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Cloning, expression and pharmacological characterization of the Melanocortin receptor subtypes

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Title (English)

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Abstract

The melanocortin receptors (MCRs) are a subfamily of G-protein coupled receptors (GPCRs). GPCRs are important targets in the drug industry, since they play a role in the regulation of many physiological functions. The MCRs bind peptides and there exist five subtypes (MC1-5) in most vertebrates. In this work, different MCRs were cloned in expression vectors, expressed in mammalian cells and pharmacologically characterized. The results are important for clarifying the functional role and evolution of the MCRs and may also contribute to the search for better drugs targeting skin cancer, obesity and anorexia.

Keywords

Melanocortin, receptor, pharmacological, primate, MC1, pig, MC4, chicken, obesity, cancer

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CLONING, EXPRESSION AND PHARMACOLOGICAL CHARACTERIZATION OF THE MELANOCORTIN RECEPTOR SUBTYPES

Lisa Johansson

sammanfattning

Melanocortinreceptorer (MCRer) är en subfamilj till G-protein kopplade receptorer(GPCRer). GPCRer är viktiga mål för läkemedelsindustrin, eftersom de spelar en viktig roll vid regleringen av många fysiologiska funktioner. Det existerar fem receptorsubtyper (MC1-5) av MCRer hos de flesta ryggradsdjur och alla subtyperna har peptider som ligander. MCRer är, bland många andra funktioner, viktiga för hud och hår pigmentering och reglering av aptit och kroppsvikt. I detta arbete har olika MCRer klonats och uttryckts mha mammalie celler och sedan karakteriserats farmakologiskt. Åtta primat MC1 receptorer som är relaterade till olika utseende på hår och hud, klonades om med en markör som möjliggör visualisering av cellernas receptoruttryck. Skillnaden i ligandbindningsaffinitet testades hos två gris MC4 receptorer, en vildtyp och en naturligt förekommande mutant, vilken är knuten till fettma hos grisar. Resultatet visade att den muterade receptorn hade en högre affinitet för de naturliga liganderna. Kycklingens MC1, MC3, MC4 och MC5 receptorer har också karakteriserats och resultatet visade en skillnad i bindningsaffiniteter och aktiveringsegenskaper, om man jämför med motsvarande humana receptorsubtyper. Resultaten är viktiga för att klargöra den funktionella rollen och evolutionen av MCRer och kan också bidra till sökandet efter bättre läkemedel riktade mot hudcancer, fettma och anorexi.

Examensarbete 20 p i Molekylär bioteknikprogrammet

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2 INTRODUCTION

2.1. G-protein coupled receptors

The G-protein coupled receptors (GPCRs) is the largest superfamily of receptors and has approximately 1000 members in mammals [1, 2]. The GPCRs has for a long time been a primary target for the drug industry and 40-50 % of drugs available today on the market have GPCRs as target and a similar percentage of all new drugs are also believed to do so. One of the reasons for this, is that GPCRs play a important role in the regulation of physiological phenomena, including sense, growth, reproduction, metabolism and homeostasis (energy balance) [3]. They also mediate the main part of transmembrane signal transduction in living cells.

The unifying properties for all GPCRs are the interaction with a G-protein (as the name implies) and the seven transmembrane(TM) regions [1, 2] and lateral translocation of GPCRs is the best-known example of GPCR-protein interaction. This leads to a bump against a heterotrimeric G-protein and a signal transduction pathway is started. The G-protein has in its inactive form a guanine nucleotide, GDP, bound. When the protein is activated the GDP switches to GTP and releases its α and βγ subunits. They can either stimulate or inhibit the activity of a wide variety of cellular effector proteins. The signaling pathway then continues with an up- or down-regulation of second messengers and eventually transcription of a specific gene or genes (gene expression) [4,5] (Figure 1).

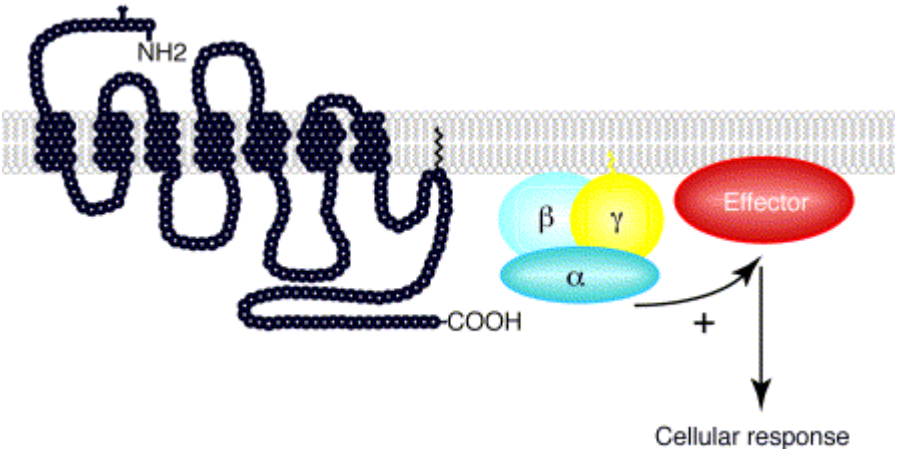

Figure 1. A G-protein-coupled receptor (GPCR) and its signaling pathways. GPCRs can couple to a variety of heterotrimeric G proteins that are assembled from α, β and γ subunits. After GPCR coupling the G protein dissociates into α and βγ subunits. The α and βγ subunits can modulate a variety of effector proteins. Adapted from Leurs et al. 1998 [4].

The ligands of the GPCRs are structurally diverse small chemicals like biogenic amines, peptides, hormones and even light (=photons). There is also a difference among the responses the receptor have upon ligand binding and activation. As mentioned above, there can either be an up- or down regulation of second messengers, but it can also be a interplay of several ligands and/or receptors in the pathway.

The only existing crystal structure of a GPCR so far is the crystal structure of bovine rhodopsin, which serve as a model for all other GPCRs. This leads to a problem when constructing new selective drugs since the GPCRs can differ quite a lot in their sequences and thereby their structure. A better understanding of the structures of the different forms of GPCRs is therefore of great importance. Mutagenesis studies and making chimeric models, are today used to see which amino acids are of importance for the structure of the receptor and binding of the ligand[2].

The GPCRs does not only transmit signals upon agonist activation. They can also spontaneously couple to a signal transduction pathway, that is, being constitutively active. They are regulated in an agonist-independent manner and can instead be down regulated by an inverse-agonist. There are a variety of human disorders caused by constitutively active GPCRs, Retinis pigmentosa, for example, where inverse-agonists can be useful drugs[4].

2.2. Melanocortin receptors

The melanocortin receptors (MCRs) are a peptide binding GPCR and have five subtypes MC1, MC2, MC3, MC4 and MC5. They are all positively coupled to the signal transduction pathway [7], that is, up regulates the intracellular levels of cyclic AMP (cAMP) after ligand activation. They are coupled to a G_s–protein which upon receptor ligand binding activates an effector protein, adenylate cyclase (AC). AC then coverts the ATP to cAMP. This second messenger then effects an enzyme, protein kinase-A (PKA) which in turn phosphorylates a transcription factor and thereby stimulates transcription.

The MCRs are probably one of the smallest proteins within the GPCR superfamily. All the subtypes have small extra celullar (EC) loops and relatively small intracellular (IC) loops. The main ligand binding areas are within the transmembrane (TM) regions TM1, TM2, TM3, TM6 and TM7 (Figure 2). These findings are based on natural mutants and mutagenesis studies[6]. This is further supported by the fact that the lowest sequence homology between the subtypes, is within the EC- loops, IC-loops and TM4 and TM5. The C-terminal of the MC1, MC3, MC4 and

MC5 receptors does not participate in ligand binding [7]. The overall amino acid sequence identity between the subtypes in human, mouse and chicken, is 37 to 60 % [7]and the amino acid identity for each subtype for human and chicken receptors can be seen in Table 1 and 2, respectively.

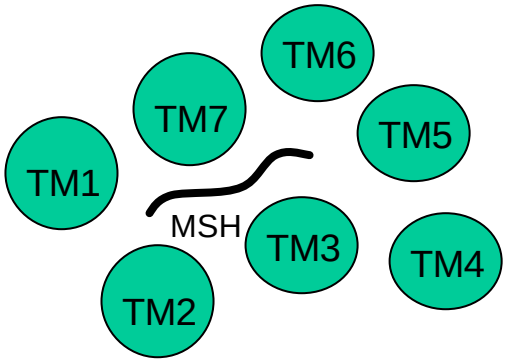

Figure 2. Schematic presentation of putative orientation of the TM regions in the MC receptors. TM1, TM2, TM3, TM6 and TM7 are making up the binding pocket for the MSH peptide[2].

Table 1. Amino acid identity (in %) between the five cloned human MC receptor subtypes [7].

Subtype	MC1	MC2	MC3	MC4	MC5
MC1	---	37.7	44.8	45.1	43.2
MC2		---	44.8	44.8	44.4
MC3			---	53.3	57.8
MC4				---	60.3
MC5					---

Table 2. Amino acid identity (in %) between the five cloned chicken MC receptor subtypes [7].

Subtype	MC1	MC2	MC3	MC4	MC5
MC1	---	38.5	46.8	46.2	49.7
MC2		---	43.7	43.2	45.8
MC3			---	59.4	64.0
MC4				---	63.7
MC5					---

2.2.1. Ligands

The melanocortins, named α, β and γ melanocyte stimulating hormone (MSH) and adrenocorticotropin (ACTH) are the endogenous ligands for the MCRs. All melanocortins are cleaved from the same precursor, proopiomelanocortin (POMC). The cleavage occurs in the anterior pituarity and intermediate lobe and results in ACTH/β-lipotropin and α-MSH/β-lipotropin respectively. POMC is also processed in the hypothalamus. The ligands control a lot of

diverse processes like adrenal steriodgenesis, skin pigmentation, inflammation, appetite regulation and body weight.

All of the subtypes bind the different ligands, except the MC2 receptor, which has a unique pharmacological profile and only binds ACTH. The rest of the subtypes bind all of the ligands with different affinity (Table 3). There is also a difference in the affinity for the ligands between species when looking at the same receptor subtype [2]. α-MSH is the most frequently used MSH analog and works as an agonist for the MCRs, that is, activates the receptor. Another widely used ligand, is the artificial ligand NDP-MSH, which has both increased stability as a peptide and increased affinity for all of the receptor subtypes, except the MC2 receptor. This is very useful in *in vitro* studies and facilitates the radio ligand binding assays [7].

Table 3. Ligand preferences of the MC receptor subtypes. Double sign (>>) means more than 50 fold affinity difference [8].

MC1	NDP>α-MSH>β-MSH>ACTH>γ1-MSH
MC2	ACTH
MC3	NDP>γ1-MSH>β-MSH>α-MSH>ACTH
MC4	NDP>>β-MSH>α-MSH>ACTH>>γ1-MSH
MC5	NDP>>α-MSH>β-MSH>ACTH>γ1-MSH

Agouti and the agouti-related peptide (Agrp) are endogenous antagonists for the MCRs. The normal function of agouti is to regulate pigmentation of the MC1 receptor and Agrp is selective for the MC3 and MC4 receptors. Both their actions have been shown to induce obesity in mice. The cyclic lactam MSH analog, Melanotan-II (MT-II), is an artificial agonist for all of the MCR subtypes with the highest affinity for MC4. It has been shown to induce anorexia in mice [7].

Table 4. Tissue distribution of the MC receptor subtypes [7].

Receptor	Tissue
MC1	Melanocytes, macrophages (brain, adipose tissue and testis)
MC2	Adrenal gland (adipose tissue)
MC3	Brain (placenta, duodenum, pancreas, stomach and heart)
MC4	Brain
MC5	Skin, adrenal gland, spleen, thymus, testis, ovary, muscle, lung, adipose tissue, liver, bone marrow, uterus, stomach, thyroid, pineal gland, mammary gland (brain)

2.2.2. The physiological properties of the melanocortin subtypes

All the subtypes have different tissue distribution (Table 4) and physiological role and here is a short description:

MC1

The MC1 receptor can be found in the melanocytes and regulates the production of black (eumelanin) pigment and red/yellow (phaeomelanin) pigment, that is, hair and skin pigmentation. The MC1 is also located in the macrophages and has an anti-inflammatory effect [9].

MC2

The MC2 receptor is located in the adrenal cortex/glands and in the adipose tissue and mediates the effect of ACTH on steroid production [7].

MC3

The physiological role of the MC3 receptor is still unclear in human. It's located in the brain, heart, duodenum and placenta and other peripheral organs [10].

MC4

The MC4 receptor is locating in the brain and is involved in the regulation of food intake and body weight and it has been shown that knock out (KO) mice are obese. When treating normal mice with an agonist, the food intake is decreased and an anorectic effect is observed. The

opposite effect is true when treating the normal mice with an antagonist [10,11]. This regulation is though, a complex system where several other hormones and receptors are involved [7, 10, 12,13].

MC5

The MC5 receptor can be found almost everywhere in the body, for example, the brain and several peripheral and internal tissues. The function in human is still unclear, but in mice it is shown to be involved in the thermoregulation and water repulsion [7].

2.3. The actual project – background and aim

2.3.1 The primate MC1 receptor

This project is a continuation of the work done by Aneta Ringholm, PhD student. She has been working with MC1 receptors from different species/taxa of the primate family (unpublished results). The different types can be considered as natural "mutants", when comparing to the human MC1 receptor, since they have had different amino acids replaced or deleted during evolution [14]. The aim of the study was to see whether there is an association between the receptor sequence and coat color phenotype (the fur) and the pharmacology behind such association.

The aim of my part of the project, was to re-clone the MC1 receptor from eight different primates with a flag, which will make it possible to see the receptor by immuno cytochemistry and then test the receptors by binding and activation assays, to see whether the flag disturbs the normal behavior of the receptor, by comparing to the previous results.

2.3.2 The pig MC4 receptor

This project is based on the work made by Dr Rothschild [15]. They have showed that a missense mutation, G -> A, in the pig MC4 receptor is associated with several performance traits, like increased back fat thickness, growth rate and food intake. The mutation is located in the seventh transmembrane region, a highly conserved region and results in the replacement of aspartatic acid (D) with an aspargine (N). This mutation could, in the pig breeding industry, be of an advantage and thereby be selected for [15].

My project is to correlate the mutation with the pharmacology of the receptor, since the article only correlates the mutation with the phenotype. This means to study the ligand binding affinity

of the receptor and then compare the results from the wild type receptor and the natural mutant form of the receptor. This study would also contribute to the search for a selective drug targeted against obesity and anorexia.

2.3.3 The characterization of the chicken MC1, MC3, MC4 and MC5 receptors

This project is a characterization of the 4 chicken receptor subtypes, MC1, MC3, MC4 and MC5 regarding the ligand binding and activation properties. Since avian are considered in evolution to lie in between "lower" vertebrates and mammals, this characterization would fill the gap, when comparing pharmacology between other characterized MCRs like, for example, zebrafish (*Danio renio*), dogfish (*Squalus acanthias*), mouse and human [16, 17].

3 MATERIAL AND METHODS

3.1. Original clones

The four chicken receptor clones cMC1, cMC3, cMC4 and cMC5 were originally a gift from Dr Sakae Takeuchi, Okayama University, Japan and sub-cloned by Natalia Poliakova, Department of Neuroscience, Uppsala University, Sweden, into a modified pCEP4 Turbo Expression vector [18]. The two pig clones pMC4 1 and 2 were supplied by Dr Rothschild, Department of Animal Science, Iowa State University, USA, and arrived in a pcDNA 3.1/V5/His-TOPO® vector (Invitrogen, Scotland). The pMC4:1 is considered as the wild type and pMC4:2 as the natural mutant. The two clones were sequenced to confirm that no contaminations had occurred.

3.2. Re-cloning of the 8 primate MC1 receptors with a flag

3.2.1. PCR amplification

The eight different primate MC1 receptors were amplified by using specific primers containing *Hind*III and *Xho*I site. The primers sequences are shown in Table 4. The PCR reaction was performed with 100 ng of the corresponding clone of primate MC1 receptor in PCR buffer (20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1 % Triton X-100), 1.2 mM dNTPs, 12.5μM of each primer and 2.5 U of Pfu in a 50 μl final volume. The conditions for the PCR were as follows: 1 min 30 s at 95°C; 35 cycles of 30 s at 95°C, 30 s at 60°C, 1 min 10 s at 72°C; and a final 5 min extension at 72°C, except for Amp1, Vvr5 and Hgr1 where the cycling conditions were: 37 cycles of 30 s at 95°C, 20 s at 59°C, 1 min 5 s at 72°C, in a GeneAmp^{R}PCR System 9700 (PerkinElmer). The PCR products were confirmed and quantified on a 1% agarose gel and purified using the QIAquick^{TM}Gel ExtractionKit (Qiagen, Hilden, Germany).

3.2.2. Cleavage, ligation and transformation

The PCR products were then cleaved with *Hind*III and *Xho*I (Amersham, Uppsala, Sweden), re-purified using the QIAquick^{TM}PCRPurificationKit and ligated into a predigested modified pCEP4 TurboExpression vector [18] using T4 DNA ligase (New England Biolab, CN, USA) at 16°C for 12 hours. The pmole ends of vector and template used were in proportion 1:4. The product from the ligation was transformed into DH5α^{TM} chemically competent cells (Invitrogen, Scotland) and the vector insert was confirmed by a colony PCR. Overnight cultures were made and Qiagen^{R}PlasmidMaxiKit (Merck, Germany) was used to purify the plasmid DNA/Receptor.

Table 5. Primer sequences used in PCR amplification of the eight different primates.

Primer	Sequence	Primate
Mne2.Hind.fwd	5'-CGA CCT AAG CTT ATG CCT GTG CAG GGA TCC-3'	Macaque, Asian langur, African colobus
Cag20.Hind.fwd	5'-CGA CCT AAG CTT ATG CCT ATG CAG GGA GCT-3'	Goeldi's monkey, Cotton top tamarin, Howlers
Vvr5.Hind.fwd	5'-CGA CCT AAG CTT ATG CCT GTG CAG GGG TCC-3'	Ruffed lemurs, Bamboo lemur
Mne2.Xho.flag.rew	5'-AAG CTC GAG CCA GGA GCA CAG CAG CAC CT-3'	Macaque, Asian langur, African colobus
Cag20.Xho.flag.rew	5'-AAG CTC GAG CAG CAC CTC CTT AAG CGT CC-3'	Goeldi's monkey
Soe296.Xho.flag.rew	5'-AAG CTC GAG CAG CAC CTC CTT GAG CGT CC-3'	Cotton top tamarin
Amp1.Xho.flag.rew	5'-AAG CTC GAG CCA GGA GCA CAG CAG CAC CTC-3'	Howlers
Vvr5.Xho.flag.rew	5'-AAG CTC GAG CAG GAG AAC AGC AGC ACC AC-3'	Ruffed lemurs
Hgr1.Xho.flag.rew	5'-AAG CTC GAG CCA GGA GAA CAG CAG CAG CTC-3'	Bamboo lemur

3.3. Transfection/receptor expression

Human Embryonic Kidney (HEK) 293 – EBNA cells (50-70% confluent cultures) were transiently transfected with 15 μg of each receptor using FuGENE™ TransfectionReagent (Roche Diagnostics, GmbH, Mannheim, Germany) diluted in OptiMEM medium (Invitrogen Corporations, Scotland) according to the manufacturers recommendations.

The cells were grown in Dulbecco's MEM/Nut Mix F-12 (without L-glutamine) (Invitrogen Corporations, Scotland) containing 10% fetal bovine serum (Biotech Line, AS, USA), 0.2 mM L-glutamine, 2.5 mg/ml G418, 2.5 μg/ml amphotericin B and penicillin-streptomycin (100 U penicillin, 100 μg streptomycin/ml) (all from Invitrogen Corporations, Scotland) until harvesting, after 48 hours. Cells were frozen in aliquots at –80°C in 25 mM HEPES-buffer (pH 7.4) containing 2.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 2 g/l bacitracin. Cells with semi-stabile expression were selected for growth in the presence of 200 μg/ml hygromycin B (Invitrogen Corporations, Scotland), starting 48 hours after transfection. The same growth medium as described above was used for the semi-stable cells and they were either harvested in the same manner or immediately used for cAMP assays.

3.4. Binding assays

3.4.1. Competition

Competition experiments were performed in a final volume of 100 µl. To each well 25 µl of the competitor (NDP, α-MSH, β-MSH from Neosystem, France) diluted in appropriate concentrations, 25 µl of hot ligand [^{125}I][Nle^{4}D-Phe^{7}] -MSH (NDP-MSH) (Euro-Diagnostica AB, Malmö, Sweden) and 50 µl of thawed diluted cells were added. The hot ligand was diluted so that the concentration per well was about 0.8 nM. The cells were incubated for 2-3 hours in room temperature and the incubation was terminated by filtration through GF7C filters (Filtermat A, Wallac Oy, Turku, Finland), which had been presoaked in 0.3% polyethylenimine, using a Tomtec (Orange, CT, USA) cell harvester. The filters were washed with 50 mM tris (pH 7.4) at 4°C and dried at 60°C. The dried filters were treated with Melitex A (Wallac Oy, Turku, Finland) melt-on scintillator sheets and the radioactivity retained on the filters counted using a Wallac 1450 Microbeta counter.

3.4.2. Saturation

Saturation experiments were performed in a final volume of 100 µl. 25 µl of cold ligand in a fixed concentration of 2 µM was added to each well for the non-specific binding. 25 µl of the hot ligand [^{125}I]-NDP-MSH was added in appropriate concentrations starting with 15 nM for the chicken MC receptor subtypes and 10 nM for the pig MC4 receptor in the first well and then diluted 2:3. Finally 50 µl of the cell suspension were added and the plates were incubated for 2 hours in room temperature. The plates were then harvested and counted in the same manner as those for the competition

3.5. cAMP assay

The experiments were performed essentially as described earlier [19]. Briefly, the cells were incubated for 2 hours with 5 µCi/ml [8-^{3}H]adenine (Amersham Biosciences, Uppsala, Sweden) and then washed and harvested in a medium composed of 137 mM NaCl, 5mM KCl, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 20 mM HEPES, 1 mM $CaCl_2$ and 10 mM glucose, pH adjusted to 7.4. The pelleted cells were resuspended in the medium as above containing 0.5 mM isobuthylmethylxantine (Sigma) and pre-incubated for 10 min at 37°C before adding the appropriate concentration of the stimulant (MT-II) in 96-well plates (Nunc, VWR International, Stockholm, Sweden). After additional 10 min of incubation in 37°C with the hormone, the reaction were stopped by rapid centrifugation, removal of supernatants, and addition of 200 µl

ice cold 0.33 M perchloric acid per well. The plates were frozen down to –20°C, thawed and the cell debri were spun down. The extent of conversion of [^{3}H] ATP to [^{3}H] cAMP was determined by Dowex / Alumina sequential chromatography [19]. [^{14}C]cAMP (Amersham Biosciences, Uppsala, Sweden) tracer in 0.75 ml 0.33 perchloric acid (about 1000 cpm) was added to each column together with the samples. The ATP / ADP and the cAMP fractions were dissolved in an appropriate volume of scintillation cocktail (Optiphase HiSafe3, Wallac, Turku, Finland) and analyzed in a Beta-counter. The conversion to [^{3}H]cAMP was calculated as the percentage of total eluted [^{3}H]ATP and was normalized to the recovery of [^{14}C]cAMP. The cAMP assay was performed in duplicates.

4 RESULTS

4.1. The primate MC1 receptors

The MC1 receptor from the eight different primates were cloned and transferred into an expression vector. The clones were transfected into mammalian cells and semi-stable expression of the receptors, due to antibiotic pressure, was achieved.

4.2. The pig MC4 receptors

The two pig MC4 clones were transiently expressed in mammalian cells and the receptors were tested in radio-ligand binding assay, using intact cells. The pigMC4:1 was considered as the wild type and the MC4:2 as the natural mutant. The saturation curves are shown in Figure 3 (the curves from the competion assay are not shown) and the K_d and K_i values obtained from both the saturation and competition assays, respectively, are shown in Table 4. Low K_d or low K_i value means high affinity of the ligand to the receptor. In both assays, ^{125}I-NDP-MSH and NDP-MSH were used as radio ligand and ligand, respectively, and in the competition assays, the αMSH and βMSH were also used as the ligands.

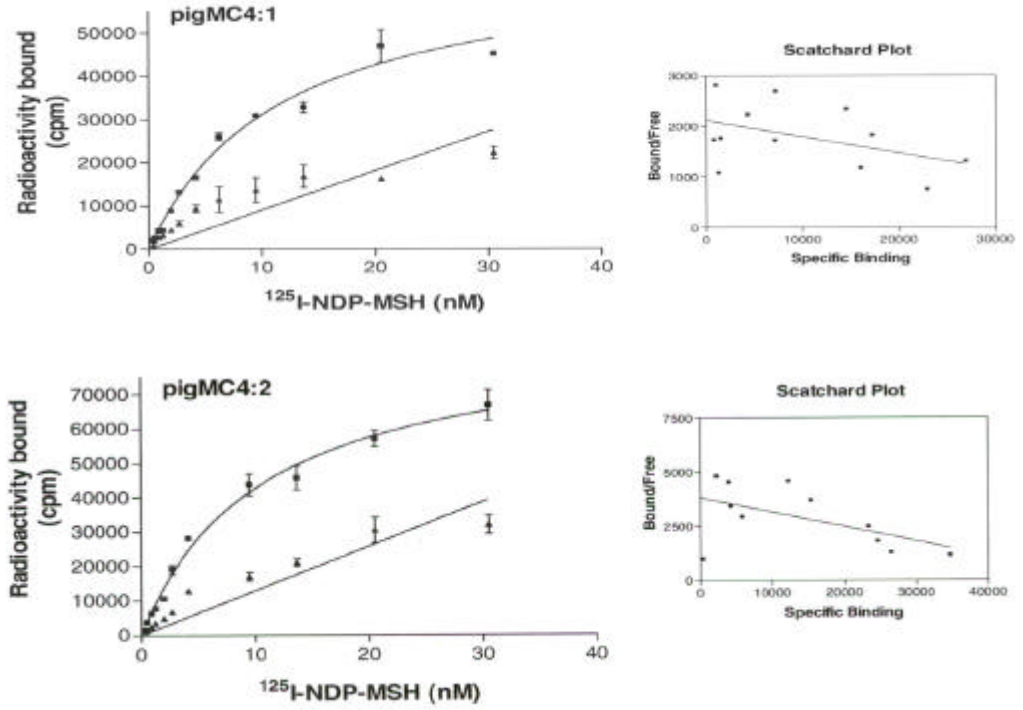

Figure 3. Saturation curves of ^{125}I-NDP-αMSH obtained from transfected HEK-293 cells. The figures show total binding (.) and non-specific binding in the presence of 2 μM cold NDP-αMSH (?) for the pig MC4:1 and MC4:2 receptors. Lines represent the computed modelled best fit of the data using one-site model. Scatchard plot for each saturation curve is shown to the right.

Table 6. K_d and K_i values obtained from the saturation and competition curves, respectively, for melanocortin peptides on the pig MC4:1 and MC4:2 receptors.

Ligand	pMC4:1 K_i (nmol/l)	pMC4:2 K_i (nmol/l)
^{125}I-NDP-MSH[a]	11.6	8.36
NDP-MSH*	0.094	0.15
	25.4	22.5
α-MSH*	340	21.8
	2082	224
β-MSH*	295	17.2
	7641	43.8

[a]K_d values (nmol/l)

K_i is calculated using the K_d for the corresponding human receptors taken from Schiöth et al, 1995, 1996 [8, 20].

* the result from both runs are shown (see discussion)

n=1 if no comments is given

4.3.The characterization of the chicken MC1, MC3, MC4 and MC5 receptors

The chicken MC4 and MC5 receptors were previously transfected and semi-stable expressed in mammalian cells. The chicken MC1 and MC3 receptors were transfected into mammalian cells and semi-stable expression was achieved due to antibiotic pressure. All of the receptors were tested in radio ligand binding assays using intact cells. The competition curves for NDP-MSH, αMSH and βMSH using ^{125}I-NDP-MSH as radio ligand are shown in Figure 4 and the saturation curves are shown in Figure 5, where the same radio ligand as above was used, and NDP-αMSH was used as the ligand. The K_d and K_i values from the assays are listed in Table 7.

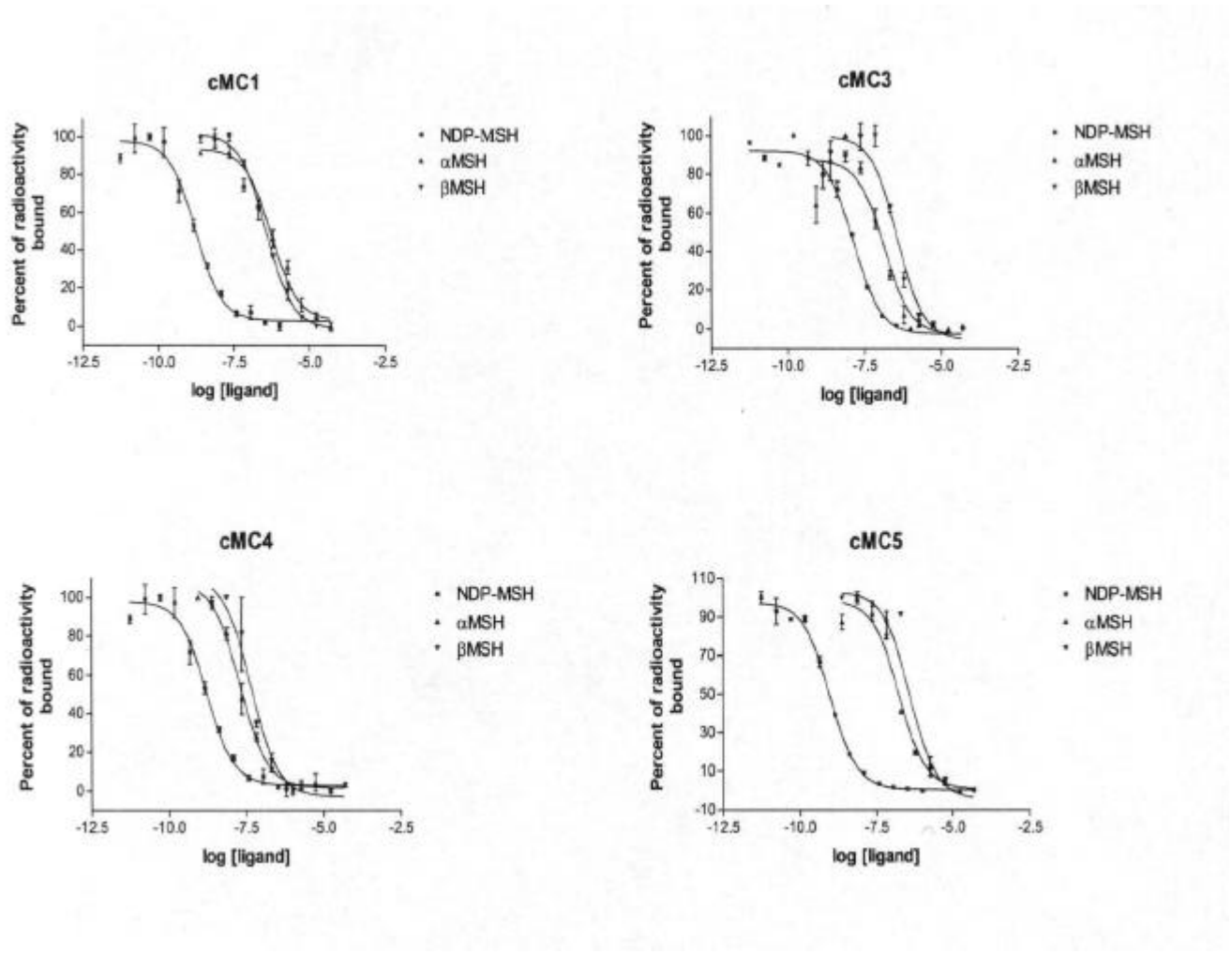

Figure 4. Competition curves of for NDP-αMSH, αMSH and βMSH obtained on transfected HEK-293 cells using a fixed concentration of 0.8 nM ^{125}I-NDP-αMSH for the chicken MC1, MC3, MC4 and MC5 receptors. Data points represents means of duplication and error bars indicate standard deviations.

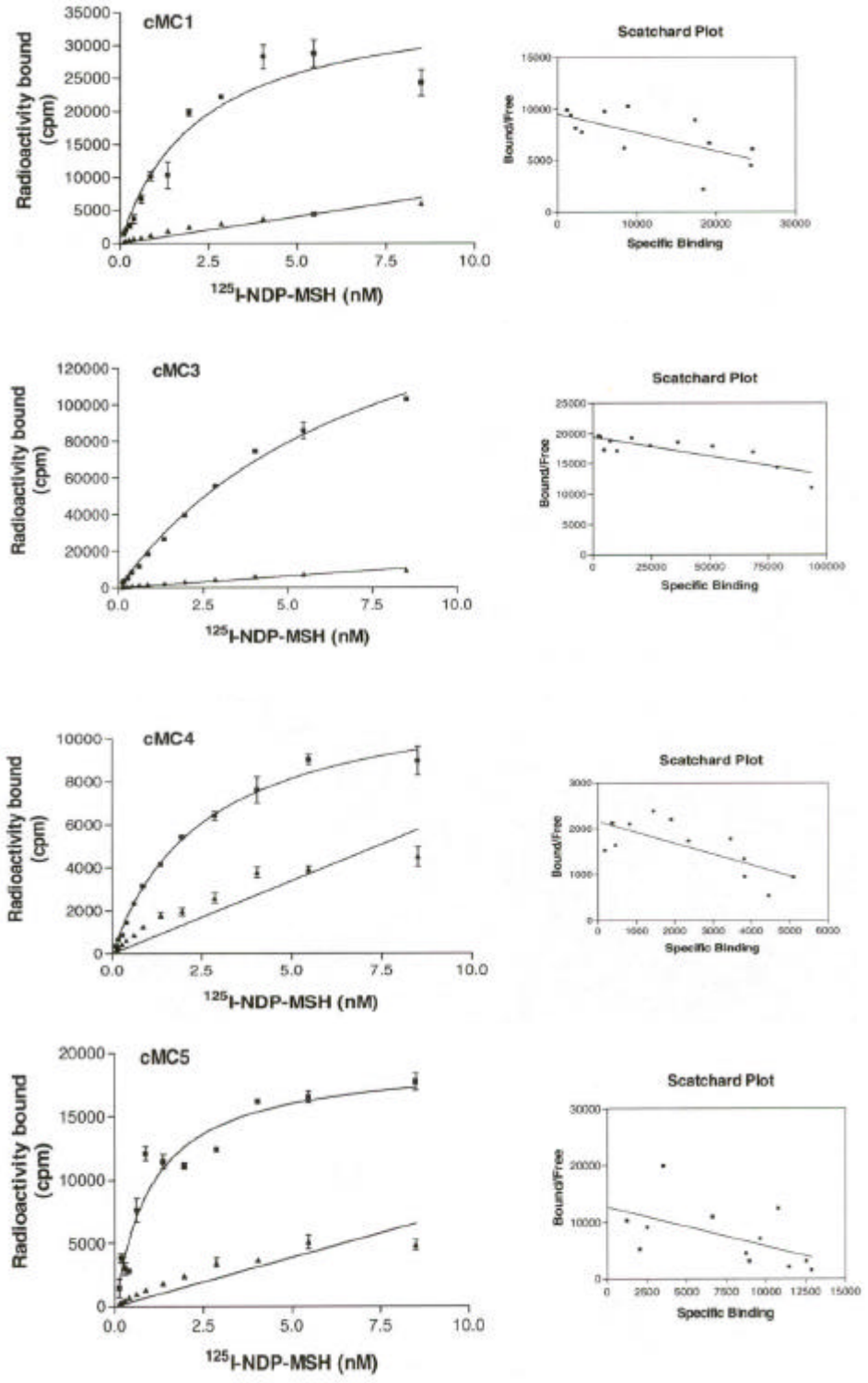

Figure 5. Saturation curves of ^{125}I-NDP-αMSH obtained from transfected HEK-293 cells. The figures show total binding (.) and non-specific binding in the presence of 2 μM cold NDP-αMSH (?) for the chicken MC1, MC3, MC4 and MC5 receptors. Lines represent the computed modelled best fit of the data using one-site model. Scatchard plot for each saturation curve is shown to the right.

Table 7. K_d and K_i values obtained from the saturation and competition curves, respectively, for melanocortin peptides on chicken MC1, MC3, MC4 and MC5 receptors.

Ligand	cMC1 K_i (nmol/l)	cMC3 K_i (nmol/l)	cMC4 K_i (nmol/l)	cMC5 K_i (nmol/l)
^{125}I-NDP-MSH[a] (n=1)	1.3	4	1.4	0.73
NDP-MSH	0.83 ± 0.28	6.28 ± 0.28	1.48 ± 0.02	0.9 ± 0.01
α-MSH	204 ± 161	52.9 ± 6.5	25.8 ± 7.85	479 ± 318
β-MSH (n=1)	244	164	40.2	370

[a] K_d values (nmol/l)

K_i is calculated using the K_d for the corresponding human receptors taken from Schiöth et al, 1995, 1996 [8, 20].

n=2 if no comments is given

The ability to stimulate production of cAMP, the intracellular response, by addition of the artificial agonist MT-II, was measured in a cAMP assay. Mammalian cells with semi-stable expression of each receptor were used in the assay. The results are shown in Figure 6 and the EC_{50}-values (ligand concentration at 50 % of maximal activation of the receptor) calculated from the assay, are listed in Table 8.

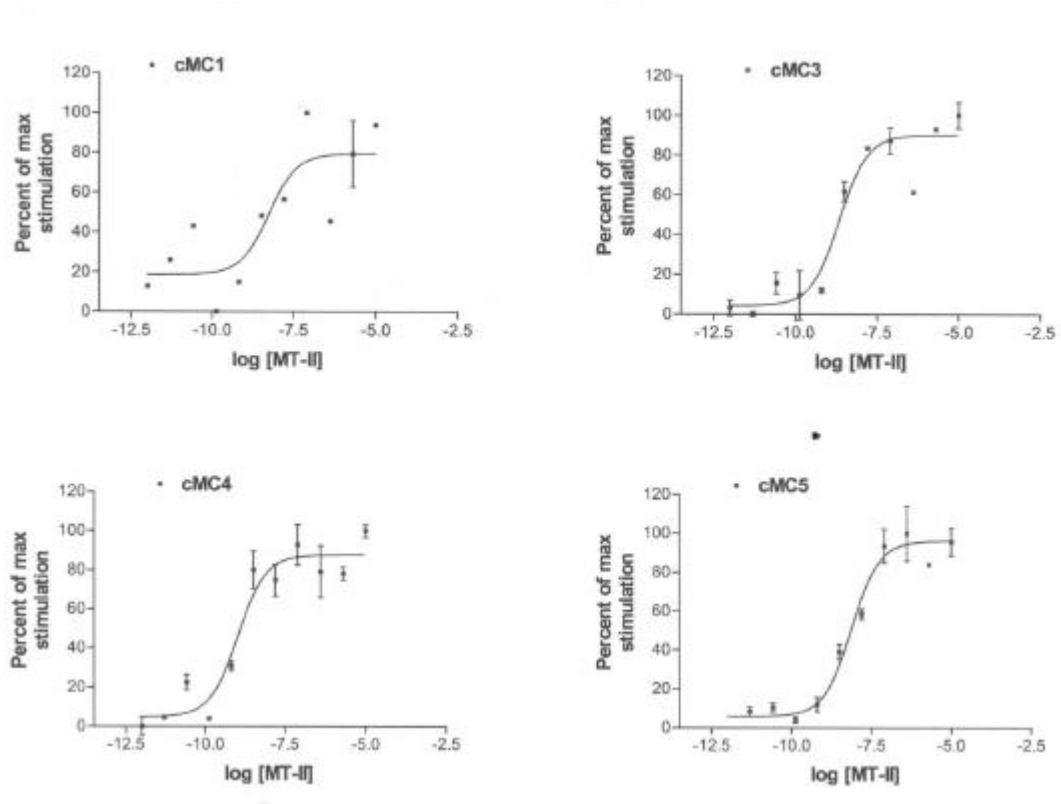

Figure 6. cAMP production curves obtained for chicken MC1, MC3, MC4 and MC5 receptors after stimulation with increasing concentrations of MT-II. Data-points represents means of duplicate and error bars indicates standard deviations.

Table 8. EC_{50}-values obtained after computer modelling analysis of the data from the cAMP assay.

cMC1 EC_{50} (nmol/l)	cMC3 EC_{50} (nmol/l)	cMC4 EC_{50} (nmol/l)	cMC5 EC_{50} (nmol/l)
5.5	2.7	1	9

5 DISCUSSION

5.1. The primate MC1 receptor

The MCR1 is involved in the hair and skin pigmentation by regulating the production of eumelanin (black pigment) and phaeomelanin (red/yellow pigment). Stimulation of the MC1 receptor by α-MSH causes increased eumelanin synthesis, while an absence of α-MSH or inhibition of MSH by the agouti protein, leads to phaeomelanin synthesis. Studies of different human populations have revealed more than 20 allelic variants of the human MC1 receptor gene and genetic studies have shown associations between various MC1 receptor alleles and red hair, pale skin and skin cancer [4, 9, 21]. The melanogenesis is expected to enhance photo protection of the skin. Understanding how these allelic variations affects ligand binding and intra cellular response, could perhaps in the future help us in developing drugs targeted against them. Then an effective protection against skin cancer could be achieved. Mutations which inactivate the MC1 receptor leads to a relative excess of phaeomelanin, that is, gives red/yellow hair and/or pale skin. However, studies on mice indicate that there are over 50 other genes involved in determining the coat color [22] and plenty of candidates for the generation of coat color diversity in primates remain to be assessed.

The MC1 receptor in different primates has recently been studied by Mundy and colleagues [14]. Primates is the family to which human belongs together with orangutans, lemurs and tamarins to mention a few species. When comparing the MC1 receptors from the diverse species in the family, to the human MC1 receptor, some mutations have occurred throughout evolution, which has resulted in a stop codon or more commonly, a replacement of an amino acid by another.

Our study is a further investigation of the work done by Mundy et al. and is a pharmacological characterization of those primate receptors. The aim of the study is to find if there is an association between the receptor sequences, pharmacology of the receptors and coat color phenotype, but no simple association has yet been found. Both binding and activation tests have been performed and of the nine different primate MC1 receptors tested, only one of them gave a significant dose-response curve in the activation assay. Three of these are probably non-functional, since they do not bind the ligand. The data from the activation assays (data not shown, work made by A. Ringholm) indicates that the other five primate MC1 receptor types may be constitutively active. When performing the activation assays there has been a problem

with different levels of cAMP between the different runs of activation assays. This can be due to varying expression levels of receptors in the cells or different amount of cells in the assays.

Therefore has my part in the project been to re-clone these eight different MC1 receptors with a flag. The flag will enable testing of the expression levels of the receptors by immuno cytochemistry. The eight clones were then transfected into mammalian cells and a semi-stable cell-line was achieved, by using antibiotic pressure.

The remaining work is now to test if the flag disturbs the receptors normal behavior in some way. This will be done by performing the binding and activation assays again and compare with the old results.

5.2. The pig MC4 receptor

The melanocortin receptors have been shown in several articles, to be involved in the regulation of energy homeostasis, feed intake and body weight. The MC3 receptor is implicated to have a role in energy homeostasis [23] and the MC4 receptor have been identified as a critical regulator of food intake and body weight [24, 25, 26]. One example of this is the MC4 receptor KO mouse that becomes obese. Another example is the injection of a MC4 selective agonist in normal mice that inhibits feed intake and a MC4 selective antagonist instead increased the food intake [7].

The regulation of the energy balance, food intake and body weight is a complex system where a lot of key hormones and their receptors are involved and the melanocortins acts in interplay with, for example, leptin, orexin and neuropeptide-Y [10]. This complexity has been a problem when designing drugs against obesity. D-Fenfluramine was once an effective weight loss drug, but had to be withdrawn from the market due to un-wanted side effects, as cardiac complications [13].

In the article by Kim et al.[15] a missense mutation in the pigMC4 receptor gene is correlated to certain phenotypic traits in the pig, such as increased back fat, growth rate and food intake. This mutation (G -> A) is resulting in the replacement of the amino acid, aspartatic acid (D) for aspargine (N) in the seventh trans membrane region of the receptor. This region has been shown to be a part of the binding pocket for the ligands. In this study only the genotype and the phenotype were correlated and the work in our lab was to see if there is a pharmacological correlation to the phenotype and genotype.

The pig MC4 receptor, wild type (:1) and natural mutant (:2), arrived in a plasmid and was transiently transfected into mammalian cells. A very low expression of the receptors was detected. This could be due to the plasmid that the receptors arrived in has not an optimized expression rate, as the expression vector normally used in the lab. Another transfection was performed using fresh cells, which grew much better and a bit higher expression of the receptor could be detected.

In the binding assays, this difference of used cells is visible in the table (Table 6) with the values from the competition runs. The upper values are from using the new cells and the lower from the runs with the old cells. This is the reason why both values are shown in the table, since calculating a mean would give a mean and a standard deviation that is misleading.

Both the wild type and the natural mutant seem to have a much higher affinity for the artificial ligand, NDP-MSH, as in other mammals. For the two endogenous ligands, α- and β-MSH, a clear difference could be detected, with the natural mutant showing a much higher binding affinity for the ligands. This is not the expected result, since a ligand activation of the receptor is shown to induce anorectic effects in mice. This might indicate that the activation is altered due to the mutation or that the low expression in some way hides the true ligand binding affinities of the natural mutant.

The significance of these results can be discussed due to the few repeats of each assay and the low expression of the receptors, as mentioned above, and it might be necessary to re-clone the receptor into another expression vector. This would also make it able to get semi-stable cell lines expressing the two receptors, wild type and mutant, and then also perform activation assays to see if the intracellular response, cAMP production, is altered due to the mutation.

If the results are correct, it indicates that the mutation and the replacement of the amino acid, has an effect on ligand binding properties and also may have an effect on the intracellular response, resulting in the changed traits in the pig. In the pig industry an important genetic tool could then be to screen for this mutation in the MC4 receptor gene, since it results in pigs with the desired phenotype, increased back fat and growth rate, of the breeding industry.

There is now also a huge interest for drugs treating obesity, not only for cosmetical reasons, since obesity is also one of the major health problems in the developed countries. A better understanding of the pharmacology behind this association, between genotype and phenotype, could contribute to a more efficient targeting of drugs against obesity and thereby avoiding the eventual side effects. Another growing health problem, in this part of the world, is self-starvation diseases, like anorexia, and drugs that would treat or in some way help people suffering from this disorder, are also of great importance.

5.3. The characterization of the chicken MC1, MC3, MC4 and MC5 receptor subtypes

In mammals, human and mouse, together with "lower" vertebrates, zebrafish and dogfish, the MCRs has been well characterized. The characterization of the chicken MCRs would fill the gap in evolution between these families and a better understanding may be achieved why certain amino acids and functions has been preserved or lost during evolution and thereby their importance for ligand binding and activation of the receptor. Also interesting is why certain subtypes seem to evolve at a slower rate during the evolution [17].

When comparing the human MCR subtypes with the chicken subtypes they differ both in their tissue distribution and amino acid sequence. There are approximately 63.0, 70.6, 76.0, 93.9 and 83.2 % amino acid identity between the human and chicken MC1, MC2, MC3, MC4 and MC5 receptor subtypes, respectively [17, 27]. The most striking difference in tissue distribution is for the MC4 receptor. This subtype is only found in the brain in humans but can in the chicken also be found in a variety of peripheral tissues. This subtype is also the receptor with the slowest evolving rate. This could indicate how important role the MC4 receptor seems to have in living organisms [28]. It has been suggested that molecules placed at a central position of the biological network are strongly constrained, whereas those in peripheral positions are less constrained [17], which would then explain this phenomena. The chicken MC3 and MC5 receptor subtype cannot be found in the brain or in the spleen and skeletal muscle, respectively, as it is in humans. This could imply a difference in function for these receptors in chicken and in human [17, 27]. The MC1 is, as in humans, expressed in the melanocytes, but instead of hair and skin, the pigmentation is visible in the colors of the feathers. Interesting is that several allelic variants are found of the chicken MC1 receptor, resulting in a constitutively active receptor that gives differences in the feather color phenotype [16].

The pharmacological characterization on the chicken MCR subtypes expressed in mammalian cells shows that they all have a preference for the artificial ligand NDP-MSH over the α-MSH, which is the same as in humans. The chicken MC1 and MC3 receptors seem to have lower affinity for all of the tested ligands, NDP-MSH, α-MSH and β-MSH, compared to the human MC1 and MC3 receptors. For the chicken MC4 and MC5 receptors the opposite is true, that is, they have a higher affinity for the ligands, when comparing to the corresponding human subtype. Other differences are the ligand binding affinity order, which differs between the chicken and the human subtypes. In humans, for all the tested ligands, the MC1 receptor has the highest affinity, followed by the MC3, MC4 and MC5 receptors, in that order. For the chicken receptors, the MC4 and MC3 show the highest affinities for α-MSH and β-MSH. For the artificial ligand, NDP-MSH, the chicken MC1 receptor together with MC5, shows the highest affinity.

When performing the activation assays the chicken MC4 receptor had the lowest EC_{50} value, which means that the MC4 requires the lowest amount of ligand to produce an effect, an intracellular response. This is in agreement with the competition experiments where the chicken MC4 subtype had the lowest values for the endogenous ligands α- and β-MSH. It also, when regarding these ligands, correlates well for the rest of the chicken subtypes. The values do not correlate well for the artificial ligand between the competition assay and activation assay, which indicates another pharmacology for this ligand in chicken.

The activation assay has only been performed once for the MT-II agonist and should be repeated to confirm the results. The K_i values are calculated using the K_d values from the human subtypes expressed in COS cells (cells from African green monkey) [8]. The saturation assay has only been performed once and should also be repeated to confirm the results and to obtain new K_d values for the chicken receptor subtypes. These K_d values can then be used for calculating more accurate K_i values. Also more ligands should be tested, for example the γ-MSH and other agonists and antagonists in the activation assay, to more complete the pharmacological profile of the chicken receptor subtypes.

These results, the difference in affinity order of the receptor subtypes for NDP-MSH and the endogenous ligands compared to the human subtypes, together with the difference in tissue distribution, further suggests that the chicken receptor subtypes could have different functions compared to the function of the human MCRs.

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