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CHIMERIC MELANOCORTIN MC3 RECEPTOR: EVIDENCE INDICATING THAT THE SECOND EXTRACELLULAR LOOP OF THE HUMAN MC3 RECEPTOR DOES NOT PARTICIPATE IN AGRP BINDING

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Abbreviations:
NDP-MSH, (Nle^4, D-Phe^7) α Melanocyte-Stimulating Hormone; AGRP, Agouti-related protein; ACTH, Adrenocorticotropic hormone; MCR, Melanocortin Receptor; EGFP, Enhanced Green Fluorescent Protein; GPCR, G protein-coupled receptor, EL extracytoplasmic loop, TM transmembrane domain.

Abstract: The melanocortin receptors are G protein-coupled receptors that have substantial structural similarities and bind the melanocortin peptides. Previous studies have shown that the endogenous antagonist Agouti-related protein (AGRP) binds three of the five known subtypes of the melanocortin receptors. In our study, we constructed a series of chimeric MC3R in order to identify the domains of MC3R that contribute to bind and interact with AGRP. Substitution of the extracytoplasmic loops (exoloops) of MC3R with homologous domains of MC1R were made. Results revealed that the first and third exoloops of the MC3R are required for the correct addressing of the receptor to the cell membrane since their substitution resulted in an intracellular retention of the chimeric receptor and thus abolished the activation of the receptor. The substitution of the second exoloop of the MC3R with the homologous loop of the MC1R did not inactivate the chimeric receptor (MC3-EL2MC1R) that correctly responded to the agonist NDP-MSH stimulation. Moreover, AGRP decreased 125I-NDP-MSH binding and intracellular cAMP production in this chimeric receptor as in the wild-type MC3R. These results confirm that the second exoloop of the human MC3R is not involved in NDP-MSH binding and suggest that it does not participate to AGRP binding.
Keywords: AGRP binding site, Melanocortin receptor, chimeric receptor.

Introduction

Pro-opiomelanocortin is post-translationally processed into a number of biologically active peptides including α, β and γ melanocyte stimulating hormone (αMSH, β-MSH, γ-MSH) and ACTH. These peptides are widely distributed in brain and pituitary. Five melanocortin receptor subtypes (MC1R-MC5R) have been cloned to date (*1-6*), belonging to the superfamily of G protein-coupled receptors. The hypothalamic melanocortinergic system

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appears to play an important role in the central regulation of food intake and energy metabolism (*7-10*).

MC1, MC3, MC4 and MC5 receptors have distinct affinity patterns for the melanocortin peptides. Sequence homology between the five receptors is only 40–60% which accounts for the lack of ligand specificity between receptors(*11*). Human MC3, MC4 and MC5 receptors bind MSH peptides with lower affinity than human MC1R (*12, 13*), whereas MC2R binds ACTH with high affinity but does not bind MSH peptides (*14*). In addition to these melanocortin peptides, two natural endogenous melanocortin antagonists, Agouti and Agouti-related protein (AGRP), have been discovered as functional modifiers of the melanocortin action. AGRP was originally identified from its sequence similarity to the agouti protein (*15-17*). It is a potent antagonist of α-MSH at the level of MC3R and MC4R but not at the level of MC1R and it is not antagonist against ACTH at the level of MC2R (*17, 18*) .

The melanocortin 3 receptor (MC3R), is known to be involved in regulation of energy homeostasis; however, there is still little information about that mechanism. Recent studies suggest that a peripheral action is one of the possible mechanisms involving the MC3R in regulating metabolism in addition of the central system. (*19*) (*20*).

Different studies using site-directed mutagenesis demonstrated that the extracellular loops and the transmembrane domains 3 and 6 of the MC1R seem to participate in the ligand binding (*21, 22*) (*23*). The third extracellular loop of the MC1 in particular appears to play a critical role in melanocortin affinity through conserved proline and cysteine residues (*24*). Other studies were performed to identify the epitopes in the MC3R which participate in ligand-binding, by using several MC1R/MC3R chimeras (*25, 26*). Saturation and competition binding studies on the expressed chimeric receptors indicated that the transmembrane domains TM1, TM2, TM3, and TM7 are the most important for ligand binding, whereas TM4 and TM5 may not contribute to the ligand-binding specificity of the MCRs (*26*). These results were confirmed using chimeric MSH peptides and chimeric MC1/MC3 receptors (*27, 28*). Lately, it has been shown that the third extracellular loop of the MC1 interacts with TM6 and TM7 which are also required for ligand-receptor binding by forming a disulfide bonds between TM6 and TM7 affecting the tertiary structure of the receptor (*29*) (*24*).

Recently, several studies were performed to define the ligand binding regions of MC4R. Studies using chimeric receptors of the human MC4R and the human MC1R demonstrated that hMC4R has at least two binding regions for AGRP. These results showed that exoloops 2 and 3 of hMC4R are important for N-terminal AGRP (87-132) binding and AGRP antagonist

action and that both the third and fourth transmembrane domains of hMC4R are crucial for AGRP (110-117) binding. The ability of NDP-MSH to bind and activate these chimeric receptors was not altered. These results suggest that AGRP has different binding sites than that of NDP-MSH (*30, 31*).

Human MC3R and MC4R exhibit 55% amino-acid identity (*32*). Knowing that exoloop 2 of the human MC3R did not participate to NDP-MSH binding (*25*) and that antagonist action of AGRP involved the exoloops 2 and 3 of the hMC4R, the aims of this study was to generate MC3/MC1 receptor chimeras to determine the participation of the different exoloop domains in the receptor activation and the potential site for AGRP binding in human MC3R.

Materials and methods

Materials. Fetal calf serum (FCS), Dulbecco's Modified Eagle Medium/ Ham's nutrient mixture F12 (DMEM/F12 1:1), LipofectAmine Plus reagent, and L-glutamine were purchased from Invitrogen (Cergy Pontoise, France); 3-isobutyl-1-methyl-xanthine (IBMX) from Sigma-Aldrich (St Quentin Fallavier, France); (Nle^4, D-Phe^7)αMSH (NDP-MSH) and ACTH from Bachem (Voisins le Bretonneux, France); human Agouti-related protein (hAGRP) (83-132) from Phoenix Europe, Karlsruhe, Germany; ^{125}I-(Lys^{11})(Nle^4-D-Phe^7)αMSH (SA:2000Ci/mmol) from Amersham Biosciences, Orsay, France.

Construction of chimeric MCRs. The full length coding sequence of the wild-type human MC3R was amplified by PCR (Polymerase Chain Reaction) from human genomic DNA using specific primers and subcloned into the pEGFP-N2 expression vector as previously described (*33*).

Three chimeric constructs of MC3R were prepared by replacement of the amino- acids of each exoloop of MC3R by those of MC1R. As represented in Figure 1, a comparison of exchange amino-acid sequences of the first, second and third exoloops between MC3R and MC1R is shown. These constructs were named MC3R-EL1MC1R (EL1), MC3-EL2MC1R (EL2) and MC3R-EL3MC1R (EL3), respectively. The chimeric receptors were generated from the cloned wild-type hMC3R-EGFP by site-directed mutagenesis as previously described (*34*) using oligonucleotides containing multiple mutated sites. Briefly, vector DNA containing the desired mutations was synthetized with *Pfu* DNA polymerase (pfu Turbo DNA polymerase, Stratagene) by PCR (conditions: 95°C for 30 sec; 94°C for 30 sec, 50°C for 1 min, and 68°C for 16 min repeated 16 cycles; 68°C for 10 min). Methylated template DNA was digested with DpnI enzyme for 1 hour at 37°C and the mixtures were then used to transform Max DH5α competent cells (Invitrogen). Inserts of plasmid DNA of several

colonies were totally sequenced confirming the presence of the desired new DNA fragment, and absence of PCR-induced errors. These plasmids were then used for transfections.

Transfection in HEK293 cells. The day before transfection, HEK293 cells (Human Embryonic Kidney) were plated on 6 well dishes at 400 000 cells/well in DMEM/F12 medium supplemented with fetal calf serum (7.5%) and 2 mM L-Glutamine. Transfections were performed using 1µg of plasmids in serum-free DMEM/F12 medium without antibiotics, using the LipofectAMINE PLUS reagent according to the manufacturer's protocol as previously described (*35*).

Northern Blot analysis. The expression of wild-type MC3R and chimeric receptors fused to EGFP was evaluated by Northern blot analysis after extraction of total RNA from the parental cell line and from transiently transfected HEK293 cells as previously described (*36*). Hybridizations were performed using the entire coding sequence of the EGFP as probe, labelled in the presence of [α-^{32}P] deoxy CTP using the Rediprime DNA labelling system (Amersham Biosciences, Orsay, France). The relative intensity of hybridization signals was quantified by image analysis and equal loading of RNA samples was confirmed by scanning the 28S negatives (SAMBA 2005, Alcatel TITN, Meylan, France).

cAMP measurement. cAMP was measured upon NDP-MSH stimulation in cells expressing the different chimeric receptors and the wild-type MC3R, forty-eight hours after transient transfection. Cells were stimulated for 20 min with increasing concentrations of NDP-MSH in the presence of 1 mM IBMX to inhibit phosphodiesterases, then recovered, and the intracellular accumulation of cAMP was measured by radioimmunological assay using ^{125}I-labelled cAMP (Beckman Coulter, Roissy, France) (*37*). In some studies, cells were stimulated with NDP-MSH or ACTH in the presence or absence of 50 nM AGRP.

Binding studies. Iodinated NDP-MSH, ^{125}I-(Lys^{11})(Nle^{4}-D-Phe^{7})αMSH was used to assess agonist binding affinity. HEK293 cells were plated in 12 well dishes at 250 000 cells/well before transfection. Forty-eight hours after transfection, the medium was removed and cells were incubated for 90 min at room temperature with 0.1 nM of ^{125}I NDP-MSH and various concentrations of unlabelled NDP-MSH in DMEM/F12 medium containing 0.5% bovine serum albumin (BSA) and 0.1% bacitracin. Binding reactions were stopped on ice by removing the culture medium and washing the cells quickly three times with 0.9% NaCl. Then, the cells were solubilized in 0.5 ml 0.5 M NaOH, 0.4% sodium deoxycholate (*38*). Radioactivity in the lysate was quantified in a Packard γ counter (PerkinElmer). Specific binding was determined by substracting the radioactivity associated with the cells in the

presence of 10^{-6}M unlabelled NDP-MSH from the total binding. The nonspecific binding accounted for less than 4% of the total binding. Scatchard analysis was performed to determine the Kd value and the number of sites per cell. Binding studies were also performed in the presence of AGRP at a concentration 100-fold higher than that of 125-I NDP-MSH.

Statistical analysis. Statistical analysis was performed with Student's t-test for comparison of two groups. Differences were considered significant when $P<0.05$

Results

To investigate the participation of the exoloops of MC3R in NDP-MSH binding and in AGRP antagonism against α-MSH action, the three chimeric human MC3R where each exoloop of MC3R has been substituted with the homologous region of MC1R (a receptor that is not inhibited by AGRP) were used.

NDP-MSH stimulated cAMP production.

Functional analysis was performed after transient expression of the chimeric receptors in HEK293 cells. The intracellular cAMP production levels were measured after stimulation by increasing concentrations of the agonist NDP-MSH. As shown in Figure 2, only the HEK293 cells transiently transfected with the wild-type MC3R or the chimeric MC3R with the substituted second extraloop of the MC1R (MC3-EL2MC1R) were able to produce intracellular cAMP in response to NDP-MSH stimulation. This production started from NDP-MSH at 10^{-11}M with a maximal response at 10^{-8}M. Although the EC50 were similar for MC3R and MC3-EL2MC1R (0.57 ± 0.09 nM and 0.59 ± 0.07 nM (n=5), respectively), the maximal stimulation above unstimulated cells was significantly higher in cells transiently transfected with wild-type MC3R compared to cells transiently transfected with MC3-EL2MC1R (120±5.3 and 97±11-fold increase above the unstimulated cells, n=5, respectively). The substitution of the first (MC3-EL1MC1R) and the third (MC3-EL3MC1R) exoloops of MC3R with those of MC1R, strongly reduced cAMP production at any NDP-MSH concentration used (Figure 2). We demonstrated previously that human wild-type MC3R fused to EGFP expressed in HEK293 cells responded equally to NDP-MSH or ACTH (*33*). In this study, only MC3R-EL2MC1R as well as wild-type MC3R were activated by both ligands (Figure 3).

Effect of AGRP (83-132) on NDP-MSH or ACTH stimulated cAMP production

AGRP (83-132) was also a functional antagonist of the hMC3R fused to EGFP expressed in HEK293 cells (*33*). The cAMP production in response to NDP-MSH or ACTH was then measured in the presence or absence of 50 nM of AGRP (83-132) using the different

chimeric MC3Rs as illustrated in Figure 3. We showed that cAMP production stimulated by 0.5nM NDP-MSH or ACTH in HEK293 cells expressing MC3-EL2MC1R was completely inhibited by 50nM AGRP. Moreover, the NDP-MSH dose-response curves for cAMP production in presence of 50 nM of AGRP were clearly shift to the right in HEK293 cells transfected with the wild-type MC3R or the MC3-EL2MC1R (Figure 4). All these results demonstrate that AGRP potently inhibits the actions of NDP-MSH at both receptors and suggest that EL2 in MC3R does not participate not only to the stimulation of the adenylate cyclase by agonist but also to the antagonist action of AGRP.

125-I NDP-MSH binding studies

The lack of cAMP production of the HEK293 cells transfected with the MC3-EL1MC1R and the MC3-EL3MC1R after agonist stimulation could be due to a defect in the ligand binding properties. Thus, we performed binding assays with 125-I NDP-MSH on transiently transfected cells expressing each chimeric receptors. As shown in Figure 5, cells expressing wild-type MC3R or MC3-EL2MC1R bound specifically 125-I NDP-MSH and the binding could be displaced by increasing concentrations of unlabelled NDP-MSH. Scatchard analysis of the binding data revealed a single class of high affinity binding sites with a similar dissociation constant (Kd : 1.34 nM and 1.6 nM for wild-type MC3R and MC3-EL2MC1R, respectively). However, the number of binding sites for MC3-EL2MC1 chimeras was sligtly reduced (70 600 sites/cell for MC3R-EL2MC1R versus 119 560 sites/cell for wild-type MC3R). No significant specific binding with 125-I NDP-MSH was obtained in HEK293 cells transfected with MC3-EL1MC1R or MC3-EL3MC1R.

Figure 6 showed that AGRP (10 nM) was able to displace the 125-I NDP-MSH binding on both wild-type MC3R and MC3-EL2MC1R confirming the results obtained on cAMP production assays.

Confocal microscopy analysis

The absence of adenylate cyclase stimulation by NDP-MSH or ACTH and absence of NDP-MSH binding in cells expressing both MC3-EL1MC1R and MC3-EL3MC1R could reflect alteration in the membrane expression levels of these receptors. Thus, we performed a confocal imaging analysis of cells transiently expressing the different chimeras. The data presented in Figure 7 (a-d) showed that wild-type MC3R and MC3-EL2MC1R are correctly expressed on the cell surface. In contrast MC3-EL1MC1R and MC3-EL3MC1R are poorly expressed at the cell membrane level and seem to be retained in intracellular compartments.

Discussion

The melanocortin receptors are the smallest G protein-coupled receptors. This family is characterized by short amino- and carboxy-terminal ends and a very small second extracellular loop. The lowest homology level between the MCR subtypes is found in the intra and extracellular loops and in the 4th and 5th transmembrane domains (TM4 and TM5) (*25*). In contrast, the highest homology level is located in the 1st, 3rd and 7th transmembrane domains (TM1, TM3 and TM7) (*26*). For many small ligands, the binding site on GPCR is localized within the seven transmembrane domain (the TM core) whereas for peptide hormones, the binding site in the receptors includes the N-terminal domain, the ELs and the superior part of the TMs (*39, 40*). In view of their apparent structural dissimilarity including their size difference and their receptor subtype specificity, it is likely that melanocortin peptides and AGRP have different receptor binding sites. It has already been demonstrated that agonists bind to hMC1R in a relatively shallow pocket formed by the TMs (*41*) and more precisely between TM1-3 and TM6-7 of this receptor (*27, 28*). Moreover, EL2 of MC3R does not participate in NDP-MSH binding (*25*) and the substitution of the EL1, EL2 or EL3 of hMC4R by homologous regions of hMC1R does not alter NDP-MSH binding and chimeric receptor activation (*30, 31*). On the other hand, the Els contribute to molecular recognition and receptor functional activity for larger peptide ligands like AGRP (87-132) (*30, 31*) suggesting that AGRP has a different binding site than that of NDP-MSH on hMC4R. However, AGRP acts as a classical competitive antagonist on hMC4R and has no allosteric effects. The presence of melanocortin ligand, NDP-MSH, at the receptor sterically prevents the binding of AGRP (*42*). In addition, the presence of a common binding pocket has ben hypothesized in the mouse MC4R where both the endogenous agonists and antagonists interact (*43*). All these observations do not necessarily rule out the presence of additional points of contact for AGRP elsewhere within MC4R (*42*). In fact, several studies suggest that AGRP possess both overlapping and unique receptor interactions as compared with melanocortin agonists (*30, 31, 43, 44*). Since hMC3R and hMC4R share 55% amino-acid identity and since AGRP is also an antagonist of the hMC3R (*16, 17*), the participation of the ELs in the AGRP binding to hMC3R was investigated. Our results demonstrate clearly that EL1 and EL3 of MC3R are very important for a correct addressing to the cell membrane of the chimeric receptors, as confirmed by confocal microscopy analysis. . If these proteins are sorted into the plasma membrane, the conclusion would be would differ from the current conclusions. In contrast substitution of EL2 of MC3R with its homologous of MC1R does

not alter the addressing to the cell membrane of the chimeric receptor. Moreover, the activation of the chimeric receptor was not modified since the affinity for NDP-MSH and the cAMP response to agonists for the human wild-type MC3R and the chimeric MC3-EL2MC1R transiently expressed in HEK293 cells are very similar. In addition, these results suggest that the chimeric MC3-EL2MC1R does not acquire MC1R like activity and retains MC3R characteristics since the affinity for NDP-MSH of MC1R was ten-fold higher than of MC3R (*12, 45*). Furthermore, AGRP antagonized the NDP-MSH or ACTH response of HEK293 cells expressing chimeric MC3-EL2MC1R as well as wild-type MC3R. Thus, these results demonstrate that the binding sites for the NDP-MSH agonist but also for the AGRP antagonist are different in the human MC3R and MC4R. EL2 is not involved in the NDP-MSH binding to MC3R and MC4R as previously demonstrated (*25, 30, 31*). However, EL2 is apparently not involved in AGRP (83-132) binding for hMC3R whereas EL2 and EL3 are important in AGRP(87-132) binding for hMC4R.

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Legends for figures

Figure 1: Representation of the human melanocortin 3 receptor. The amino-acid sequences of the EL1, EL2 and EL3 of hMC3R and hMC1R (above) are shown for comparison.

Figure 2: cAMP accumulation assays, Intracellular cAMP production in HEK293 cells transiently expressing hMC3R-EGFP and different chimeric receptors (EL1, EL2 and EL3) in response to increasing concentrations of NDP-MSH. Each point represents the mean ± SD of one experiment performed in triplicate. The experiment was repeated at least three times. Basal level of cAMP production in the absence of agonist stimulation was between 2.85 and 5 pmoles/10^6 cells depending on the experiments.

Figure 3: Effect of AGRP on chimeric receptors, Intracellular cAMP production in HEK293 cells transiently expressing hMC3R-EGFP and different chimeric receptors in response to 0.5 nM ACTH or NDP-MSH concentrations in presence or absence of 50 nM AGRP. Each point represents the mean ± SD of one experiment performed in triplicate. The experiment was repeated at least three times.

Figure 4: AGRP inhibition of NDP-MSH stimulated cAMP production, in HEK293 cells transiently expressing hMC3R-EGFP and chimeric MC3-EL2MC1 receptors in the presence or absence of 50nM AGRP. Each point represents the mean ± SD of one experiment performed in triplicate. The experiment was performed two times.

Figure 5: ^{125}I-NDP-MSH binding assays, Inhibition of binding of ^{125}I-NDP-MSH to HEK293 cells transiently expressing hMC3R-EGFP and different chimeric receptors by increasing concentrations of unlabelled NDP-MSH. Each point represents the mean ± SD of one experiment performed in triplicate. The experiment was performed two times.

Figure 6: Effect of AGRP on ^{125}I-NDP-MSH binding, Inhibition of binding of ^{125}I-NDP-MSH to HEK293 cells transiently expressing hMC3R-EGFP or MC3-EL2MC1R by 10 nM of unlabelled AGRP. Each point represents the mean ± SD of one experiment performed in triplicate. The experiment was repeated three times.

Figure 7: Confocal microscopy observation, Fluorescent confocal microscope images of HEK293 cells transiently expressing hMC3R-EGFP or different chimeric receptor fused to EGFP, after stimulation with UV (488nm).

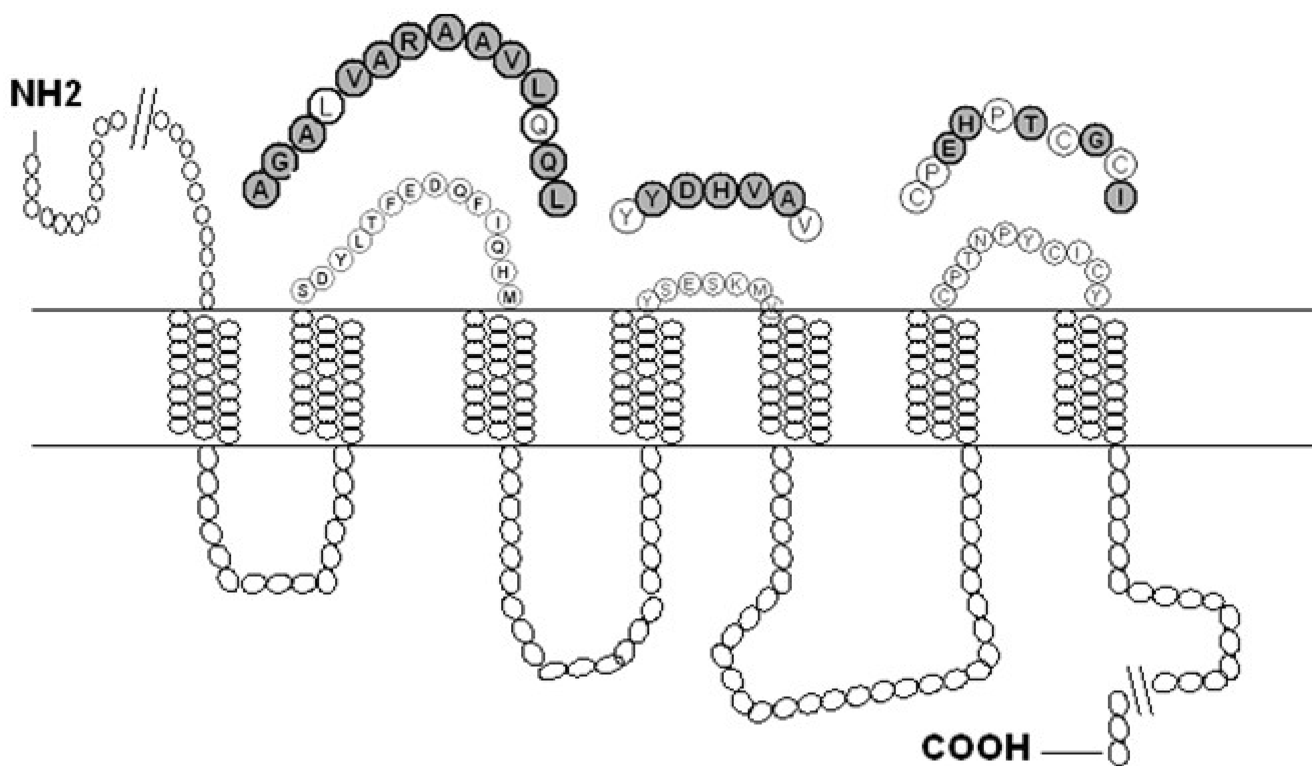

Figure 1

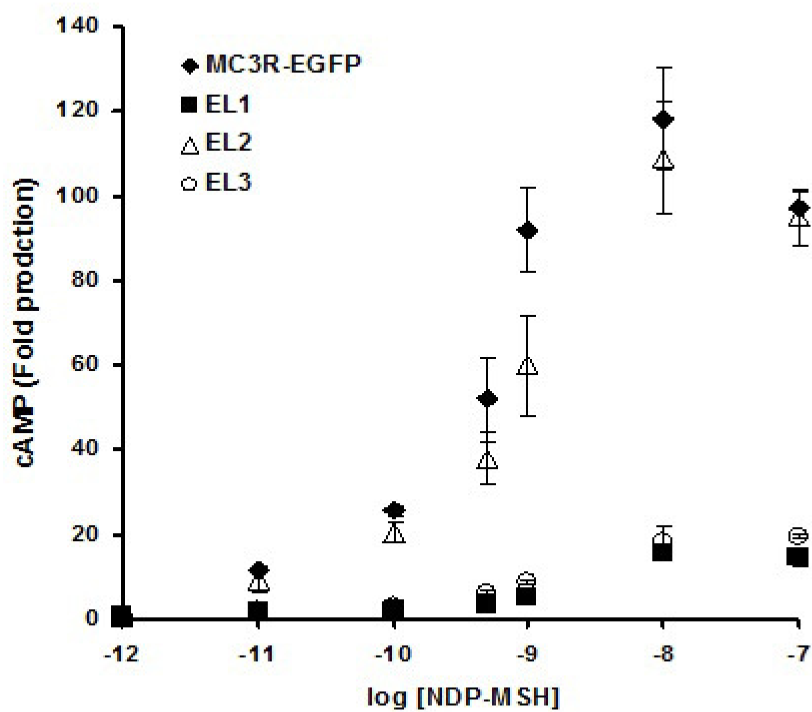

Figure 2

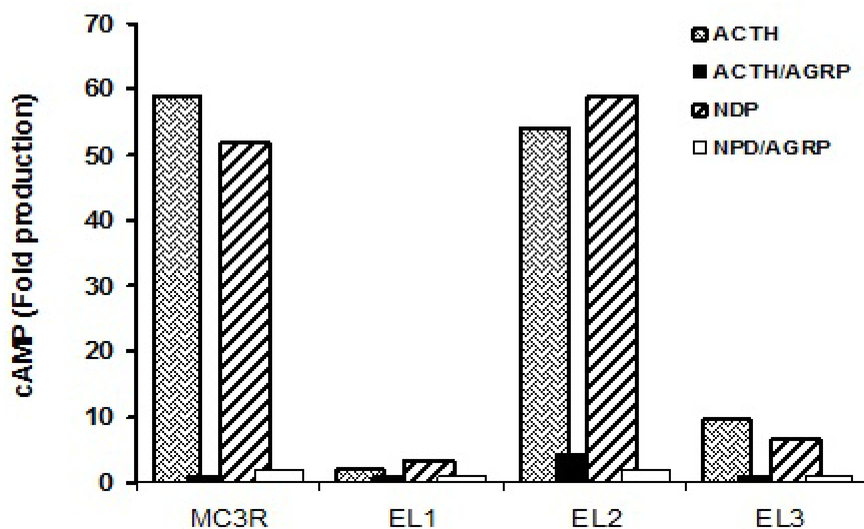

Figure 3

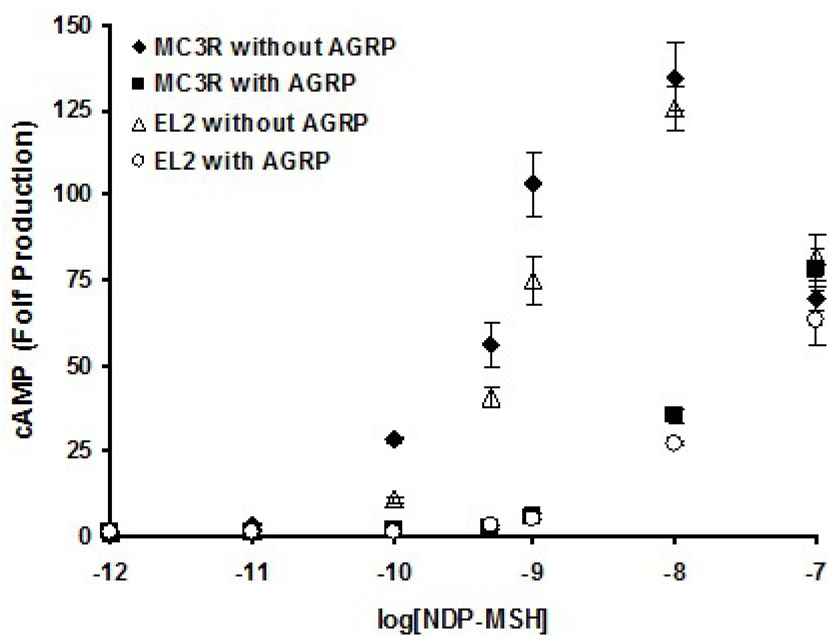

Figure 4

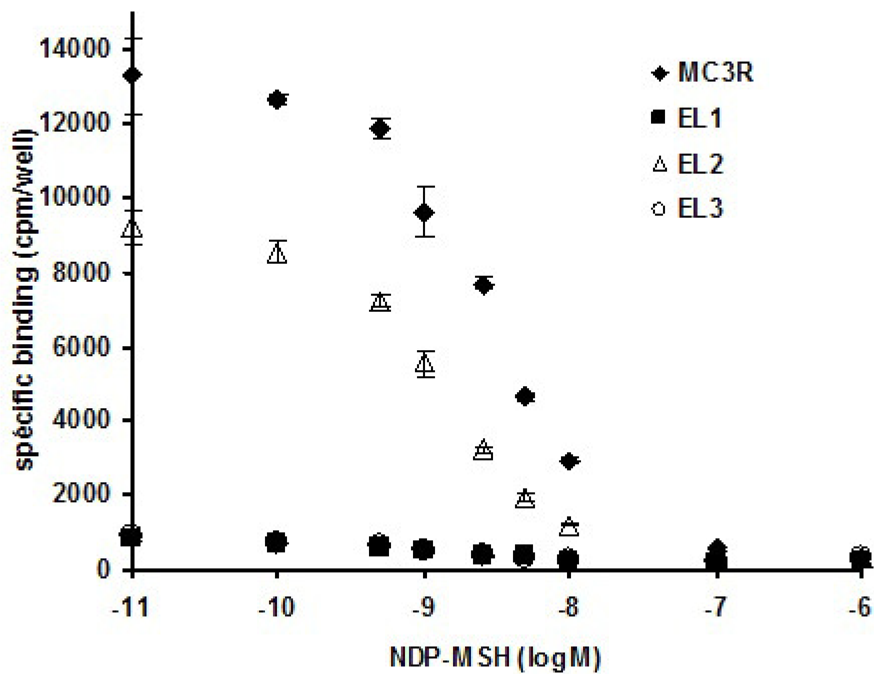

Figure 5

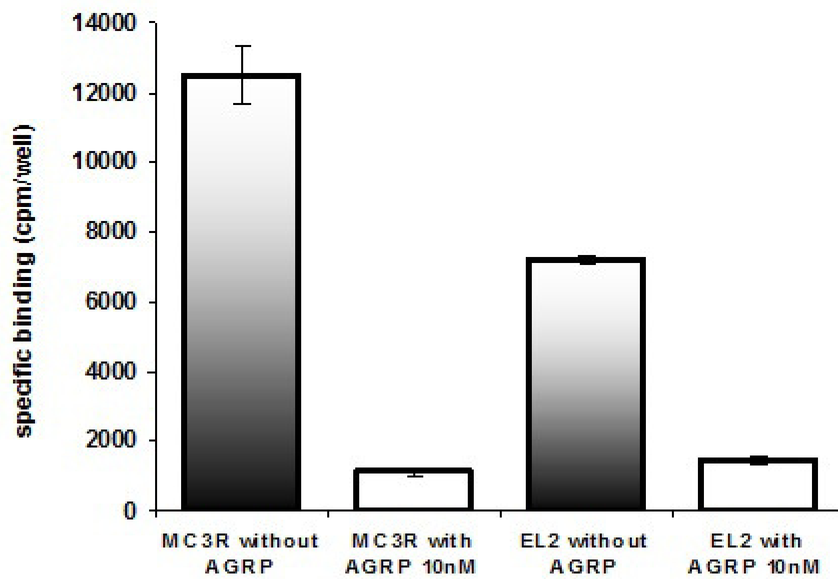

Figure 6

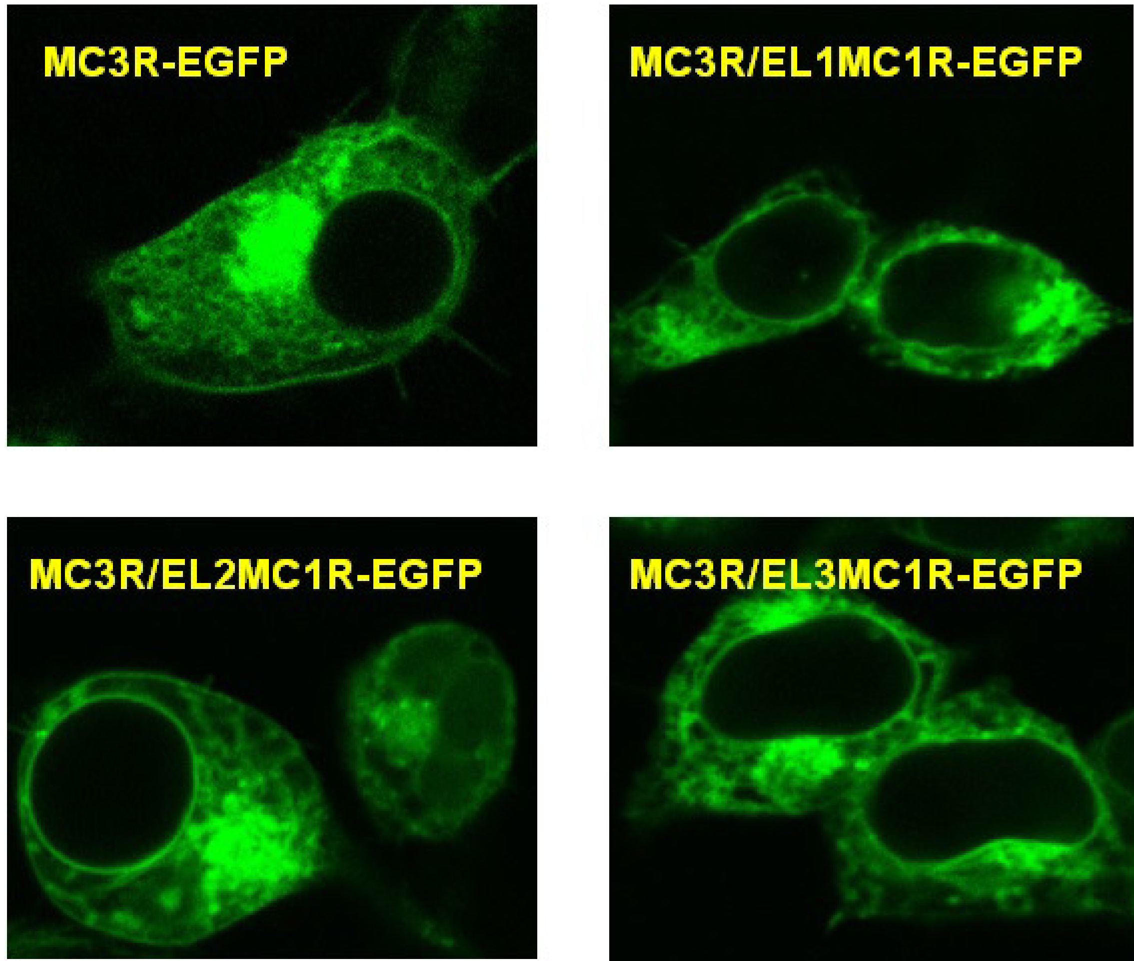

Figure 7