, Sweden). Melanocortin mimetics, N-[(3R)-1,2,3,4-tetrahydroisoquinolinium-3-ylcarbonyl]-(1R)-1-(4-chlorobenzyl)- 2-[4-cyclohexyl- 4-(1H-1,2,4-triazol-1ylmethyl)piperidin-1-yl]-2-oxoethylamine (THIQ), I-THIQ, [^{125}I]THIQ, 1-[(D-1,2,3,4-tetrahydroisoquinoline-3-carbonyl)-4-chloro-D-phenylalanyl]-4-cyclohexyl-piperazine ditrifluoroacetate (MSY-3) and 1-(D-1,2,3,4-tetrahydroisoquinoline- 3-carbonyl-4-chloro-D-phenylalanyl)-4-[*R,R*-2-(1,2,4-triazol-1-yl)cyclohexyl]-piperazine ditrifluoroacetate (MSY-6) were synthesized as described in (paper II, Mutulis *et al.*, 2004) by Dr. Felikss Mutulis, Dr. Ilze Mutule, Dr. Svetlana Yahorova. LC/MS was performed on a Perkin Elmer instrument PE SCIEX API 150EX equipped with a Turboionspray Ion Source and a Dr Maisch ReproSil–Pur C18-AQ, 5 m, 150×3mm HPLC column using a gradient formed from water and acetonitrile, with 5mM ammonium acetate additive (paper II). hAGRP(83–132) was from Phoenix Pharmaceuticals Inc. (Belmont, CA, USA). PFASTBac1, DM5, DhlO-bac, restriction enzymes, synthetic Sf900 II medium and cell culturing reagents were from Invitrogen LifeTechnologies. Sf9 cells (Chlorocebus Aethiopus 1999-06-07) were from ATCC (Manassas, VA, USA). Guanosine-5'-*O*-3-thiotriphosphate (GTPγS) and all other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

3.2. Cell cultures and expression of receptor clones for binding studies

Sf9 cells were grown in 50–100 ml Sf-900 II medium at 27°C in small spinner bottles (250 ml) as described (O'Reilly *et al*., 1992). Recombinant viruses (clones v506-1 (MC_1) (Lindblom *et al.,* 2001), vVPH4 (MC_3), v119-7 (MC_4) and v113-9 (MC_5), done by Dr. Michael Szardenings (paper III) were added to the cell culture ($2–3 \times 10^6$ cells/ml) and the incubation continued for additional 72 h before harvest. B16 melanoma cells were grown as described (Schiöth *et al*., 1997). The human MC_4R was cloned into the expression vector pCMV/neo, and was a gift from Dr. Ira Gantz (Gantz *et al.,* 1993). The receptors were expressed in COS-1 (CV-1 origin, simian virus 40) cells and grown in Dulbecco's modified Eagle's medium with 10% foetal calf serum. Eighty percent confluent cultures were transfected with the DNA mixed with liposomes in serum-free medium. After transfection, the serum-free medium was replaced by serum-containing medium, and the cells were cultivated for about 48 h (Schiöth *et al*., 1996). Most of the work with cell cultures was done by Dr. Ramona Petrovska (Uppsala University, Sweden).

3.3. Membrane preparations

Cells were collected by centrifugation at $800 \times g$ for 5 min and Dounce homogenised (5 times by 10 stokes with 30 s intervals) in ice-cold homogenisation buffer containing 20 mM Na-HEPES, 0.1 mM phenylmethanesulfonyl fluoride, 0.25 mM benzamidine, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml soybean trypsin inhibitor, pH 7.4 at concentration $5 \cdot 10^8$ cells/50 ml. The homogenate was centrifuged at $700 \times g$ for 5 min at 4°C, the pellet was then rehomogenised and centrifuged again. The combined supernatants were collected, sedimented at $70{,}000 \times g$ (60 min at 4°C) and washed once in new buffer and re-centrifuged. The final pellet was resuspended in the homogenisation buffer at a protein concentration 1–3 mg of protein/ml and aliquots were stored at –80°C. Protein was determined using the Bradford method (Bradford, 1976) with bovine serum albumin as standard.

3.4. Radioligand Binding

Assays were (unless otherwise stated) 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-MSH or [^{125}I]THIQ were 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-MSH were carried out for 3 h at 25°C and terminated by rapid filtration through GF/B glass-fibre filters (Whatman Int. Ltd., Madistone, UK) pretreated with 0.3% polyethyleneimine and 1 mg/ml BSA using a Brandel cell harvester with three washings of 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 and washings using ice-cold 10 mM Na-acetate buffer (pH 4.5), which was found to be optimal to wash out non-specific binding of this radioligand from the filter. Non-specific binding was determined in the presence of 3 μM I-NDP-MSH or I-THIQ.

Kinetic association experiments were started by 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 pre-incubation of the membrane suspension with [^{125}I]NDP-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 addition of non-labelled NDP-MSH (final 3 μM or 100μM) or I-THIQ (final 3μM) or hAGRP(83–132) (final 0.8 μM or 10 μM), or by 100-fold dilution of the reaction medium. At timed intervals aliquots were filtered on GF/B filters and washed with the corresponding buffer and bound radioactivity was determined as described above.

3.5. Data analysis

Binding data were analyzed by computer modeling, fitting them using nonlinear least-squares regression to appropriate formulas assuming that ligands bound to one, two or more independent binding sites or to the developed equations for tandemly coupled receptors (see Appendix and Discussion section in paper IV). Data of association kinetics were fitted to equation (6), which assumes that association occurs in an exponential fashion to *n* independent sites:

$$B(t) = B_{nonsp} + \sum_{i=1}^{n} B_i (1 - e^{-k_i^{obs} t}) \qquad (6),$$

where *B(t)* is the binding at time *t*, B_{nonsp} the non-specific binding, B_i the equilibrium binding and $k^{obs}{}_i$ the observed association rate constant for site *i*.

Dissociation kinetic data were fitted to equation (7), which assumes that dissociation occurs in exponential fashion from *n* sites:

$$B(t) = B_{nonsp} + \sum_{i=1}^{n} B_i e^{-k_i^d t} \quad (7),$$

where $B(t)$ is the binding at time t, B_{nonsp} the non-specific binding, B_i the binding at t=0 and k^d_i the observed dissociation rate constant for site i.

Data are presented as mean ± S.D. of 2–4 independent determinations. Statistical comparison of non-linear regression models were performed by an F-test using the extra sums of squares principle, as described (Draper and Hunter, 1967). Non-linear least-squares regressions were done using GraphPad PRISM™ 4.03 (GraphPad Software, San Diego, CA, USA).

4. MAIN RESULTS AND DISCUSSIONS

4.1. Search for selective ligands for MC receptors [I]

The challenge of studies on receptors of peptides is development of methods and techniques to determine the biological properties of native peptides and find new small non-peptide compounds which do not bare shortcomings of peptide hormones as poor enzymatic stability, bioavailability, and receptor specificity. A classic approach is to generate new, small molecule ligands by determination of the minimally active peptide fragment for the receptors. Early studies with melanocortin peptides involved selective removal (truncation) of N- and/or C-terminal residues of the common peptides with the determination of binding and/or functional activity of the truncated analogs. The found minimal sequence required for biological activity was Ac-His-Phe-Arg-Trp-NH_2 for α -MSH and Ac- $_D$-Phe-Arg-Trp-NH_2 for NDP-MSH. There has been suggested also that a bioactive conformation contains a β-turn around that core peptide sequences, which is stabilized by the presence of the $_D$-Phe residue, with the His, $_D$-Phe, and Trp side chains in close proximity on one surface of the peptide and the Arg side chain on the opposite surface of the molecule (Sugg *et al.,* 1988).

In the present study (paper I) a set of small melanocortic peptidomimetics and peptoids was generated. Peptoids have in their structure amino acid residues with N-substitueted glycines, which are protease resistant, lack asymmetrical carbons and hydrogen bond donors. Subset selection was based on efforts to mimic the β-turn structure found in classical studies of melanocortins. Compared to primary structure based peptoid imitations of natural peptide sequences, pharmacophoric groups (i.e., imidazole, benzene ring, guanidine group, indole) in these molecules were placed closer, simply by reducing the number of bonds between them and so trying to mimic their placement into hypothetical β-turn structures (Fig. 5 in paper I). Thirty-three structures has synthesized by our colleagues and we have tested them on human melanocortin receptors $MC_{1,3–5}$. Most of the compounds displayed low micromolar activity with preference for diamines, guanidino and 2-naphthyl derivatives compared to monoacetylated, amino and 3-indolyl counterparts. For 15 compounds we have found that one or several MC receptor subtypes have affinity lower than 10 μM. Highest subtype selectivity had compound 12c of the paper I, which had more than 13-fold higher affinity for MC_1R (1.56±0.26 μM) in comparison with affinities to other MC receptor subtypes. The NMR study of this compound showed presence of four possible rotamer comformations at its tertiary amide bonds with quite equal distribution (1:1:0.9:0.6). This showed similarity of the secondary structure of our compounds to ‘classical’ peptoids, and indicated the importance of stretched conformations for the activity of the MC receptor ligands.

Another important point of that study was to improve the reliability of radioligand binding assays for MC receptor subtypes. We have used membrane preparations instead of intact cell binding experiments earlier used for MC receptor characterization. We have prepared membranes from insect *Sf9* cells, which expressed corresponding MC receptor subtypes due to baculovirus infection system. There have been also several earlier attempts to characterize MC receptors in membrane preparations of *Sf*9 cells, but they have been unsuccessful, probably due to failure to find optimal experimental conditions (discussed also in paper III).

4.2. The new non-peptide radioligand for MC_4 receptor [II]

The Merck research group has reported about a finding a potent and receptor selective small molecule agonist (THIQ), for the MC_4R (Sebhat *et al.,* 2002) (**3** in Fig. 4). This compound, based on the 4-substituted 4-cyclohexylpiperidine template was the first public disclosure of a highly potent non-peptide ligand selective for the MC_4R. In the [^{125}I]NDP-MSH displacement assays it showed IC_{50} = 0.92 nM for human MC_4R, having at least 250-fold selectivity over other MC receptor subtypes. Dr. Mutulis with his colleagues have synthesized halogen derivative of THIQ (I-THIQ, in our paper II it has been referred also as I-Mex2) that retained high affinity and selecticity for MC_4R in competition with [^{125}I]NDP-MSH. The subnanomolar affinity of the compound suggested to check its usability as specific radioligand for MC_4R. The synthesised [^{125}I]THIQ indicated clear specific binding to recombinant human MC_4Rs expressed in insect *Sf*9 cells. However, the more detailed analysis of [^{125}I]THIQ binding data revealed that experimental conditions found to be suitable for [^{125}I]NDP-MSH binding, could not be used for the new radioligand. [^{125}I]THIQ had high tendency to absorb on the surface of plastics used for the preparation of solutions and silanization of the surfaces did not significantly reduce this process (our unpublished data). Effective factors to reduce the non-specific binding were to use higher concentrations of BSA in solutions and decrease the pH of the buffer. Optimization of the buffer conditions revealed that the minimal absorption of the [^{125}I]THIQ was at pH 4.5. Therefore, we have done ligand dilutions in low concentration of acetate buffer with pH 4.5, but incubations in high concentration of HEPES buffer with pH 7.4, while washing of filters was done again with acidic acetate buffer. In addition to subtype selectivity, the [^{125}I]THIQ has been also as a valuable tool for understanding ligand binding mechanisms to MC_4R and development of two tandem site model, presented in the paper IV.

4.3. Evidences for tandemly arranged ligand binding sites [III, IV]

[^{125}I]NDP-α-MSH has been widely used in drug screening, but little attention has been given on its kinetics and mechanisms in binding to MC receptors. The data of the paper III show that the binding of peptides to the different MC receptors are governed by complex regulations, which cannot be described by simple reversible bimolecular reactions. Although association kinetics of [^{125}I]NDP-MSH followed the regularities of simple bimolecular reactions, the dissociation of [^{125}I]NDP-MSH from the melanocortin receptors was heterogeneous. Only a part of the specifically bound [^{125}I]NDP-MSH could be released from the MC_1, MC_3 and MC_4 receptors at a measurable rate, while another part remained firmly bound. In the paper IV, where only MC_4 receptors were studied, was found that the dissociation of [^{125}I]NDP-α-MSH depends on the time of preincubation. After short preincubation, when the binding of [^{125}I]NDP-MSH had not reached the half of the maximal binding, the addition of non-radioactive I-NDP-MSH only stopped further association without causing any dissociation. With longer preincubation times, the radioligand binding levels increased and dissociation down to steady state levels could be followed. On the other hand dissociation of our new radioligand [^{125}I]THIQ from the complex with MC_4R initiated by non-radioactive I-THIQ was also heterogeneous, but complete. Usage of I-NDP-MSH or hAGRP(83–132) as initiator of [^{125}I]THIQ dissociation revealed considerably slower second phase of the radioligand release. It is important to note that the rate of slow component of [^{125}I]THIQ release initiated by I-NDP-MSH it was comparable with the rate of fast component of [^{125}I]NDP-α-MSH release.

Displacement studies (in paper III) with 11 different MC peptides gave grossly different shapes of their competition curves. Some peptides yielded shallow curves that resolved into apparent two-site fits, but other peptides yielded super-steep curves with Hill coefficients up to 1.6 (*Table* 1. in paper III). Shallow and biphasic competition curves are usually connected with the presence of two or more independent binding sites for the ligand, but in our case the proportion of high- and low-affinity binding sites varied for different peptides and receptor subtypes. Therefore it would be proposed that the binding sites are dynamically formed and regulated, which was also supported by the experiments using masks. In these cases we have used one specific ligand at concentration sufficient for blocking of the high affinity sites and leaving the low affinity sites open and for other ligands usual competition experiments with [^{125}I]NDP-α-MSH were carried out and obtained similar heterogeneous curves as without masks. These data suggest that the MC receptors are subjected to homotropic allosteric regulation. In the case of a positive co-operative interaction the binding of a ligand to one site induce increase in affinity for adjacent site(s) and ligand binding curves have Hill-coefficients larger than

unity. Ligand binding with negative co-operativity causes decrease of affinity for adjacent site(s) and Hill-coefficients smaller than unity. Steric considerations and BRET analysis of Rluc and GFP tagged receptors proposed that these interacting sites are located on different subunits of receptor complexes. Addition of data of [^{125}I]THIQ binding and using peptic and low molecular weight ligands together, revealed that the binding to MC_4R proceeds consecutively to two tandemly arranged interconnected binding sites and allowed us to present corresponding model (Scheme 8).

$$RR \underset{k_{-1}}{\overset{k_1}{\underset{-L}{\overset{+L}{\rightleftharpoons}}}} LRR \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} R^*LR \underset{k_{-3}}{\overset{k_3}{\underset{-L}{\overset{+L}{\rightleftharpoons}}}} R^*LRL \qquad (8)$$

This minimal model proposes that MC_4 receptors exist as preformed dimers RR and after binding of the first ligand L, conformational transformations of the complex R*LR occurs before the binding of the second ligand. When both receptor units have bound [^{125}I]NDP-MSH, the radioligand can be released only from one unit. The [^{125}I]NDP-MSH bound to the remaining unit stays practically irreversibly bound due to a very slow retransformation rate of the transformed complex. The considerably faster binding of [^{125}I]THIQ did not allow accurate kinetic differentiation of the two binding sites. However, addition of NDP-MSH to the preformed [^{125}I]THIQ-MC_4R complex drastically retarded the release of [^{125}I]THIQ from the complex, blocking conformational transformations in the complex by binding into the second binding site. The mathematical drivations of the scheme (8) have been presented as Appendix to paper IV. The complex mechanism of the ligand binding to MC_4Rs caused the situation where the apparent potencies of the same ligand determined in displacement experiments differed more than three orders of magnitude, depending on the experimental conditions and the radioligand used (Fig. 6 in paper IV).

5. CONCLUSIONS

- Based on *Sf*9 insect cell membrane preparations, a reliable radioligand binding assay for the melanocortin receptors (MCR) has been developed.
- The presence of Ca^{2+} at least in submillimolar concentration was required to achieve high affinity specific binding of [^{125}I]NDP-MSH to MCRs subtypes.
- Among thirty-three compounds having both peptide and peptoid features in their structure were tested on human $MC_{1,3-5}R$ subtypes. Fifteen of them had higher affinity than 10 μM. One of them was specific for MC_1R, having more than 13-fold higher affinity (1.56±0.26 μM) in comparison to other MCR subtypes.
- New, MC_4R-specific high affinity non-peptide radioligand 1-(D-1,2,3,4-tetrahydroisoquinoline- 3-carboxy-D-4-^{125}iodophenylalanyl)-4-cyclo-hexyl-4-[(1,2,4-triazol-1-yl)methyl]piperidine trifluoroacetate ([^{125}I]THIQ) was synthesised and used for characterization of the receptors.
- Kinetic and equilibrium studies of [^{125}I]NDP-MSH binding to different MCR subtypes indicated that this process is governed by the complex regulation, which cannot be described by the simple reversible bimolecular reaction.
- Comparative kinetic studies of [^{125}I]NDP-MSH and [^{125}I]THIQ binding to MC_4Rs were base for the presentation of the new model of ligand binding to two tandemly arranged interconnected binding sites.
- The complex mechanism of the ligand binding to MC_4Rs caused the situation where the apparent potencies of the same ligand determined in displacement experiments differed more than three orders of magnitude, depending on the experimental conditions and the radioligand used.

6. SUMMARY IN ESTONIAN

MELANOKORTIINI RETSEPTORITE ALATÜÜPIDELE LIGANDIDE SEOSTUMISE REGULATSIOONI KINEETILISED ASPEKTID

Melanokortiini retseptorite (MCR) osalus paljude oluliste füsioloogiliste protsesside regulatsioonil on viimasel ajal saanud tõestuse ja see on tõstnud selle retseptori farmaatsiatööstuse üheks tähtsaks ravimimärklauaks. Samas on aga väga vähe teada nendele retseptoritele ligandide sidumise regulatsioonist, sekundaarsete virgatsainete moduleerimisest ning biokeemilise vastuse genereerimisest. Käesolevas töös seati eesmärgiks uurida ligandide seostumise regulatsiooni erinevate melanokortiini retseptorite alatüüpide korral ning töötada välja metoodikad nendele ligandide sidumisomaduste määramiseks.

Esimese ülesandena uuriti, millised ligandi struktuurielemendid on vajalikud efektiivseks ja alatüüpspetsiifiliseks seostumiseks melanokortiini retseptoritega. Varieerides erinevate funktsionaalsete rühmade paiknemist ligandi peptiidses struktuuris prooviti mimikeerida β-pööret, mis on olulise tähtsusega melanokortiinsete ligandide sidumisel. Ligandide seostumise efektiivsuse määramiseks töötati välja uus membraanne katsesüsteem, mis kasutas Sf9 rakkudesse ekspresseeritud MCR alatüüpe. Uuritud ligandide hulgast leiti 15 uut struktuuri, mille afiinsus oli submikromolaarses suurusjärgus.

Järgmisel etapil sünteesiti uus selektiivne MC_4 retseptori jaoks kõrge afiinsusega mittepeptiidne radioligand (1-(D-1,2,3,4-tetrahüdroisokinoliin-3-karboksü-D-4-^{125}jodofenüülalanüül)- 4-tsüklo-heksüül-4-[(1,2,4-triasool-1-üül)metüül]piperidiin trifluoroatsetaadi, ehk [^{125}I]THIQ, mis oleks alternatiiviks klassikaline peptiidse radioligandi ([^{125}I]NDP-MSH) jaoks.

Kolmandas ja neljandas artiklis püüti välja selgitada nende radioligandide seostumise regulatsiooni kineetilisi aspekte MCR alatüüpidele. Võrreldes [^{125}I]NDP-MSH sidumist MCR alattüüpidele leiti, et spetsiifilise sidumise saavutamiseks on kõigi alatüüpide korral vajalik vähemalt mikromolaarse Ca^{2+} juuresolek. Samas sõltusid [^{125}I]NDP-MSH väljatõrjumiskõverate kujud teiste MCR-spetsiifiliste ligandide poolt nii ligandi struktuurist kui ka retseptori alatüübist. Seejuures näidati, et tegemist on kompleksse kineetilise protsessiga, milles olulist rolli mängib homotroopiline kooperatiivne regulatsioon. Seda kooperatsiooni uuriti edasi kineetiliste metodidega MC_4 retseptorite korral, kus lisaks [^{125}I]NDP-MSH sidumiskineetikale uuriti ka mittepeptiidse radioligandi ([^{125}I]THIQi) seostumine kineetikat. Sidumise heterogeensus ja peptiidsete ligandide mõju [^{125}I]THIQ dissotsiatsiooni kiirusele viitasid mitme, omavahel seotud sidumiskoha esinemisele. Kogutud andmete põhjal esitati mudel, kus MC_4 retseptorid esinevad dimeerina, ning nende sidumiskohad paiknevad tandemis, kus ühele ligandi sidumine mõjutab oluliselt teise sidumisomadusi.

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Mutulis F., Mutule I., Liepinsh E., Yahorau A., Lapinsh M., **Kopantshuk S.,** Veiksina S., Rinken A., Wikberg J.E. (**2005**) N-alkylated dipeptide amides and related structures as imitations of the melanocortins' active core. *Peptides*, 26(10): 1997–2016.

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Scientific publications

1. Kopanchuk S., Veiksina S., Petrovska R., Mutule I., Szardenings M., Rinken A and Wikberga J.E.S., (2005) Co-operative regulation of ligand binding to melanocortin receptor subtypes: Evidence for interacting binding sites. *Eur. J. Pharmacol*., 512(2-3), 85–95.

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Teaduspublikatsioonid

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