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Authors: Cathrine Laustrup Møller, Kirsten Raun, Marianne Lambert Jacobsen, Thomas Åskov Pedersen, Birgitte Holst, Kilian W. Conde-Frieboes, Birgitte Schjellerup Wulff

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Cathrine Laustrup Møller[1,2], Kirsten Raun[1], Marianne Lambert Jacobsen[1], Thomas Åskov Pedersen[1], Birgitte Holst[2], Kilian W. Conde-Frieboes[3] and Birgitte Schjellerup Wulff[1].
[1]Diabetes Biology and Pharmacology, Novo Nordisk A/S (Novo Nordisk Park, 2760 Maaloev, Denmark), [2]University of Copenhagen, Faculty of Health Science, Department of Neuroscience and Pharmacology (Blegdamsvej 3B, 2200 Copenhagen N) [3]Diabetes Protein Engineering, Novo Nordisk A/S (Novo Nordisk Park, 2760 Maaloev, Denmark),

Email:
Cathrine Laustrup Møller: ACML@novonordisk.com (corresponding author)
Kirsten Raun: KIRA@novonordisk.com
Marianne Lambert Jacobsen: MLJA@novonordisk.com
Thomas Åskov Pedersen: TQPE@novonordisk.com
Birgitte Holst: HOLST@sund.ku.dk
Kilian W. Conde-Frieboes: KCF@novonordisk.com
Birgitte Schjellerup Wulff: BSW@novonordisk.com

Telephone: +45 44448888 (Novo Nordisk Park, 2760 Maaloev, Denmark)
Fax: +45 4466 3939 (Novo Nordisk Park, 2760 Maaloev,Denmark)

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[1]Diabetes Biology and Pharmacology, Novo Nordisk A/S (Novo Nordisk Park, 2760 Maaloev, Denmark), [2]University of Copenhagen, Faculty of Health Science, Department of Neuroscience and Pharmacology (Blegdamsvej 3B, 2200 Copenhagen N) [3]Diabetes Protein Engineering, Novo Nordisk A/S (Novo Nordisk Park, 2760 Maaloev, Denmark),

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The melanocortin receptors (MCRs) belong to the G-protein coupled receptors (family A). So far, 5 different subtypes have been described (MC1R- MC5R) and of these MC2R and MC5R have been proposed to act directly in adipocytes and regulate lipolysis in rodents. Using ACTH and α-melanocyte stimulating hormone (α-MSH) generated from proopiomelanocortin (POMC), as well as synthetic MSH-analogues to stimulate lipolysis in murine 3T3-L1 adipocytes it is shown that MC2R and MC5R are lipolytic mediators in differentiated 3T3-L1 adipocytes. Involvement of cAMP, phosphorylated extracellular signal-regulated kinase (ERK) 1/2, protein kinase B (PKB), adenosine 5' monophosphate-activated protein kinase (AMPK) and Jun-amino-terminal kinase (JNK) in MCR mediated lipolysis were studied. Interestingly, results obtained in 3T3-L1 cells suggest that lipolysis stimulated by α-MSH, NDP-α-MSH, MT-II, SHU9119 and PG-901 is mediated through MC5R in a cAMP independent manner. Finally, we identify essential differences in MCR mediated lipolysis when using 3T3-L1 cells compared to primary adipocytes.

INTRODUCTION

The melanocortin system acts through multiple pathways relevant for the prevention of obesity and obesity-related complications (Cone 1999; Marks *et al.* 2002; Spiegelman & Flier 2001). The system is acknowledged to have an important function in regulation of satiety and energy expenditure (Yaswen *et al.* 1999; Spiegelman & Flier 2001; Cheung *et al.* 1997; Azzara *et al.* 2002). MC4R knockout mice exhibit a well-described phenotype defined by increased lean body mass and fat mass, hyperphagia and disturbances in the metabolic response to overnutrition (Mountjoy *et al.* 1994; Huszar *et al.* 1997; Tschop & Heiman 2001). Furthermore, loss of MC4R function is the most frequent monogenetic alteration in severely obese humans (Krude *et al.* 1998) and in severe, early onset childhood obesity the frequency of mutations in the MC4R locus is 4-6% (Farooqi *et al.* 2003). Besides the effect on satiety and energy expenditure, the melanocortin system is believed to influence insulin release and insulin sensitivity (Fan *et al.* 2000; Huo *et al.* 2009). The melanocortin peptides and their receptors are also suspected to have a direct lipolytic effect on adipose tissues in rodents (Cho *et al.* 2005; Boston 1999; Spirovski *et al.* 1975). However this effect is controversial in humans (Hoch *et al.* 2007). The effect of melanocortins on adipocytes have by some researchers been explained by neuronal regulation of lipolysis (Brito *et al.* 2007), since MC4R mRNA has been identified in sympathetic neurons connected to white adipose tissue (WAT), indicating that central MC4R might stimulate lipid mobilization in the periphery (Song *et al.* 2005). However, studies in differentiated murine 3T3-L1 adipocytes suggest a direct lipolytic effect stimulated by melanocortin peptides ACTH and α-MSH (Bradley *et al.* 2005; Cho *et al.* 2005),

which supports the earlier identification of MC2R and MC5R mRNA in this cell line (Boston & Cone 1996).

MC1R action is primarily connected to melanocytes (Wikberg *et al.* 2000) but it is also expressed in human subcutaneous fat where the receptor has been found to elicit cell proliferation (Hoch *et al.* 2007). MC2R is primarily expressed within the adrenal cortex, but has also been identified in mouse white adipose tissue (WAT) (Boston 1999) and is found to induce lipolysis in WAT from both mouse and rat (Bradley *et al.* 2005; Boston 1999). Indeed, the MC2R was suggested to stimulate lipolysis through activation of hormone sensitive lipase in 3T3-L1 adipocytes (Cho *et al.* 2005). The expression of MC2R in human adipose tissue remains controversial (Abdel-Malek *et al.* 2000; Wikberg *et al.* 2000). The receptor has by some researchers been identified in human subcutaneous WAT (Smith *et al.* 2003), although the lipolytic function remains unclear. MC3R is located in the brain where it is expressed in the hypothalamus (Marks *et al.* 2006) especially in the arcuate nucleus (Roselli-Rehfuss *et al.* 1993; Mountjoy *et al.* 1994; Mountjoy & Wild 1998). MC3R knockout mice display reduced lean body mass, an increased fat mass and are slightly hypophagic compared to wild type controls (Marks *et al.* 2006), but the receptor has not been ascribed lipolytic importance. MC4R is widely located throughout the CNS and high levels have been found especially in hypothalamic regions involved in regulation of feeding behaviour and energy expenditure, such as the paraventricular nucleus of the hypothalamus (Mountjoy *et al.* 1994; Mountjoy & Wild 1998). MC4R mRNA has also been identified in human adipose tissue, where it might contribute to the control of energy balance and body weight (Chagnon *et al.* 1997) although this involvement remains controversial. The function of MC5R is poorly understood despite its extensive distribution in the periphery (Bednarek *et al.* 2007). MC5R has been identified in exocrine gland tissue and are involved in glandular secretion (van der Kraan *et al.* 1998). Besides the expression in 3T3-L1 cells, MC5R has also been identified in human adipose tissue and associated with obesity phenotypes (Chagnon *et al.* 1997).

The G_s transduction pathway is established to be a validated path of signalling for all MCRs (Barrett *et al.* 1994; Mountjoy *et al.* 1992) all though other signalling pathways have been suggested (Buch *et al.* 2009; Konda *et al.* 1994; Daniels *et al.* 2003; Rodrigues *et al.* 2009). The natural occurring melanocortin peptide family consists of α-, β- and γ-melanocyte stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH). These peptides are generated from a common precursor proopiomelanocortin (POMC) and function as MCR agonists. It has previously been shown, that MC2R binds ACTH and none of the other POMC derived peptides (Baumann *et al.* 1986; Boston & Cone 1996). When a MCR is stimulated, it couples to $G\alpha_s$, which activates intracellular adenylyl cyclase and increases cAMP production. α-MSH stimulated phosphorylation of extracellular signal-regulated kinase (ERK) has previously been linked to lipolysis in 3T3-L1 cells and MC5R has been established to activate phosphatidylinositol 3-kinase dependent ERK after stimulation by α-MSH (Rodrigues *et al.* 2009). In addition, cAMP dependent AMPK has been linked to MC5R mediated fatty acid oxidation stimulated by α-MSH in skeletal muscle (An *et al.* 2007). The essential function of central MC4R in regulation of satiety and energy expenditure raises questions of other potential metabolic effects of the melanocortin system. So far, the attention of this system in metabolic research has been focused on the CNS. A systemic abundance of other melanocortin peptides than ACTH has been suggested by several researchers (Yaswen *et al.* 1999; Shishioh-Ikejima *et al.* 2010; Zemel & Shi 2000). Furthermore, peripheral effects of melancortin receptor antagonist agouti-related protein (AgRP) supports the assumption that a systemic melanocortin system indeed exists (Doghman *et al.* 2007). However, it is not fully known where and how the melanocortin peptides act peripherally. Identification of pharmacological selective ligands with physiological relevance in the periphery is an essential aspect not yet clarified. MC5R has been shown to be expressed in various peripheral tissues relevant for lipid and glucose metabolism (An *et al.* 2007; Boston 1999), for which reason identification of peripheral MC5R selective agonists are highly relevant. Different non-selective and selective MCR agonists and antagonists have been described in various studies, addressing the role of MCR receptor

pharmacology. However, one single focussed study which correlates binding affinities of these known MCR ligands on mouse receptors with their ability to induce lipolysis in adipocytes has not previously been shown. In this study, mouse MCR binding affinities of known human agonists are linked to MCR mediation of lipolysis in murine 3T3-L1 cells and the signalling pathways involved. We find that lipolysis in 3T3-L1 cells stimulated by α-MSH, NDP-α-MSH, MT-II, SHU9119 and PG-901 are mediated through MC5R independent of cAMP signalling. Finally, we identify essential differences in MCR mediated lipolysis between 3T3-L1 cells and primary adipocytes from isolated mouse epididymal adipose tissue.

MATERIALS AND METHODS

Test Compounds

NDP-α-MSH (BACHEM), ACTH (BACHEM), α-MSH (BACHEM), melanotan (MT-II), SHU9119, PG-901, LY2112688 (prepared in-house using standard peptide synthesis protocol) and isoproterenol (Sigma).

Materials and Cell Lines

Trizol (Invitrogen) and 1-bromo-3-chloro-propane (Sigma) were employed in the extraction of RNA from adipose tissue. RNeasy mini-kit (Qiagen) and RNase-free DNase set (Qiagen) were used in RNA extraction from cells and tissue. A RNA ladder was used to visualize intact RNA (Invitrogen). iScript^{TM} cDNA synthesis kit (Biorad) was employed when synthesizing cDNA. Platinum qPCR supermix (Invitrogen), Primers (scanprimer/oligonucleotides; DNA-Technology) and LNA probes (Exiqon ProbeLibrary) were used in real time PCR. B16-F12 mouse melanoma cells (ECACC) expressing MC1R, mouse MC3R cDNA transfected CHO-K1 cells (Euroscreen), mouse MC4R cDNA transfected BHK570 cells (in house produced) and mouse MC5R cDNA transfected CHO-K1 cells (PerkinElmer Products) were employed in binding studies. I^{125}-NDP-α-MSH (PerkinElmer) was used as radioligand in binding assays. 97% Ovalbumin (Sigma), Tween-20 (Merck-Schuchardt), HEPES (Sigma) and hydroxypropyl-β-cyclodextrin (HP-β-CD) (Acros Organics) were used in all binding buffers and Bacitracin (Sigma) was used in MC4R binding buffer. Confluent 3T3-L1 cells were harvested using versene (Lonza) and inducers of adipogenesis in 3T3-L1 cells were Troglitazone (Sigma), IBMX (Sigma), Dexamethazone (Sigma) and human insulin (Novo Nordisk). Cells were stained using Oil Red O (Sigma). NEFA-HR (2) kit from Wako was employed when measuring release of non-esterified fatty acid (NEFA) as an index of lipolysis. Collagenase Type II (Sigma C-6885) was used in degradation of epididymal WAT. Non-metabolizable adenosine phenylisopropyl adenosine (PIA) (Sigma) and adenosine deaminase (ADA) (Roche) was used when measuring NEFA from isolated adipocytes. FlashPlate® cAMP assay (Life Science Products) was employed to determine cyclase activity in stimulated 3T3-L1 cells. Invitrogen cell extraction buffer, protease inhibitor (Sigma) and 4-(2-Aminoethyl) benzenesulfonylfluoride (AEBSF) (Calbiochem) was used in protein purification. In immunoblotting Bis Tris gels (4-12%, Invitrogen), NuPAGE MOPS SDS Running buffer (Thermo Scientific), antioxidant (Thermo Scientific), full range rainbow marker (Amersham), iBLOT gel transfer stacks (Invitrogen), Starting Block T20 buffer (Thermo Scientific), TBS-T (Ambresco) and Amersham Detection Reagents were used. Primary antibodies for ERK, PKB, AMPK and JNK were purchased from Cell signalling. Secondary antibodies from Thermo Scientific and MEK inhibitor (U0126) from Calbiochem were used.

Cell Culture

B16-F12 melanoma cells expressing MC1R was cultured in DMEM with glutamax and 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2. Mouse MC3R and MC5R cell lines were cultured in Ham's F12 medium 10% FCS, 1% pen/strep and 0.4 mg/ml G418 at 37 ºC and 5% CO_2. Mouse MC4R cell line was cultured in DMEM, 10% FCS, 1 mg/ml G418 and 1% pen/strep at 37 ºC and 5% CO_2.

Culturing and differentiation of 3T3-L1 Cells

3T3-L1 pre-adipocytes were grown to 100% confluence in DMEM (2% FBS and pen/strep). Confluent 3T3-L1 cells were harvested and dispersed in 96 well collagen coated plates (35000 cells/well; 100 µl/well). The next day (day 0) induction of adipogenesis was initiated by giving DMEM (FBS + pen/strep) supplemented with 1µM troglitazone, 0.5 mM IBMX, 0.25 µM dexamethazone and 100 nM human insulin. At day 2 and 4, DMEM (FBS + pen/strep) in addition of insulin was given. At day 6, DMEM (FBS + pen/strep) was given alone. At day 8, the cells were washed and DMEM (+ 2% fatty acid free bovine serum albumin) was added before over night incubation. At day 9, the cells were fully differentiated and ready for experiments.

RNA Extraction and cDNA Synthesis

RNA was extracted from non-differentiated 3T3-L1 cells (day 0), differentiated 3T3-L1 cells (day 9) and epididymal adipose tissue dissected from lean C57bl/J6 mice (average 21.8 g, 8 weeks). After euthanasia of the animals adipose tissue were dissected, frozen (-80°C) and stored until RNA extraction. Mouse adipose tissue was homogenised for 20-30 seconds using 1 ml of Trizol/100 mg of tissue. 200 µl of 1-bromo-3-chloro-propane was added to 1 ml of homogenate. The concentration and purity of the collected eluate was analysed using a NanoDrop. iScript™ cDNA synthesis reactions were incubated 5 minutes at 25 ºC, 30 minutes at 42 ºC and 5 minutes at 85 ºC .

Real Time PCR

Primers for MC1-5Rs and the reference genes cyclophilin B (CYCB) and hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1) were designed in mouse.
*MC1R (*NM_008559): 5'-AGAAGCTGGGGGCTGATAC-3';
3'-AGGAAGTAGGAAGCGGTCTGT-5'.
*MC2R (*NM_008560.2): 5'-TGGAAAAGTTCTCAGCACCAC-3;
3'-TCTTTGTGTGGAAGGAT CTGG-5'.
*MC3R (*NM_008561.3): 5'- GCTCAGACGGGACGTAAA AG-3;
3'-CTTACAGACGAAGCCAAGCAC-5'.
*MC4R (*NM_016977): 5'-GCTGCAGGAAGATGAACTCC-3;
3'-TC CAGAGGTGGAGGGAAGTAT-5'.
*MC5R (*NM_013596.2): 5'-CGGAGCAGAGCAGAATGG-3;
3'-AGGGTGGAGGAGG AGTTCA-5'.
*HPRT1 (*NM_013556.2)*:* 5'-TCCTCCTCAGACCGCTTTT-3;
3'-CCTGGTTCATCATCGTAATC-5'.
*CYCB (*NM_011149)*:* 5'-CACCAATGGCTCAGTTCTT-3;
3'-CTCCACCTTCCGTACCACAT-5'. Standard curves for each primer set were initially run. Each reaction was prepared by mixing 10 µl Platinum qPCR supermix, 0.4 µl of forward- and reverse primer, 0.08 µl probe and 4.12 µl nuclease free H_2O (total volume of 15 µl). Synthesized cDNA was diluted 1:10, after which 5 µl of cDNA and 15 µl of PCR mastermix were added to a 96 well plate and centrifuged (30 seconds/300 rpm). Samples were run in triplicates.

Binding Studies

Test compounds were dissolved to a 4 mM concentration in dimethyl sulfoxide (DMSO) and diluted from 100 µM to 1 pM in the assay in a receptor specific binding buffer. *MC1R buffer:* 25 mM HEPES (pH = 7.4), 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM EDTA, 0.1% ovalbumin, 0.005% Tween-20 and 5% HP-β-CD. *MC3R and MC5R buffer:* 25 mM HEPES (pH 7.4), 1 mM $CaCl_2$, 5 mM $MgSO_4$, 0.1% ovalbumin, 0.005% Tween-20 and 5% HP-β-CD. *MC4R buffer:* 25 mM HEPES (pH = 7.0), 1 mM $CaCl_2$,1 mM $MgSO_4$, 1 mM EGTA, 0.1% ovalbumin, 0.02%, Bacitracin and 0.005% Tween-20. The binding assay was performed in duplicates in a total volume of 100 µl directly on cells in suspension (50 µl added, 50.000 cells/well). A fixed concentration of ^{125}I-NDP-

α-MSH (250 pM in a volume of 25 µl) and varying concentrations of non-labelled competing test compound (in a volume of 25 µl) were added to the cells, incubated and gently shaken for 1 hour at room temperature. The incubation was stopped by filtration through 0.5% polyethylenimine (PEI)-treated filter plates. Filters were washed with cold 0.9% NaCl and air-dried at 50°C for 1-2 hours. 50 µl scintillation liquid was added to each well of dried filter plates and counted.

Isolation of Mouse Epididymal Adipocytes

Epididymal adipose tissue was dissected from C57bl/J6 mice (average 20 g, 8 weeks). After euthanasia (CO_2 and cervical dislocation) the adipose tissue was dissected and transferred to vials (in 1.5 ml PBS). The tissue was cut in to homogenous pieces in 2 ml Krebs buffer (25 mM HEPES, 4% human serum albumine (HSA), 1 mM glucose, 2 mg/ml collagenase) and shaken for 1 hour at 36.5°C in a shaking water bath (190 rpm). The suspension was filtrated, washed twice in 20 ml HEPES Krebs Ringer buffer (1% HSA) after which the fat percentage was measured. The adipocytes were resuspended to a concentration of 2% in washing buffer + 5 mM glucose, 100 nM PIA, 0.25 µl/ml ADA (stock 10 mg/ml) and dispersed in 96 w plates.

NEFA Assay in 3T3-L1 Cells and in Isolated Epididymal Adipocytes

Induction of NEFA release from 3T3-L1 adipocytes and primary cells was measured as an index of MCR agonist induced lipolysis and β-adrenergic agonist isoproterenol was used as a positive control. Previous lipolysis studies have shown a similar dose response curve between glycerol and NEFA when using positive control isoproterenol, for which reason only one measure was chosen (data not shown). The release of NEFA proved to be a reproducible measure of lipolysis, though potential re-esterification of NEFAs would underestimate the degree of MCR mediated lipolysis. Indeed, a potent release of NEFA was stimulated by positive control isoproterenol in both 3T3-L1 cells and mouse primary adipocytes. All test compounds were dissolved to 4 mM in DMSO and diluted to between 10 µM and 1 pM in HBSS buffer (2% bovine serum albumine (BSA). The use of ADA and PIA are in some assays recommended when comparing a basal level of lipolysis with a stimulated state. In 3T3-L1 studies the basal state of the cells was not used as reference. Moreover, NEFA top and bottom values in stimulated 3T3-L1 cells were identical between assays which supported the assumption that adenosine had an equal effect and did not interfere with the results obtained. ADA and PIA were employed when measuring release of NEFA in isolated epididymal adipocytes since some variation was found in basal NEFA levels between experiments. The NEFA level reached a dose response maximum between 2-3 hours, for which reason 3 hours were chosen as the optimal time span (data not shown). The release of NEFA was also measured after stimulation with 10 nM test compound in the presence or absence of a MEK inhibitor (10 µM). The MEK inhibitor was preincubated for 30 minutes after which test compounds were added to the cells. Plates were incubated for 30 or 60 minutes before release of NEFA was measured.

cAMP measurements

cAMP was measured using the FlashPlate™ method. 50 µl 3T3-L1 adipocytes ($2x10^6$ cells/ml) in suspension were dispersed and 50 µl stimulation buffer was added per well. 50 µl test compound was added in each well, incubated and gently shaken for 30 min at room temperature after which 100 µl detection mix was added in all wells (see description in FlashPlate protocol). Test plates were gently shaken for 30 minutes and transferred to an anti-cAMP coated 96 well plates, plates were shaken 30 minutes at room temperature, incubated 1.5 hour at room temperature and counted according to the FlashPlate protocol.

Protein Purification and Western Blotting

3T3-L1 cells were cultured as described and stimulated with ACTH, α-MSH, MT-II, SHU9119, PG-901, LY2112688 (300 nM) and NDP-α-MSH (3 nM and 300 nM) in 12 well plates. After stimulation the cells were washed in ice cold PBS and placed on dry ice. Protein was extracted

using 120 µl Invitrogen cell extraction buffer + 6 µl protease inhibitor per well, incubated (30 minutes on ice) and transferred to tubes. Tubes were centrifuged for 10 minutes (10.000g, 4°C) and the supernatant was used for determination of protein content using spectrophotometry (562 nm). 25 µg protein (in a total of 20 µl) and 6 µl marker was loaded and run (30-45 minutes at 150 V and 30-45 minutes at 180 V). Gels were transferred to membranes, washed, blocked (1 hour) and incubated over night (4°C) with primary antibody. Membranes were washed (3 x 10 minutes) and secondary antibody was added to the membranes (1 hour at room temperature). Amersham Detection Reagents were used when developing blots.

Data Analysis and Statistics

Data obtained from the binding assays were analysed using Graphpad Prism. The ΔΔCt Method was used to compare mRNA expression between a gene of interest and a calibrator. Normalisation of Ct-values was done using an average of reference gene Ct-values (CYCB and HPRT1). Two-way ANOVA was used to calculate statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, no * indicates insignificance).

RESULTS

MC2R and MC5R are induced during adipogenesis

The scope of this study was to create an overview of the binding of existing MCR agonists and antagonists to mouse MCRs and to correlate binding affinities with their ability to induce lipolysis in adipocytes. Initially, the mRNA expression of MC1-5R was compared in non-differentiated 3T3-L1 cells, differentiated 3T3-L1 cells and mouse epididymal WAT (figure 1) by use of real time PCR. MC2R was induced 300 fold in differentiated 3T3-L1 adipocytes compared to non-differentiated cells. MC2R was expressed 400 fold higher in mouse epididymal WAT compared to non-differentiated 3T3-L1 cells. MC5R was expressed by a 1500 fold increase in differentiated 3T3-L1 cells compared to non-differentiated cells. However, the receptor was virtually non detectable in the non-differentiated state. In mouse epididymal WAT MC5R was expressed by a 65 fold increase compared to non-differentiated 3T3-L1 cells, and thus at considerably lower levels than in the differentiated 3T3-L1 cells. MC1R, MC3R and MC4R mRNA seem to be more abundantly expressed in the non-differentiated state compared to differentiated 3T3-L1 cells and mouse adipose tissue.

Binding Studies

MC2R is acknowledged only to be activated by ACTH (Baumann *et al.* 1986), for which reason binding studies were not performed on this receptor. In order to assess MCR agonist specificity, competition binding curves of ACTH, α-MSH, NDP-α-MSH, MT-II, PG-901, SHU9119 and LY2112688 were obtained using cells over-expressing murine MC1R (mouse melanoma cells), murine MC3R and MC5R (recombinant CHO-K1 cells) and murine MC4R (recombinant BHK570 cells) (table 1). Results obtained in binding studies supported present literature at human MCRs stating that NDP-α-MSH, α-MSH and MT-II are non-selective agonists that bind to all MCRs, although α-MSH had a relatively lower affinity for mouse MC1R compared to human (mouse IC_{50} = 316 ± 93 nM vs. human IC_{50} = 5.97 nM) (Haskell-Luevano *et al.* 1997). Furthermore, we found that neither MC1R, MC3R nor MC5R had a high affinity for LY2112688, whereas MC4R had a high affinity for LY2112688 supporting results found in human (Greenfield *et al.* 2009). PG-901 is in the literature described as a human MC5R agonist, which also functions as an antagonist at human MC3R and MC4R (Grieco *et al.* 2002), which correlates to results obtained on mouse MCRs. Mouse MC5R showed a high affinity for PG-901 (IC_{50} = 67 ± 15 nM) but also for ACTH (IC_{50} = 21 ± 9 nM). SHU9119 is from the literature known to be an antagonist on human MC3R and MC4R and an agonist on human MC1R and MC5R (Hruby *et al.* 1995). Results obtained using SHU9119 on mouse MCRs confirmed human data, though we found a relatively lower affinity for

mouse MC1R and mouse MC5R (table 1) compared to human MC1R (K_i = 0.714 ± 0.161 nM) and human MC5R (K_i = 1.12 ± 0.31) (Hruby *et al.* 1995).

MC2R and MC5R are likely mediators of NEFA release

In order to establish the lipolytic potential of the various MCR ligands, release of NEFA from 3T3-L1 cells differentiated into adipocytes, was measured after treatment with MCR agonists. The β-adrenergic agonist isoproterenol was employed as a positive control. NDP-α-MSH, α-MSH, ACTH and PG-901 were established as full agonists reaching an E_{max} comparable to isoproterenol (E_{max} = 600 μM) (figure 2.A and 2.B). Moreover, MT-II and SHU9119 were both established as partial agonists (E_{max} = 400 μM) (figure 2.A and 2.B). PG-901 and SHU9119 potently induced NEFA release (EC_{50} = 0.58 ± 0.01 nM and EC_{50} = 0.27 ± 0.05 nM) (table 2), excluding MC3R and MC4R as possible candidates for stimulation of lipolysis, as both compounds have an antagonistic effect on MC3R and MC4R. Furthermore, as described in the previous paragraph PG-901 is a selective MC5R agonist indicating that MC5R is involved in lipolysis in 3T3-L1 cells. LY2112688 produced a NEFA release with an E_{max} under 400 μM, and had a lower potency compared to the other compounds employed (EC_{50} = 5270 ± 1073 nM). Because of the weak potency of LY2112688, a well-defined E_{max} was not established. These results add more credit to the theory that MC4R is not involved in the lipolysis in 3T3-L1 cells. ACTH stimulates a potent release of NEFA (EC_{50} = 0.74 ± 0.08 nM). Our binding studies showed that ACTH has a relative high binding affinity to all MCR receptors except MC1R (table 1). This supports that MC5R or MC2R rather than MC1R are likely mediator of lipolysis in 3T3-L1 cells. Since MC2R only binds ACTH and not α-MSH or α-MSH analogues (Schioth *et al.* 1997), additional receptors besides MC2R must be involved in the stimulation of NEFA release from 3T3-L1 adipocytes. In summary, based on binding studies using cells overexpressing MC1R, MC3R, MC4R and MC5R and NEFA release measured in differentiated 3T3-L1 adipocytes, MC2R and MC5R are the most likely mediators of lipolysis.

cAMP independent signalling downstream of MCR stimulation in 3T3-L1 adipocytes

We used the previously described MCR agonists to induce intracellular cAMP, in order to verify this signal transduction pathway as a key mediator of MCR signalling in stimulation of lipolysis. To our surprise this was not the case. As in lipolysis assays we used the β-adrenergic agonist isoproterenol as a positive control, which induced cAMP generation with an E_{max} = 14 nM and EC_{50} = 36 ± 6 nM. ACTH induced cAMP with an E_{max} = 11 nM and EC_{50} = 27 ± 4 nM, whereas α-MSH only induced a weak response in a subset of experiments (figure 3). In contrast NDP-α-MSH, PG-901, MT-II, SHU9119 and LY2112688 stimulation did not induce cAMP in differentiated 3T3-L1 cells. Surprisingly, this result suggests that lipolysis stimulated by the α-MSH analogues is cAMP independent. In order to dissect the downstream signalling pathways involved in MCR mediated NEFA release, we assessed levels of ERK 1/2 phosphorylation in stimulated 3T3-L1 adipocytes using western blotting. ERK 1/2 phosphorylation was induced after stimulation of NDP-α-MSH, α-MSH, PG-901 and LY2112688 compared to the basal levels (figure 4). SHU9119 and MT-II were not employed, since these compounds did not add any further information compared to the selected analogues. The phosphorylation of ERK 1/2 was highest at t = 5 minutes for all test compounds, after which the phosphorylation decreased (t = 15 minutes). The phosphorylation of pERK 1/2 is increased dose dependently in differentiated 3T3-L1 cells with NDP-α-MSH, α-MSH and PG-901 as potent inducers where high phosphorylation is reached at a low concentration (app. 10 nM) (figure 4.B). LY2112688 induces ERK 1/2 phosphorylation at a relatively higher doses (maximum density is reached at 1 μM) compared to the other compounds (figure 4.B). The melanocortin mediated stimulation of pERK was inhibited by the preincubation of a MEK inhibitor (U0126) see figure 4.C. However, when adding the MEK inhibitor to the 3T3-L1 cells in the lipolysis assay, NEFA release was not decreased suggesting that ERK phosphorylation was not necessary for MCR mediated lipolysis (figure 5). We also examined levels of phosphorylated PKB, AMPK and JNK

after stimulation with MCR agonists however no increase in phosphorylation was detected compared to the basal levels (data not shown).

NEFA Release in Adipocytes Isolated from Mouse Epididymal Adipose Tissue

To further establish MCR subtypes mediating lipolysis in mouse adipose tissue, release of NEFA was measured from primary mouse adipocytes isolated from epididymal fat pads, after stimulation with test compounds and isoproterenol (figure 6). ACTH was established as a full agonists reaching an E_{max} comparable to isoproterenol (E_{max} = 700 µM). However, ACTH stimulated release of NEFA from primary mouse adipocytes was very potent compared to isoproterenol (isoproterenol EC_{50} = 24 ± 17 nM and ACTH EC_{50} = 0.007 ± 0.001 nM) (figure 6). α-MSH induced a NEFA release with an E_{max} of 600 µM and EC_{50} = 0.63 ± 0.10 nM, whereas incubation with NDP-α-MSH, PG-901, MT-II, SHU9119 and LY2112688 did not result in NEFA release from isolated adipocytes. This suggests essential differences between primary murine adipocytes and differentiated 3T3-L1 adipocytes. In order to disclose whether MC5R selective compound PG-901 and non-selective NDP-α-MSH bind to MCRs on adipocytes isolated from epididymal WAT, an inhibition study was performed (figure 7). The release of NEFA stimulated by α-MSH was decreased with increasing dose of NDP-α-MSH and PG-901 (IC_{50} = 0.23 ± 0.03 nM and IC_{50} = 7.32 ± 2.28 nM) indicating that these peptides in fact bound to MC5R but antagonized the effect of α-MSH on lipolysis in primary adipocytes.

DISCUSSION

Melanocortin receptors are important regulators of energy metabolism and mediate signals of feeding behaviour within the CNS. However, the physiological relevance of melanocortin receptors in the periphery is another aspect of the melanocortin system that has not been fully clarified. MC5R has been shown to be expressed in various peripheral tissues relevant for lipid and glucose metabolism (An *et al.* 2007; Boston 1999). This study supports the assumption that the melanocortin system is involved in peripheral regulation of adipocyte metabolism and presumably has direct lipolytic capacity in addition to neuronal regulation. We show that selective MC5R agonists potently induce lipolysis in differentiated murine 3T3-L1 cells however this effect is not obtained in mouse primary adipocytes. 3T3-L1 cells are frequently used in studies of adipose tissue biology and are thought to represent a valid model of preadipocyte differentiation. 3T3-L1 cells are morphological indistinguishable from fibroblasts but committed to adipocyte differentiation. However, differentiated 3T3-L1 cells have a quite different morphology compared to real adipocytes. 3T3-L1 cells differentiated to adipocytes have a multilocular morphology whereas primary adipocytes are unilocular. ACTH and α-MSH were the only test-compounds which stimulated NEFA release in primary adipocytes. MC5R expression was detected in epididymal adipose tissue using real time PCR (figure 1), but MC5R selective test compounds does not stimulate lipolysis. It is likely that MC5R binds NDP-α-MSH, PG-901, MT-II and SHU9119 in primary adipocytes but possess a different ability to induce intracellular signalling compared to 3T3-L1 cells. This hypothesis is supported by figure 7, where primary adipocytes were stimulated by a fixed concentration of α-MSH and increasing doses of PG-901 and NDP-α-MSH respectively. The relative release of NEFA stimulated by α-MSH was decreased indicating that PG-901 and NDP-α-MSH in fact are bound to MC5R but operate as antagonists and lack the ability to induce intracellular signalling mediating lipolysis. Furthermore, NDP-α-MSH antagonizes α-MSH more potently than PG-901 (figure 7). It has previously been suggested, that functional interaction between melanocortin agonists exists, which possibly gives some melanocortin agonists the ability to act as antagonists (Boston & Cone 1996; Mountjoy 2010). The result that MC5R selective agonist PG-901 as well as non-selective NDP-α-MSH in fact operate as antagonists in primary cells raises new questions with regard to peripheral effects of melanocortin peptides and use of MC4R agonists as an anti-obesity target. It is highly relevant to examine these interactions of melanocortin

agonists in order to develop MCR candidates with improved selectivity properties, resulting in compounds with a better safety profile or even dual functionality.

The difference in lipolysis seen between differentiated 3T3-L1 cells and primary adipocytes might also be caused by a difference in expression of MC5R accessory proteins or additional factors necessary for the activation of the receptor. In the absence of MC2R accessory protein (MRAP), MC2R is known to be retained in the endoplasmatic reticulum (ER) where it is degraded. However, in the presence of MRAP, MC2R is glycosylated and translocated to the plasma membrane where it is capable ACTH binding (Sebag & Hinkle 2009). Novel research has suggested that all MCRs may employ an accessory protein. MRAP2 has been identified as a MRAP homologue, which might modulate the functionality of MC1R, MC3R, MC4R and MC5R (Chan *et al.* 2009). Furthermore, prolylcarboxypeptidase have been shown to regulate food intake by inactivating α-MSH centrally. It is plausible that similar mechanisms might regulate effects of the melanocortin system in the periphery (Wallingford *et al.* 2009).

Surprisingly, we find that NEFA release stimulated by NDP-α-MSH, PG-901, MT-II and SHU9119 is cAMP independent and relays on other, yet to be identified signalling pathways. ACTH and α-MSH were the only test compounds which induced cAMP in 3T3-L1 cells (figure 3). Phosphorylated ERK increased after stimulation of all test compounds in 3T3-L1 cells compared to the basal levels (figure 4.A), however when using a MEK inhibitor a decrease in NEFA release was not seen, which suggests that ERK is not an essential mediator of MCR induced lipolysis (figure 5). Elegant studies by the Collins lab have shown that β_3-adrenergic receptor agonists regulate lipolysis through both cAMP-dependent protein kinase (PKA) and ERK (Robidoux *et al.* 2006). We have used the same MEK inhibitor incubation time (1 hour) and same concentration of MEK inhibitor (10 μM) as employed by Robidoux *et al.* A higher concentration of the MEK inhibitor could have been tested in 3T3-L1 cells (10-50 μM). However, increasing doses of MEK inhibitor U0126 might also increase the risk of cell damaging. Furthermore the dose used was demonstrated to block the phosphorylation of ERK completely (figure 4.C), indicating that a higher dose should not make a difference. The possibility that cAMP independent signalling pathways are employed by the melanocortin system might be relevant in other biological systems and need to be further examined. Indeed, examination of signalling pathways employed by other known lipolytic hormones might increase the understanding of melanocortin receptor stimulation of lipolysis. Prostaglandin E2 (PGE2) has been shown to mediate an antilipolytic effect in adipose tissue through the binding to the $G\alpha_i$-coupled EP3 receptor (Vassaux *et al.* 1992). Furthermore, deorphanized GPR109A, GPR109B and GPR81 have been shown to have antilipolytic effects and to be regulators of other metabolic pathways in adipocytes (Ahmed *et al.* 2009). These novel findings confirm the complexity of adipocyte lipolysis and its importance for the regulation of energy metabolism. Additional studies in other adipose tissue depots than epididymal WAT would be of great value. Indeed, novel research suggests essential differences in adipocyte function and lipolytic response between depots such as subcutaneous and visceral WAT (van, V *et al.* 2002; Dicker *et al.* 2009). Lastly, experiments such as siRNA mediated knock-down of the MCRs would be needed to further characterize melanocortin receptor involvement in lipolysis.

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- α-MSH, NDP-α-MSH, MT-II, SHU9119 and PG-901 stimulation of lipolysis in differentiated murine 3T3-L1 is mediated through MC5R independently of cAMP.

- Essential differences in MCR mediated lipolysis are found between 3T3-L1 cells and murine primary adipocytes.

- Lipolysis stimulated by α-MSH is antagonized by PG-901 and NDP-α-MSH in primary adipocytes. This indicates that in some cases the agonist NDP-α-MSH works as an antagonist.

- This study supports a peripheral regulation of adipocyte metabolism by the melanocortin system in addition to neuronal regulation.

Table 1

Table 1. Binding (IC_{50}) of melanocortin peptides to mouse MCRs: Indicated values are an average from three independent experiments (±SEM). IC_{50} values were calculated from competition binding curves of ACTH, α-MSH, NDP-α-MSH, MT-II, PG-901, SHU9119 and LY2112688 obtained on cells expressing MC1, 3, 4 and 5R as described using a fixed concentration of I^{125}-NDP-α-MSH and increasing amounts of non-labelled competing peptides (1 pM - 10 μM).

	mMC1R	***mMC3R***	***mMC4R***	***mMC5R***
Test compound	IC_{50} (nM)	IC_{50} (nM)	IC_{50} (nM)	IC_{50}
ACTH	560 ± 242	14 ± 2	3.03 ± 0.92	21 ± 9
α-MSH	316 ± 93	18 ± 1	19 ± 1	96 ± 36
NDP-α-MSH	0.20 ± 0.05	0.89 ± 0.17	0.27 ± 0.10	0.38 ± 0.07
MT-II	8.23 ± 2.35	3.06 ± 0.78	0.37 ± 0.08	43 ± 11
PG-901	2748 ± 438	2.23 ± 0.41	0.44 ± 0.23	67 ± 15
SHU9119	110 ± 49	1.80 ± 0.31	0.12 ± 0.03	32 ± 8
LY2112688	820 ±104	27 ± 6	0.10 ± 0.06	1239 ± 93

Table 2. EC_{50} values from stimulation of NEFA release from 3T3-L1 adipocytes: NEFA release from 3T3-L1 adipocytes in response to ACTH, α-MSH, NDP-α-MSH, MT-II, PG-901, SHU9119, LY2112688 and Isoproterenol (1 pM - 10 μM) was measured as described in Figure 1.

	3T3-L1 cells
Test compound	EC_{50} ± SEM (nM)
Isoproterenol	1.20 ± 0.12
ACTH	0.74 ± 0.08
α-MSH	1.20 ± 0.26
NDP-α-MSH	0.053 ± 0.004
MT-II	2.35 ± 0.88
PG-901	0.58 ± 0.01
SHU9119	0.27 ± 0.05
LY2112688	5270 ± 1073

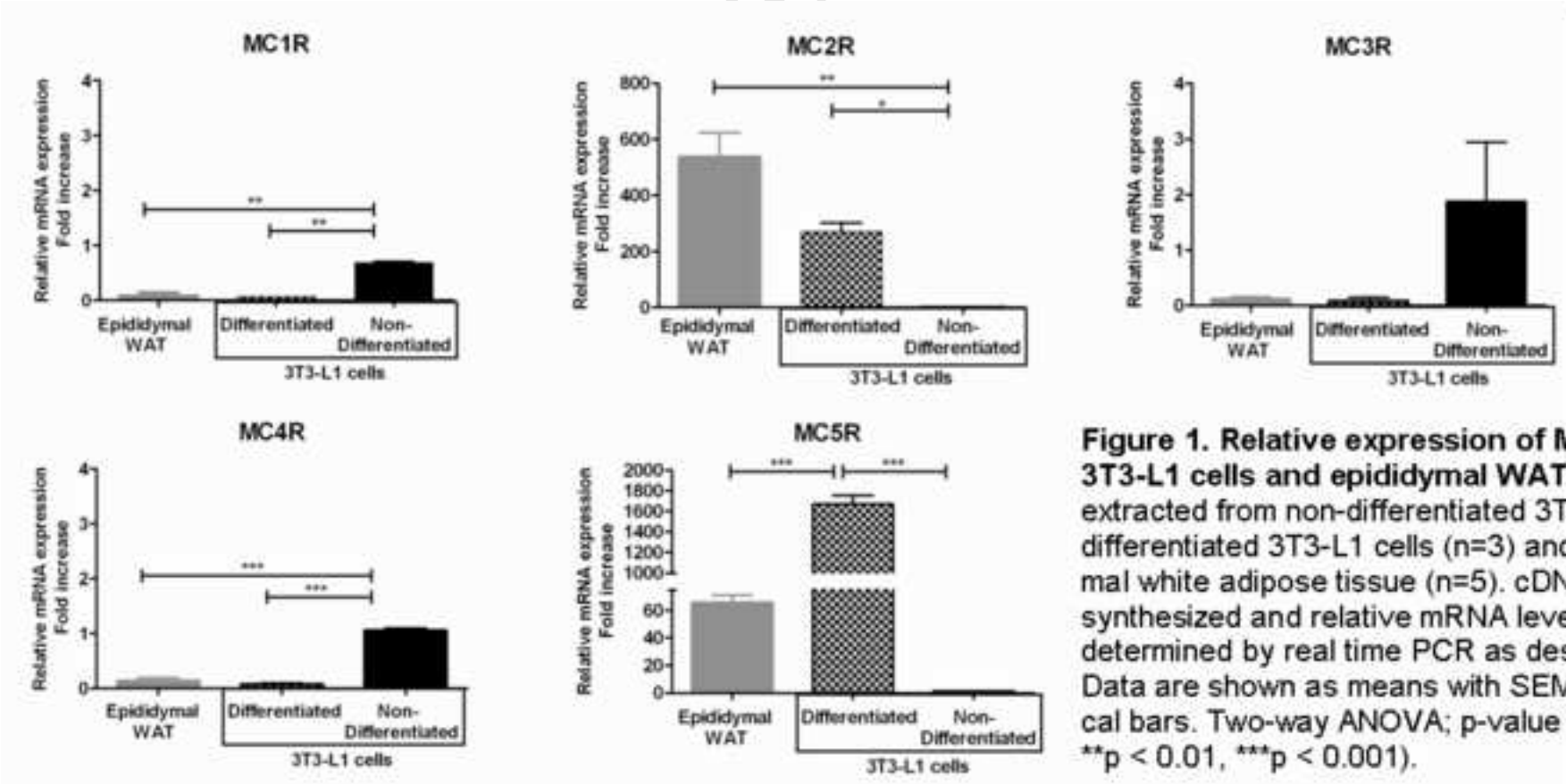


Figure 1. Relative expression of MCRs in 3T3-L1 cells and epididymal WAT: RNA was extracted from non-differentiated 3T3-L1 cells, differentiated 3T3-L1 cells (n=3) and epididymal white adipose tissue (n=5). cDNA was synthesized and relative mRNA levels were determined by real time PCR as described. Data are shown as means with SEM as vertical bars. Two-way ANOVA; p-value (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Figure 2. Stimulation of NEFA release from 3T3-L1 adipocytes: Cells were incubated with increasing concentrations of ACTH, α-MSH, NDP-α-MSH, MT-II, PG-901, SHU9119, LY2112688 and Isoproterenol (positive control) (1 pM - 10 μM) for 3 hours and NEFA release measured as described. The mean (±SEM) of three independent experiments performed in triplicate are shown.

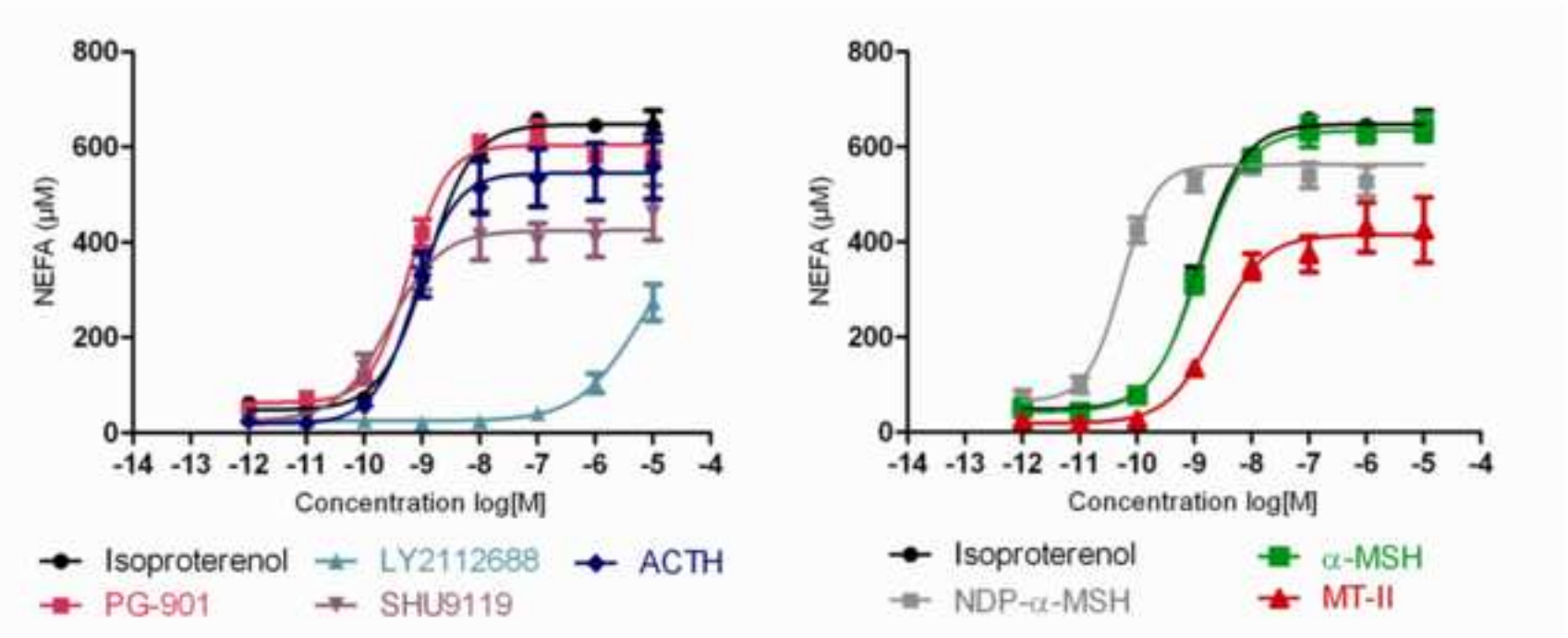
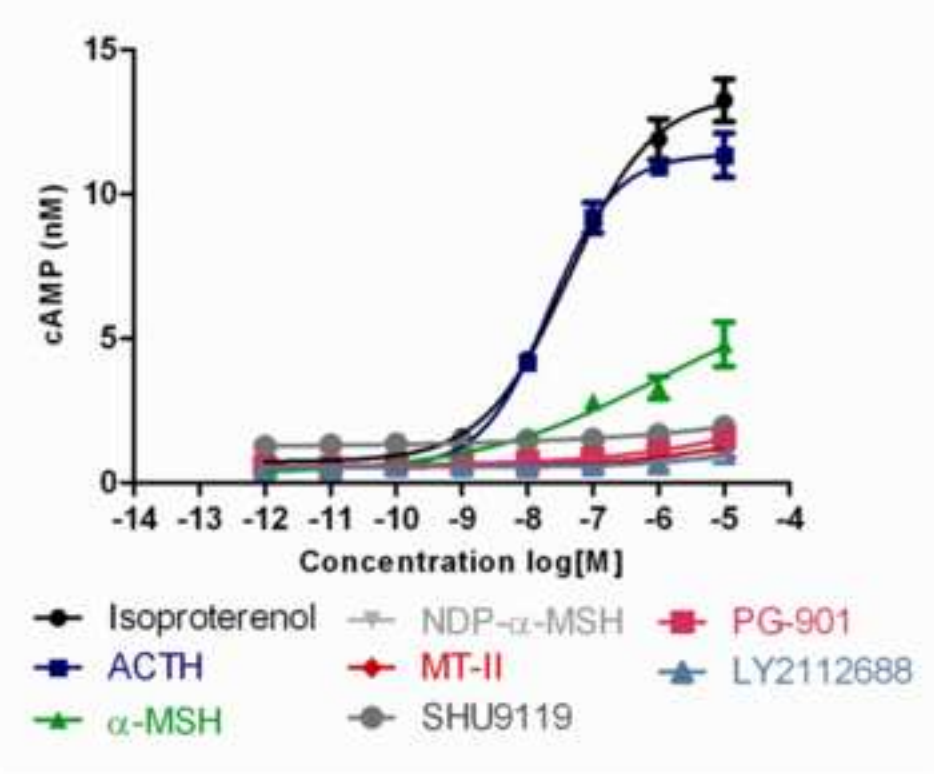


Figure 3. cAMP response to stimulation with melanocortin peptides: Murine 3T3-L1 adipocytes were incubated with increasing concentrations of peptide as in Figure 1 and intracellular cAMP concentration was determined as described. Three experiments were carried out in duplicates and results from one representative experiment with duplicate wells (mean ±SD) are shown. Only Isoproterenol (EC_{50} = 36 ± 6 nM) and ACTH (EC_{50} = 27 ± 4 nM) potently induced an increase of cAMP.



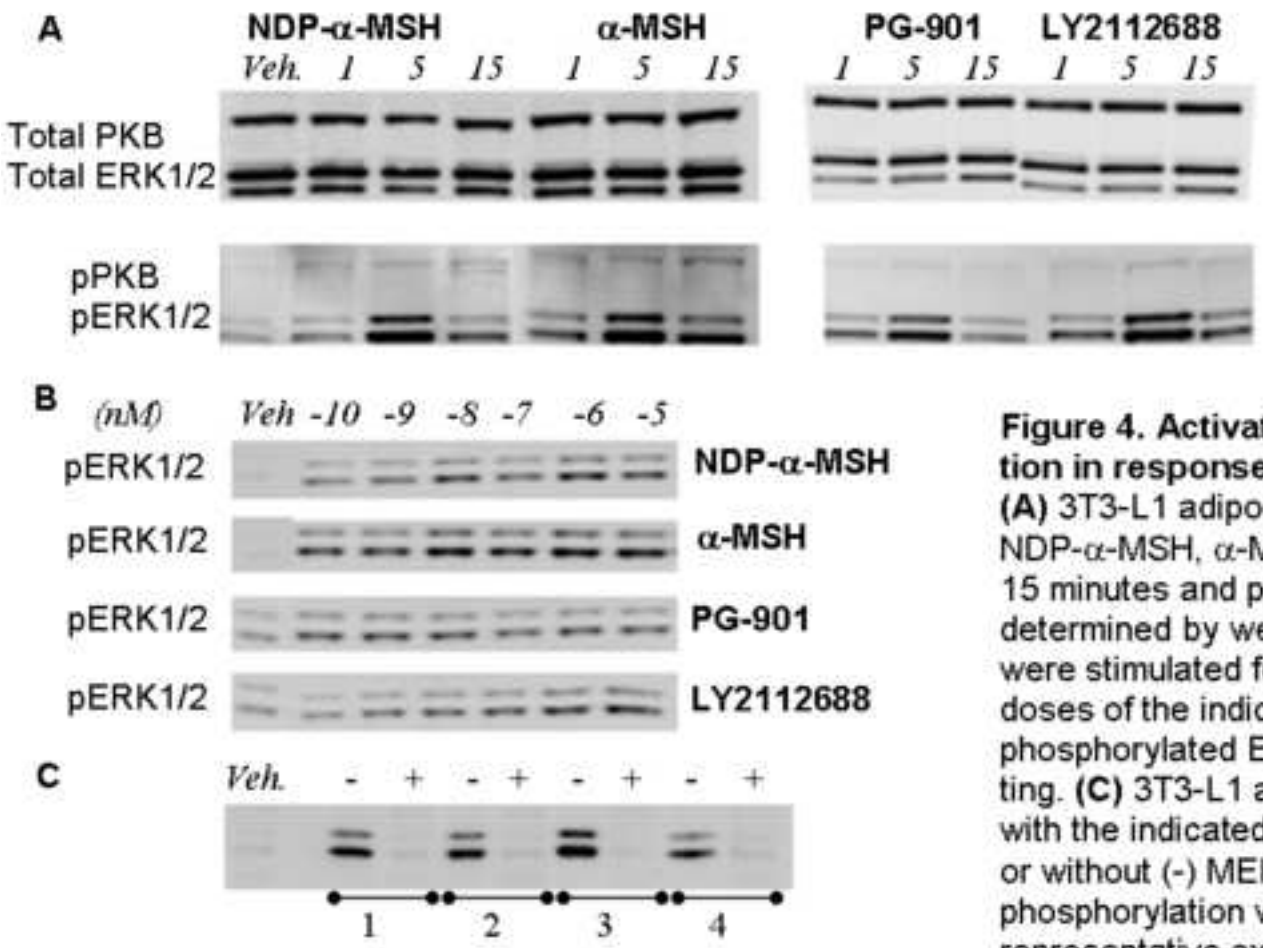


Figure 4. Activation of ERK and PKB phosphorylation in response to melanocortin peptide stimulation: **(A)** 3T3-L1 adipocytes were stimulated with 300 nM NDP-α-MSH, α-MSH, PG-901 and LY2112688 for 1, 5 or 15 minutes and phosphorylation of ERK1/2 and PKB was determined by western blotting. **(B)** 3T3-L1 adipocytes were stimulated for 1, 5 and 15 minutes with increasing doses of the indicated melanocortin peptide. Total and phosphorylated ERK1/2 was determined by western blotting. **(C)** 3T3-L1 adipocytes were incubated for 5 minutes with the indicated melanocortin peptide as in (A) with (+) or without (-) MEK-inhibitor U0126 (10 μM) and pERK phosphorylation was determined by western blotting. A representative experiment out of 3 performed is shown.

Figure 5

Figure 5. Melanocortin peptide stimulated lipolysis is unaffected by MEK inhibitor: NEFA release from 3T3-L1 adipocytes were determined after stimulation with 10 nM PG-901 or NDP-α-MSH in the presence or absence of a MEK inhibitor (10 μM U0126) at t=30 and 60 minutes. The experiment was carried out twice in triplicates with similar results. Results from one experiment are shown (mean ±SD).

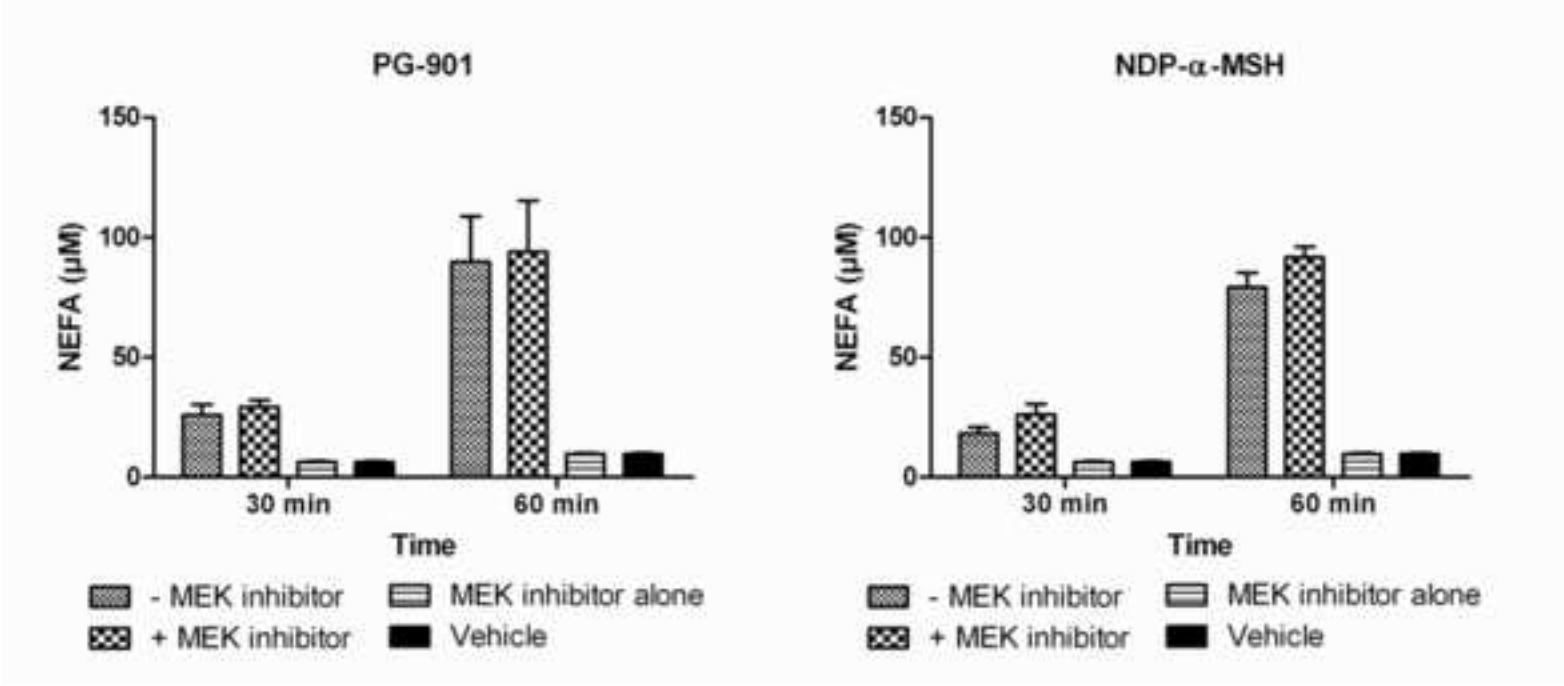


Figure 6. Stimulation of NEFA release from primary murine adipocytes: Primary adipocytes were isolated from mouse epididymal adipose tissue and incubated with increasing concentration (1 pM – 10 μM) of ACTH, α-MSH, NDP-α-MSH, MT-II, PG-901, SHU9119 and LY2112688. NEFA release was measured as described. Two experiments were carried out in duplicates and results from one representative experiment with duplicate wells (mean ±SD) are shown. Isoproterenol (positive control) EC_{50} = 24 ± 17 nM, ACTH EC_{50} = 0.007 ± 0.001 nM and α-MSH EC_{50} = 0.63 ± 0.10 nM.

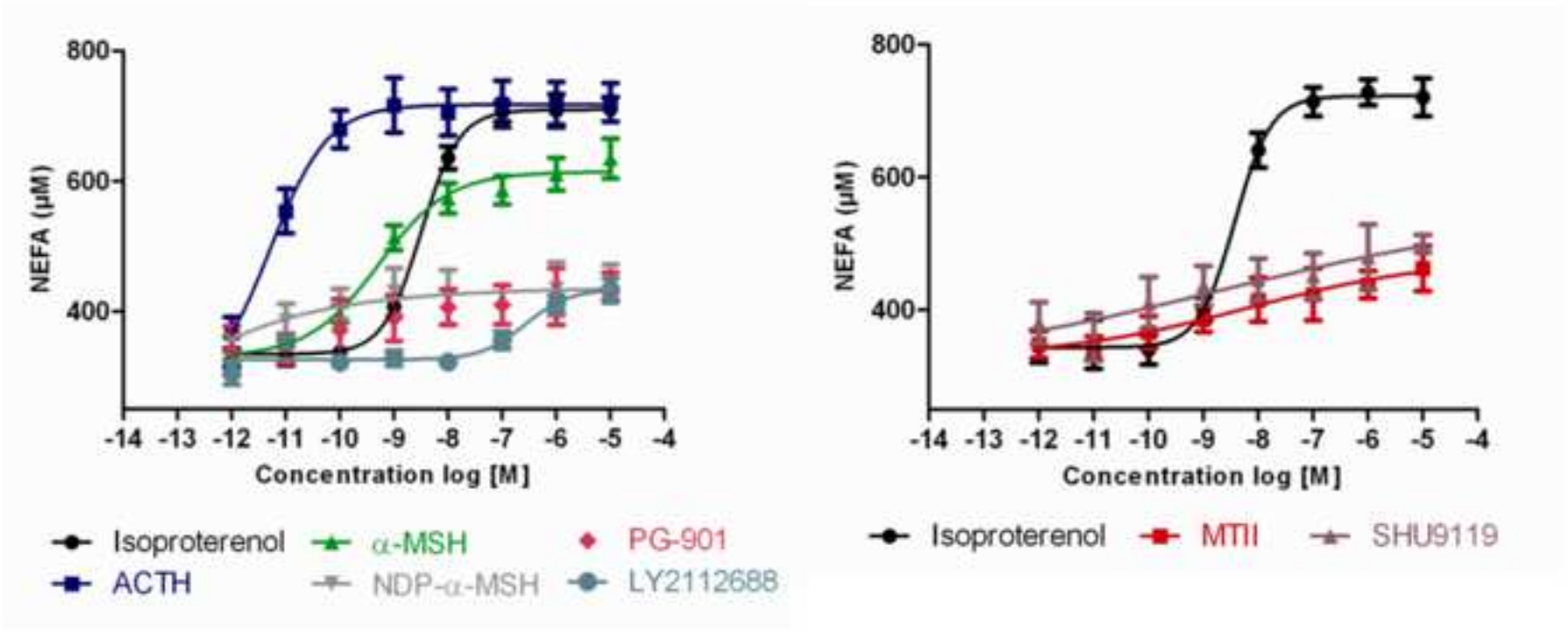


Figure 7. NDP-α-MSH and PG-901 acts as MSH antagonists on primary murine adipocytes: Mouse primary adipocytes isolated from epididymal adipose tissue were incubated with a 100nM α-MSH and increasing concentrations of NDP-α-MSH or PG-901 (1 pM - 10 μM). The results are shown as average values from two independent experiments carried in duplicates (±SD). α-MSH stimulated NEFA release was inhibited by NDP-α-MSH and PG-901 with IC_{50} values of 0.23 ± 0.03 nM and 7.32 ± 2.28 nM, respectively.