Effects of melanocortin receptor modulation on hypertrophic responses in cardiomyocytes

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After cardiac stress such as that occurring during myocardial infarction the heart undergoes pathological remodelling consisting of cardiomyocyte hypertrophy, interstitial fibrosis, contractile dysfunction, cell death and ventricular dilatation. It is due to this remodelling that the survivability is 10% at 10 years post-infarction. In this study we explored the possible cardioprotective effects of melanocortin receptor activation in an initial search for a treatment for cardiac remodelling. ELISA tests were used to study the signalling cascade of melanocortin receptor activation and qPCRs were used to study gene expression changes. Using an established rat cardiomyocyte cell line and primary mouse cardiomyocytes it was established that activation of all melanocortin receptors results in a cardioprotective response through the downregulation of pro-hypertrophic genes. We were also able to determine through independent activation of the receptors for melanocortins that the receptors for melanocortin 1 and 5 were the ones responsible for the cardioprotective response, with melanocortin receptor 5 activation providing the strongest cardioprotective effect. Further study of these receptors and their cardioprotective effects could lead to a treatment for cardiac hypertrophy.

Key words: Cardiac Hypertrophy, Melanocortins, Cardiomyocytes

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1. Introduction

1.1Forms of hypertrophy and heart failure

Globally, heart failure has already become an epidemic. It is an increasingly common condition in the developed world and its associated comorbidities are a rising cause of death. After diagnosis of heart failure, the 5 year survival rate is 50% and drops to only 10% at 10 years post-diagnosis (Roger, 2013). It is therefore of crucial importance to study the underlying causes and mechanisms that lead to cardiac failure to improve the symptoms and prognosis of affected patients.

Cardiac hypertrophy is associated with heart failure and with negatively progressing outcomes of the disease. Left ventricular hypertrophy was observed in up to 20% of the general population in the nineties and has since been shown to rise up to 30% (Cramariuc and Gerdts, 2016). This is a worrying trend as there is no good treatment after the onset of cardiac hypertrophy and it is a herald of worsening symptoms and disease.

The main reason for the lack of treatment of hypertrophy is that cardiomyocytes finish differentiating in neonates and thereafter any increase in the size of the cardiac walls is through an increase in size of those cardiomyocytes. Once the heart starts deteriorating there is not much the body can do to revert it (Engel, 2005). Hypertrophy is a response to increased heart wall stress, in an attempt to increase the capacity of the heart and maintain the increased workload hypertrophy takes place. This hypertrophy can be benign, such as that seen in athletes who perform extended exercise sessions, or pathological. Non-pathological hypertrophy, also known as physiological, is characterized by an increased contractile function without fibrosis or cell death and is reversible and does not lead to heart failure. A summary of non-pathological cardiac hypertrophy subtypes can be found on Figure 1. It is common amongst high-performance athletes, with eccentric hypertrophy appearing in endurance training and concentric hypertrophy appearing in strength training (Mihl et al., 2008; Carabello, 2002).

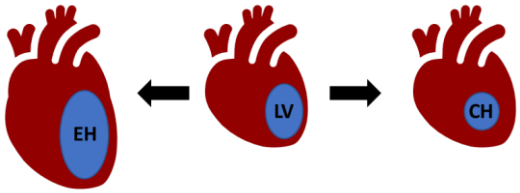

Figure 1. Graphical summary of hypertrophy subtypes. The abbreviations stand for Eccentric Hypertrophy (EH), Left Ventricle (LV), Concentric Hypertrophy (CH). Cardiac hypertrophy can take place as an adaptation to volume overload, in which case the LV experiences elongation of the cardiomyocytes and increase of the ventricular volume while retaining the same wall thickness. If the primary stimulus is a pressure overload however, the heart will respond through the proliferation of cardiomyocytes and the thickening of the cell walls.

Pathological hypertrophy on the other hand is commonly a result of disease and is maladaptive. Pathological hypertrophy appears after abnormal haemodynamic stress (e.g. systemic hypertension and aortic stenosis (Samak et al., 2016)) and leads to imbalanced hypertrophy and can progress to heart failure. It is therefore important to know what mechanisms trigger this maladaptation to find potential ways of reverting it once it occurs (Samak et al., 2016; Shimizu and Minamino, 2016).

Heart failure has many clinical phenotypes, and a useful distinction is between heart failure with preserved ejection fraction (HFpEF) and with reduced ejection fraction (HFrEF). HFpEF is mostly observed in women (Abebe et al., 2016) and is associated with valvular and hypertensive heart pathologies, obesity and diabetes. It has been reported that oestrogen has a protective effect on endothelial dysfunction and furthermore has anti-proliferative effects on vascular smooth muscle, which explains why HFpEF is more common in older women (Simmonds et al., 2020). HFrEF is described when the ejection fraction decreases below 40 %. HFrEF is mostly present in male patients (Abebe et al., 2016) and presents with ischemic heart disease and dilated cardiomyopathy. HFrEF is associated with cardiac hypertrophy and worsened clinical outcomes. HFrEF incurs in cardiomyocyte loss through apoptosis and necrosis. After ischaemia there is an initial cell death which causes inflammation and further cell death. This pronounced cell death and inflammatory situation promotes further cardiac remodelling and enhances fibrosis.

Pathological hypertrophy is initially compensatory but quickly progresses to include ventricle dilation and cardiomyocyte lengthening and contractile dysfunction. Hypertrophy is also exacerbated by increased fibrosis, cardiomyocyte death and changes in Ca^{2+} metabolism. HFrEF is further characterized by a fibrotic increase as a result of cardiomyocyte death. HFpEF fibrosis however is caused by increased deposition of

collagen type I (which confer increased tensile strength) to the detriment of collagen type III (which is more flexible).

1.2 Markers and mediators of hypertrophy

Pathological hypertrophy is characterized by a switch to a foetal gene expression profile, this consists of increased expression of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), myosin heavy chain and skeletal muscle α-actin (αSKA). This switch to foetal gene expression also includes a shift into carbohydrate-based energy production instead of the traditional lipid-based metabolism (Samak et al., 2016). Hypertrophy is also linked with inflammation and expression of pro-inflammatory cytokines (IL-6 (Interleukin 6), Interleukin-1β, Interleukin 1 receptor antagonist and TNFα) (Kuusisto et al., 2012). Conversely, physiological hypertrophy does not involve an increase in these markers and may even present with a decrease in their expression.

BNP is a hormonal peptide secreted by cardiomyocytes when they are stretched due to increased ventricular volume. Once released, it binds to the atrial natriuretic factor receptor in a fashion similar to ANP. Its biological activity differs from ANP; BNP has a lower affinity, but longer biological half-life and it causes a decrease in vascular resistance and central venous pressure. In the heart, the effect of natriuretic factor receptor activation leads to a protective effect against hypertrophy as blocking of this receptor leads to cardiac fibrosis (Oliver et al., 1997). It has been shown that congestive heart failure is linked with a sustained increase in plasma BNP, myocardial hypertrophy and left ventricle stiffness (Tamura et al., 2000).

One of the most well-studied hypertrophic agents is angiotensin II. The pro-hypertrophic effect of angiotensin II is mediated through an increase in the expression of TGF-β (Schultz et al., 2002). It is known that *Tgfb1* mRNA levels are increased upon pressure overload or drug-induced hypertrophy.

Alpha smooth muscle actin (αSMA) is a marker for cardiac remodelling and its expression is highest during embryonic development and is reactivated later in life when there is production of new cardiomyocytes (Nagamoto et al., 2000; Ma et al., 2012). Both αSMA and αSKA are the predominant actin isoforms in cardiac sarcomeres. Increase in αSKA expression has been linked to cardiac hypertrophy and it constitutes a well-accepted marker for cardiac hypertrophy in both humans and rodents (Driesen et al., 2009; Black et al., 1991).

TGF-β is associated with fibrosis and hypertrophy in mouse hearts when it is overexpressed (Dobaczewski et al., 2011). TGF-β, however, is known to be one of the most pleiotropic peptides and it can exert many different effects in different tissues or in the same tissue when under different conditions or concentrations (Letterio and Roberts, 1998). TGF-β upregulation through angiotensin II signalling is known to take place but it is yet unknown whether this upregulation results in the expression of functional TGF-β or if another signal is still needed to produce the functional peptide (Campbell and Katwa, 1997). Decrease in TGF-β expression cannot be definitively linked to a protective effect on the heart. Among other mechanisms, it is known that TGF-β inhibits nitric oxide synthase 2 (NOS2) expression (Umar and Van Der Laarse, 2010; Lowenstein and Padalko, 2004).

Nitric oxide (NO) is a mediator in cardiac signalling and is known to have a protective effect on cardiomyocytes. *In vivo*, it is known that pressure overload induces NOS2 activity in cardiomyocytes which produces an increase in NO levels. In heart failure, the levels of NO exceed those with protective effects and become toxic, leading to a production of reactive oxygen and nitrogen species (Umar and Van Der Laarse, 2010; Nathan, 1997). The increase in NOS2 expression mediated by cytokines in cardiac failure leads to apoptosis of cardiomyocytes.

It is known that angiotensin II promotes the activation of fibroblasts to promote their switch to myofibroblasts and their production of extracellular matrix. The effect of angiotensin II is mediated through production of TGF-β. Endothelin I is also involved in extracellular matrix production and myoblast differentiation, downstream of the TGF-β effect. Furthermore, angiotensin II induces endothelin I expression through activation of extracellular signal-regulated kinase (ERK) and reactive oxygen species. Because angiotensin II is higher on the signalling cascade, it is considered to have greater hypertrophic effect than endothelin I (Leask, 2010).

Agonists of β_1-adrenoceptor such as isoprenaline also cause cardiac hypertrophy. Isoprenaline is routinely used to induce hypertrophy and fibrosis both *in vivo* and *in vitro* (Weil et al., 2006; Yin et al., 2009). Phenylephrine also causes re-expression of embryonic cardiac genes β-myosin heavy chain and ANP). Both, isoprenaline and phenylephrine, cause cardiac hypertrophy through a signalling cascade involving ERK in the same manner as angiotensin II and endothelin I. Due to the shared signalling and identical outcomes, all four are used interchangeably as hypertrophic agents (Yue et al., 2000).

Type 3 collagen (Collage, type 3, alpha 1 or Col3A1) is a homotrimeric protein that constitutes a major structural component of blood vessels. Its expression is increased in cardiac hypertrophy caused by pressure overload as it presents with increased deposition of extracellular matrix.

C-C motif chemokine Ligand 2, also known as monocyte chemoattractant protein 1, is a chemokine that recruits monocytes, memory T cells and dendritic cells to the sites of inflammation.

IL-6 is produced in response to tissue damage to produce a pro-inflammatory response which limits host damage. This pro-inflammatory response, if maintained, leads to chronic inflammation and fibrosis. This general aspect of IL-6 effect is also replicated in the heart where reduced IL-6 expression is linked with protective effects after acute damage such as myocardial infarction while a sustained IL-6 response results in maladaptive fibrosis (Fontes et al., 2015).

1.3 Melanocortin receptors in the body

Melanocortins are a family of peptide hormones that originate from the cleavage of a single precursor protein known as proopiomelanocortin (POMC). Melanocortins include adrenocorticotropic hormone (ACTH) and melanocyte-stimulating hormones (α, β and γ-MSH), an overview of the cleavage products of POMC can be found in Figure 2. Melanocortins mediate their biological actions via five different melanocortin receptors (Mc1R-Mc5R) that are found throughout the body.

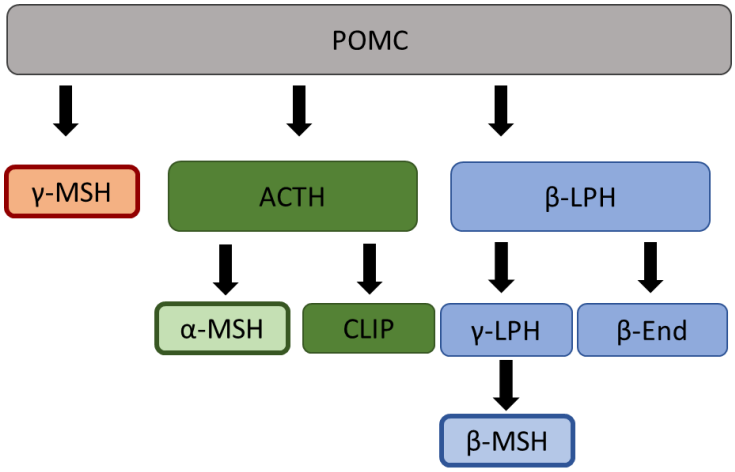

Figure 2. Overview of the cleavage products of POMC. Outlined in bold are the main melanocortin stimulating peptides. POMC is first cleaved into γMSH, ACTH and β-LPH (Beta Lipotropin). ACTH is then further cleaved into αMSH and CLIP (Corticotropin-Like Intermediate Peptide). B-LPH is cleaved into γ-LPH (Gamma Lipotropin) and β-End (Beta Endorphin. γ-LPH is processed into β-MSH.

Melanocortin receptors are G-protein-coupled receptors that take part in very diverse processes including pigmentation, inflammation, energy homeostasis and stress response (Cooray and Clark, 2011). Melanocortin effects in the body are still being unveiled, with new effects in different tissues being discovered continuously. The wide range of activities and pathways in which melanocortins are involved is explained by the existence of accessory proteins and the ability of the melanocortin receptors to recognise multiple agonistic and antagonistic ligands.

A brief overview of the known roles of melanocortins will be useful to understand the role of these proteins in the organism: Mc1R is responsible for the expression of eumelanin and the darkening of skin and hair (Cone, 2006), Mc2R activation leads to the expression of steroidogenesis in the adrenal gland (Thistlethwaite et al., 1975), Mc3R and Mc4R are mainly located in the nervous system and have effects on obesity and pain (Mountjoy, 2010; Feng et al., 2005; Starowicz et al.) and Mc5R was linked to thermal regulation and hair lipid production (Trotta et al., 2018; Abdel-Malek, 2001). Melanocortin receptors are known to be expressed also in the heart but their functional role is still unclear (Chhajlani, 1996; Getting et al., 2004; Trotta et al., 2018).

Mc1R has been thoroughly studied for its effect on human pigmentation. Studies have for the most part centred on allelic variations of this locus as it accounts for striking mutations such as red hair pigmentation and the linked increase in melanoma risk. Mc1R however also has an effect on many paracrine systems, like most G-protein-linked receptors it can engage in many different signalling pathways. Whether through promiscuous coupling with different G-proteins, ligand-induced interactions with β-arrestins or other scaffolding proteins (Herraiz et al., 2017). Most of the known effects of Mc1R signalling start with G-protein-dependant adenyl cyclase activation.

Mc3R has been found to play a role in energy metabolism as loss of function mutations cause increased fat content and decreased muscle mass without significant changes in overall weight (Humphreys et al., 2011). Furthermore, Mc3R has also been shown to increase expression after increased sodium intake.

Mc4R is known to protect from obesity as deficiency in this gene produces increased body fat, hyperphagia and hyperinsulinemia (Gonçalves et al., 2018). It has been proven that different Mc4R agonists produce different downstream signalling cascades, showing that each agonist produces a unique response although they all bind to the same receptor. This property hasn’t been yet confirmed for other MCRs (Girardet and Butler, 2014).

1.3.1 Melanocortins used in this research

Alpha melanocyte-stimulating hormone (α-MSH) was used as a general activator for all melanocortin receptor subtypes. Although generally considered a pan-agonist, α-MSH has a different affinity for all melanocortin receptors, ranging from very strong to almost negligible. In decreasing α-MSH affinity order the melanocortin receptors are Mc1R, Mc3R, Mc5R, Mc4R and Mc2R (Cooray and Clark, 2011).

All MCR subtypes were individually activated in this study except for Mc2R. The agonists used to activate the melanocortin receptors were LD211 (a synthetic Mc1R agonist), γ-MSH (a natural Mc3R agonist), THIQ (a synthetic Mc4R agonist) and PG 901 (a synthetic MC5 agonist). A summary for all the melanocortin receptor binding relevant to this study can be found in Figure 3

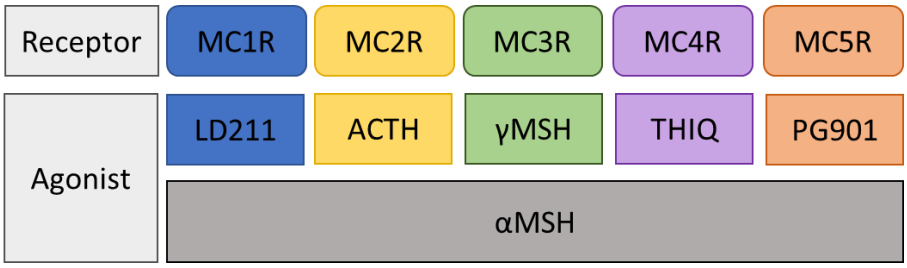

Figure 3. Summary of melanocortin receptors and their agonists. αMSH is a pan-agonist that activates all melanocortin receptors at some level. In this study both the pan-agonist and the specific agonists for Mc1R, Mc3R, Mc4R and Mc5R were used.

2. Results

2.1 Melanocortin signalling studies

The project was started by studying the possible signalling pathways activated by different melanocortin receptor agonists. For this purpose, the rat cardiomyocyte cell line H9c2 was first used and assayed for the phosphorylation status of different mitogen-activated protein kinases such as ERK and JNK using a commercial ELISA kit.

The first experiment was performed to test the effects of α-MSH at a fixed 0.1 nM (-10 M) concentration in H9c2 cells. The first time the effects of α-MSH at differing times were studied, there was no change in any of the kinases studied, these results can be found in Figure 4 (a-c). This lack of response went against what was expected as there had been some preliminary results previously carried out in the research group that hinted at some kind of effect; therefore, the experiments were repeated. To improve the quality of the data the 5-minute time point was removed and the space in the ELISA plate previously

taken by that time point was spread within the other times. The reasoning behind the removal of this time point is that the whole assay process and sample collection causes a delay between wells which exceeds 5 minutes and therefore can't be fully corrected by staggering the treatment initiation times to account for the collection delays. As can be seen in Figure 4 (d-f), there was no significant response to α-MSH. There appears to be no effect caused by α-MSH at -10M on the levels of pERK. In 4e there appears to be a tendency towards a decrease of pJNK with increasing time, the same tendency seems to take place in 4f. These downward tendencies however are not significant and were completely non-existent in the first -10M α-MSH study at Figure 4 a-c. The lack of response in these initial studies prompted a shift in what angle to study the signalling cascade from.

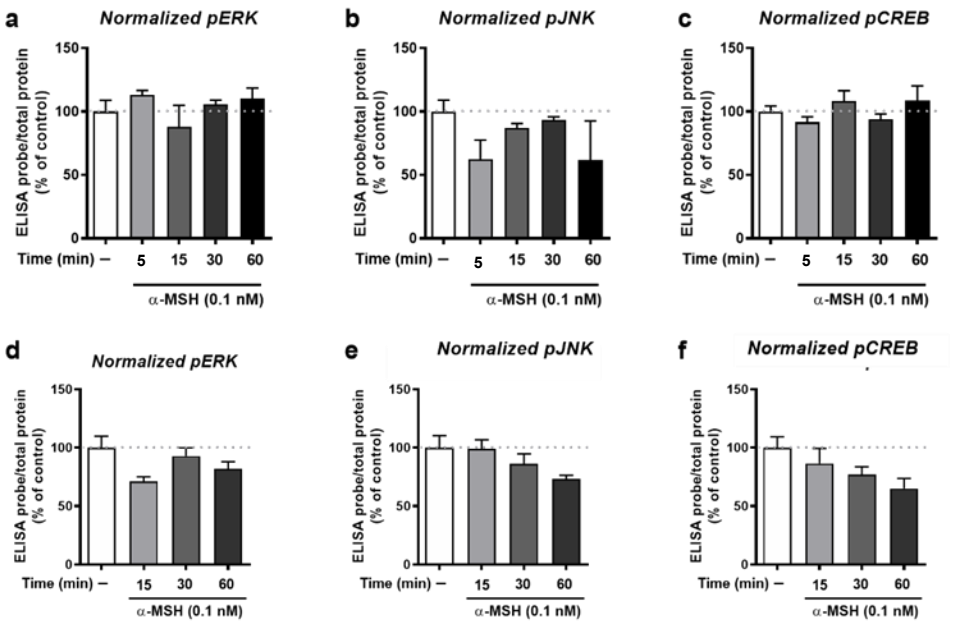

Figure 4. The effects of αMSH on p-ERK, pJNK and p-CREB in H9c2 cells. Results from phosphorylated ERK (a), JNK (b) and CREB (c) at different incubation times (5, 15, 30 and 60 min) after a treatment with a single concentration of 0.1 nM of α-MSH. Results are shown normalized by the total protein ammount as assayed by a BCA total protein assay and as a % of the control, presented are 4 replicates per timepoint. Data is presented as mean ± SEM. Results from phosphorylated ERK (d), JNK (b) and CREB (c) at different incubation times (15, 30 and 60 min) after a treatment with a single concentration of 0.1 nM of α-MSH. Results are shown normalized by the total protein ammount as assayed by a BCA total protein assay and as a % of the control, presented are 5 replicates per timepoint. Data is presented as mean ± SEM.

One hypothesis for the varying results between replicates was that the cell line failed to correctly replicate the signalling cascade that would have been seen in wild-type cells and was instead producing inconsistent phosphorylation of the cascade intermediaries. To test

this hypothesis and to confirm whether this lack of response was characteristic of cardiomyocytes, a second round of experiments were carried out as replicates of the first but using primary neonatal mouse cardiomyocytes. In these tests all parameters, except the cell type, were the same.

There appeared to be a tendency towards a decrease in pJNK and pERK at 5 and 15 minutes due to α-MSH (Figure 5a, b). Yet the decrease was not significant, at this point we resolved to test whether or not there was a confounding effect exerted by contradictory effects of different melanocortin receptor subtypes. The first melanocortin subtype agonist tested was PG 901. The effects of this melanocortin agonist had much stronger and clearer effects than those of α-MSH. In Figure 5d PG 901 was seen to cause a decrease in ERK phosphorylation which was most marked at the 60-minute time point. Figure 5e shows a marked decrease in pJNK levels at 15 and 60 minutes, the strong effects of this Mc5R activation were masked in the previous α-MSH experiments.

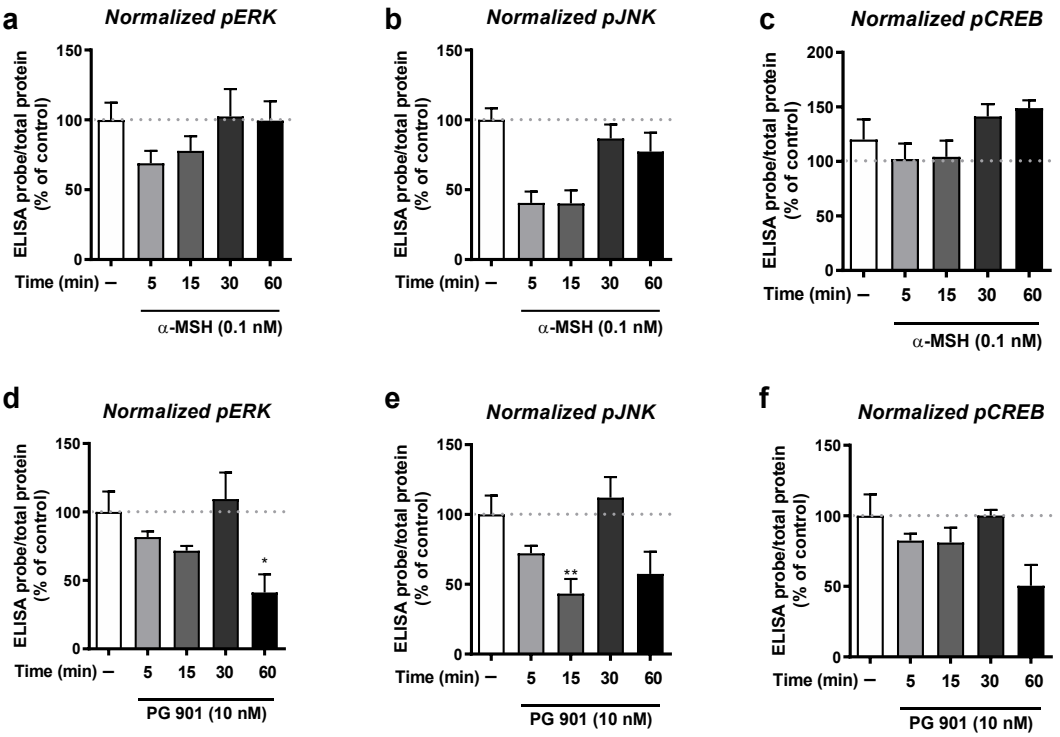

Figure 5. The effects of αMSH on p-ERK, pJNK and p-CREB in mouse neonatal primary cardiomyocytes. Results from phosphorylated ERK (a), JNK (b) and CREB (c) at different incubation times (5, 15, 30 and 60 min) after a treatment with a single concentration of 0.1 nM of α-MSH. Results are shown normalized by the total protein ammount as assayed by a BCA total protein assay and as a % of the control, presented are 5 replicates per timepoint. Data are presented as mean ± SEM. Results from phosphorylated ERK (d), JNK (e) and CREB (f) at different incubation times (5,15, 30 and 60 min) after a treatment with a single concentration of 10 nM of PG 901. Results are shown normalized by the total protein ammount as assayed by a BCA total protein assay and as a % of the control, presented are 5 replicates per timepoint. Data are presented as mean ± SEM. One asterisk above a column denotes a P value lower than 0.05 when comparing that column to the control using a one-way ANOVA and a later Tukey multiple comparison test. Two asterisks denote a P value lower than 0.01 and three denote a P value lower than 0.001.

Mc1R activation through LD211 can be seen in Figure 6. The decreases in pJNK levels at 5 and 15 minutes seen in 6b appear in the same line as the decreases seen in 5b and 5e. Based on this it was postulated that both Mc1R and Mc5R were chiefly responsible for the melanocortin effects upon the myocardial tissue and appeared to be exerting an effect in the same direction and through similar signalling pathways.

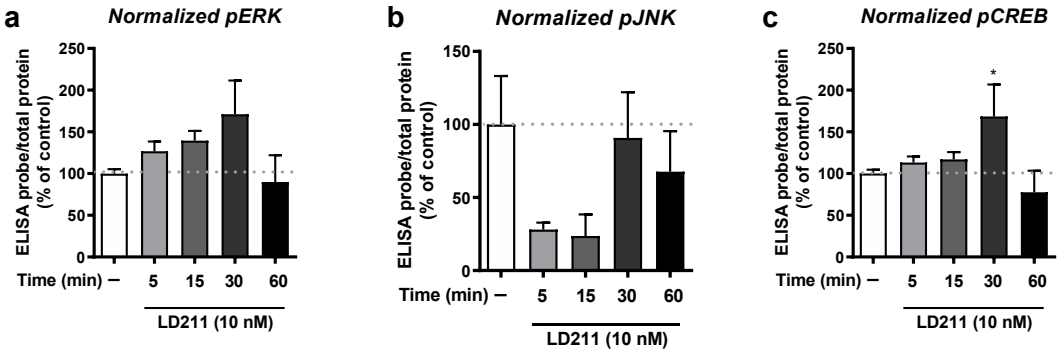

Figure 6. The effects of LD211 on p-ERK, pJNK and p-CREB in mouse neonatal primary cardiomyocytes. Results from phosphorylated ERK (a), JNK (b) and CREB (c) at different incubation times (5, 15, 30 and 60 min) after a treatment with a single concentration of 10 nM of LD211. Results are shown normalized by the total protein ammount as assayed by a BCA total protein assay and as a % of the control, presented are 5 replicates per timepoint. Data are presented as mean ± SEM. One asterisk above a column denotes a P value lower than 0.05 when comparing that column to the control using a one-way ANOVA and a later Tukey multiple comparison test. Two asterisks denote a P value lower than 0.01 and three denote a P value lower than 0.001.

To continue with the study of the effects of melanocortins, the focus of the research moved to quantifying changes in mRNA levels of hypertrophy and inflammation markers with qPCR.

2.2 Gene expression studies

2.2.1 α-MSH treatments in H9c2 cell line

Due to the high volume of qPCR results produced, only those with statistically significant changes or those having importance in comparisons between cell types was included in this section. All other qPCR results are presented in the Annex 1. Applied concentrations were the same as in signalling experiment but the treatment times used were increased since changes in gene expressions take longer to appear than phosphorylation of kinase in cell signalling transduction.

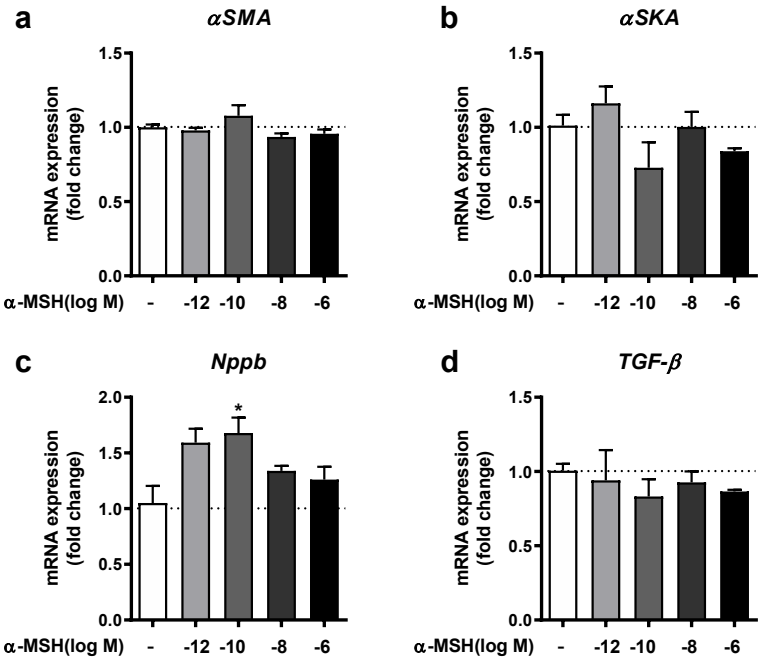

Figure 7. Compilation of results from qPCR tests performed on H9c2 cells after 3 hours of incubation with α-MSH. The genes assayed were αSMA (alpha smooth muscle actin), αSKA (alpha skeletal muscle actin), Nppb (ventricular natriuretic peptide) and TGF-β (transforming growth factor beta). One asterisk above a column denotes a P value lower than 0.05 when comparing that column to the control using a one-way ANOVA and a later Tukey multiple comparison test. Data are presented as mean ± SEM, presented are 5 replicates per study condition and 6 for the controls.

In the first batch of qPCR results, a variable concentration of α-MSH was used to induce a response in H9c2 cells after 3h of incubation. Shown in Figure 7, the pan-agonist has no effect on the hypertrophic markers αSMA, αSKA or TGF-β. The treatment however caused an increase in Nppb expression at the -10 M concentration.

After the concentration series, a time series with a fixed 10 nM (-8 M) concentration of α-MSH was performed. In Figure 8, a decrease in the expression of both αSMA and αSKA can be seen after 1h of incubation with α-MSH. The expression change in Nppb observed in 8c went against the change seen in the 7c as there is a decrease at 1 h and a smaller, non-significant decrease at 3 h. TGF-β expression is also decreased after both 1 h and 3 h treatment. From these results, it is clear that α-MSH evokes inconsistent responses as was seen in the ELISA tests.

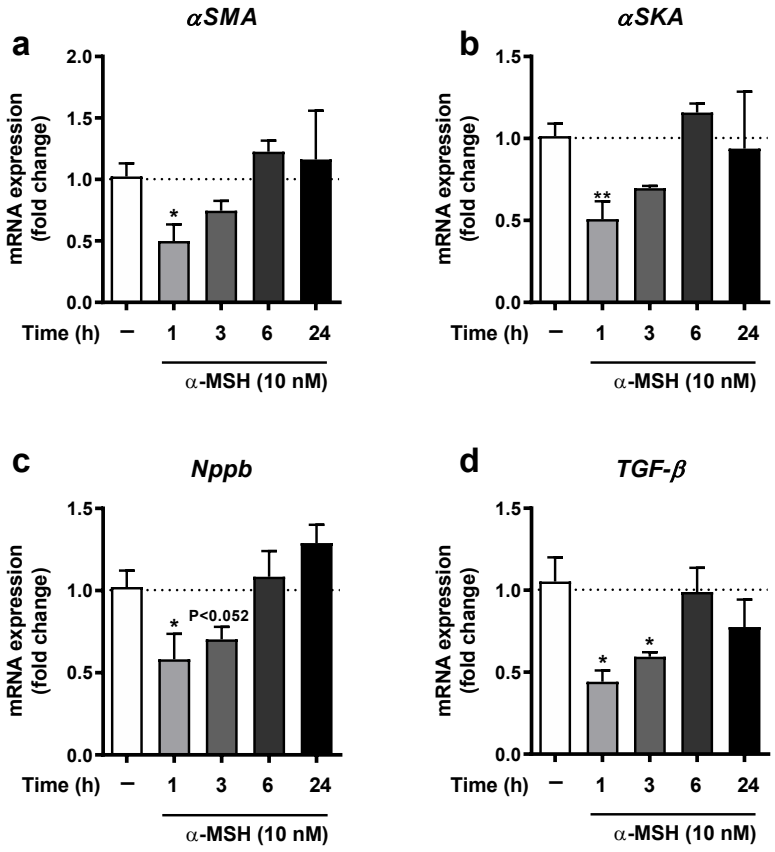

Figure 8. Compilation of results from qPCR tests performed on H9c2 cells after incubation with -8 M α-MSH at different times. The genes assayed were αSMA (alpha smooth muscle actin), αSKA (alpha skeletal muscle actin), Nppb (ventricular natriuretic peptide) and TGF-β (transforming growth factor beta). One asterisk above a column denotes a P value lower than 0.05 when comparing that column to the control using a one-way ANOVA and a later Tukey multiple comparison test. Two asterisks denote a P value lower than 0.01. In a column in graph 5c the P value is shown as although not showing statistical significance it showed a clear decrease and was just on the border of being considered significant. Data are presented as mean ± SEM, presented are 5 replicates per study condition and 6 for the controls.

2.2.2. α-MSH treatments in primary neonatal mouse cardiomyocytes

The same time and concentration series performed in H9c2 cells were performed in primary cardiomyocytes. The tests on primary cells were started by performing α-MSH concentration series after 3 h of incubation. In Figure 9, a decrease in interleukin 6 (IL-6) expression was observed after treatment with 10 nM (-8 M) concentration of α-MSH. TNFα expression was on the other hand increased after treatment with 0.1 nM (-10 M) αMSH.

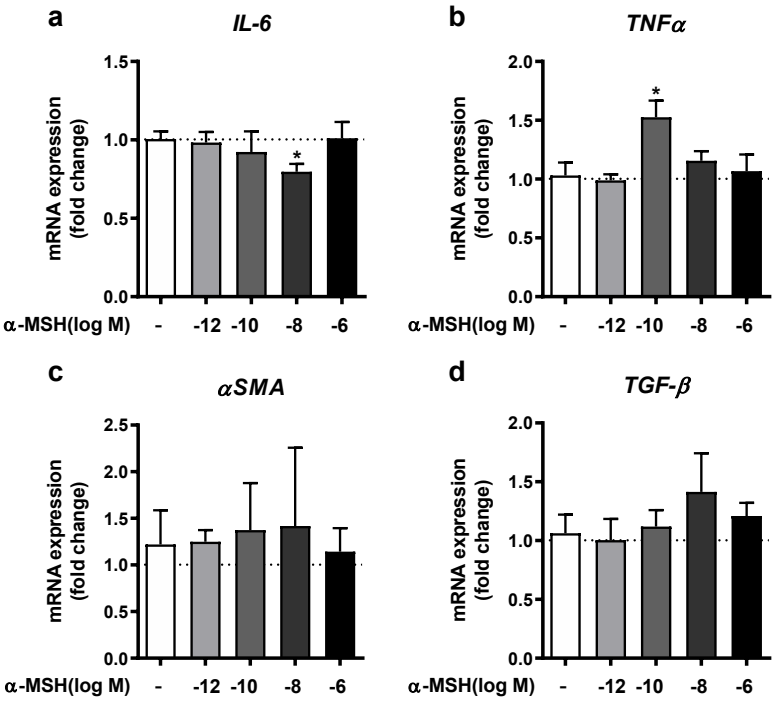

Figure 9. Compilation of results from qPCR tests performed on primary neonatal mouse cardiomyocytes after 3 hours of incubation with α-MSH. The genes assayed were IL-6 (interleukin 6), TNFα (Tumour necrosis alpha), αSMA (Alpha smooth muscle actin) and TGF-β (Transforming growth factor beta). One asterisk above a column denotes a P value lower than 0.05 when comparing that column to the control using a one-way ANOVA and a later Tukey multiple comparison test. Data are presented as mean ± SEM, presented are 5 replicates per study condition and 6 for the controls.

In Figure 10, a very strong decrease in αSMA can be seen both after 1 and 3 hours of incubation with 10 nM (-8 M) α-MSH. This decrease is in line with effect seen in the H9c2 cells. Furthermore, TGF-β expression showed a decrease after both 1 and 3 hours of incubation with α-MSH, which parallels the effect seen on H9c2 cells. Nppb expression was not significantly affected by α-MSH treatment.

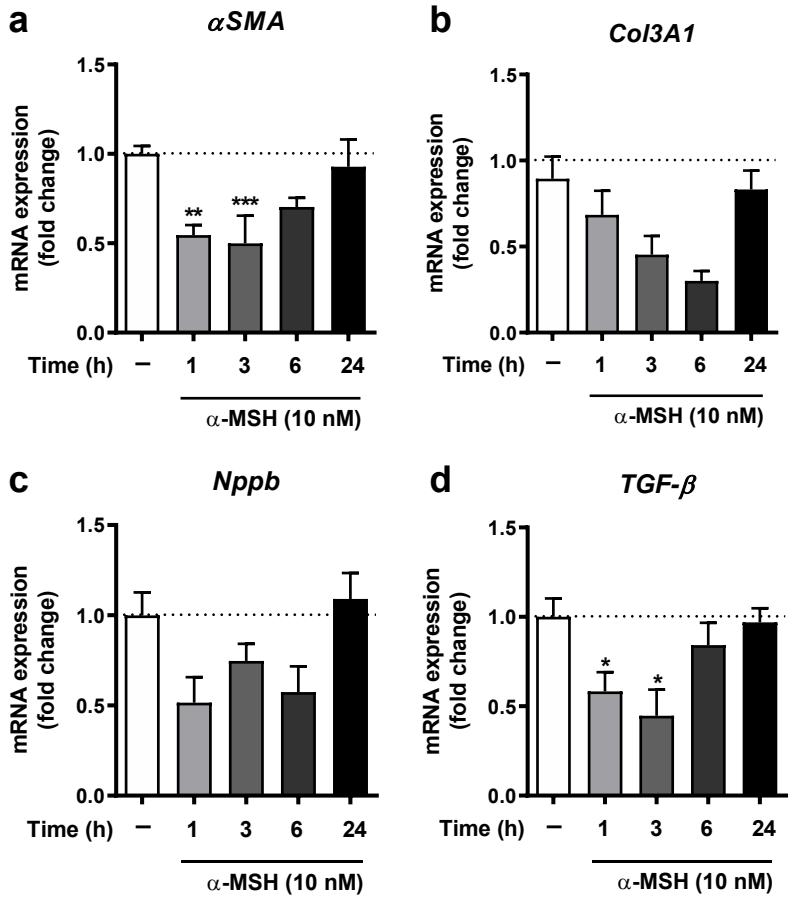

Figure 10. Compilation of results from qPCR tests performed on primary neonatal mouse cardiomyocytes after incubation with -8 M α-MSH at different times. The genes assayed were αSMA (alpha smooth muscle actin), Col3A1 (collagen 3A1), Nppb (ventricular natriuretic peptide) and TGF-β (transforming growth factor beta). One asterisk above a column denotes a P value lower than 0.05 when comparing that column to the control using a one-way ANOVA and a later Tukey multiple comparison test. Two asterisks denote a P value lower than 0.01 and three denote a P value lower than 0.001. Data are presented as mean ± SEM, presented are 5 replicates per study condition and 6 for the controls.

2.2.3 Subtype specific melanocortin receptor agonist treatments in H9c2 cell line

H9c2 cells were used to evaluate the effects of individual melanocortin receptor activation. In Figure 11, non-significant decreases were observed in αSMA and αSKA after treatment with the Mc5R agonist PG 901. Furthermore, PG 901 clearly reduced TGF-β expression, while the other melanocortin receptor agonists did not cause any observable changes.

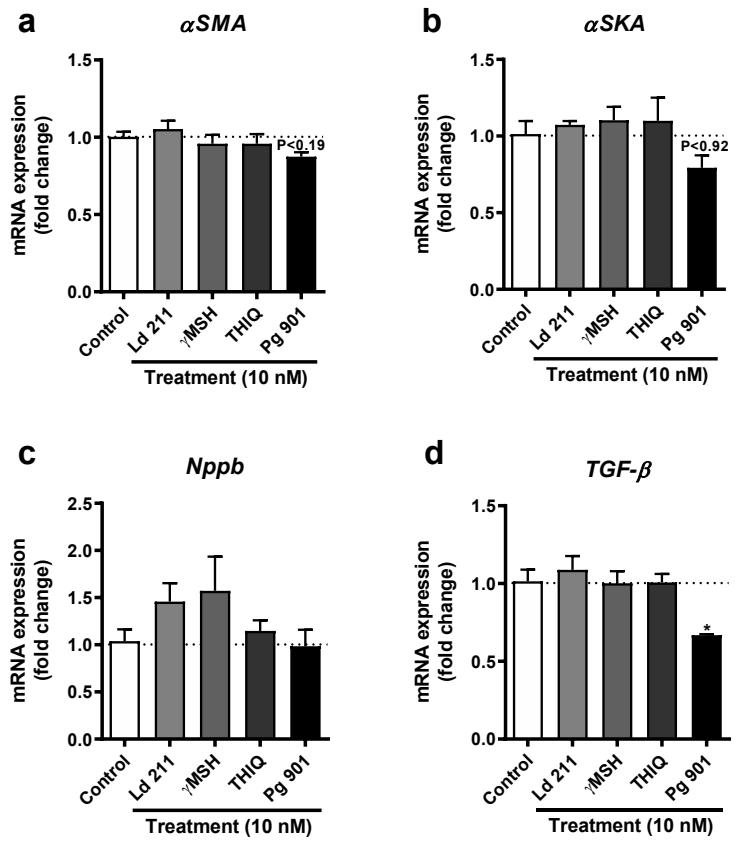

Figure 11. Compilation of results from qPCR tests performed on H9c2 cells after incubation with -8 M of different melanocortin receptor agonists for 3h. The genes assayed were αSMA (alpha smooth muscle actin), αSKA (alpha skeletal actin), Nppb (ventricular natriuretic peptide) and TGF-β (transforming growth factor beta). One asterisk above a column denotes a P value lower than 0.05 when comparing that column to the control using a one-way ANOVA and a later Tukey multiple comparison test. In the PG 901 columns in graph 12a and 12b the P value is shown as the results were in line with those expected and were used to reach the study conclusions, the exact P value was added for transparency. Data are presented as mean ± SEM, presented are 4 replicates per study condition and 6 for the controls.

In Figure 12, an increase in IL-6 expression can be seen after incubation with γ-MSH while a decrease can be seen after incubation with PG 901. NOS2 expression was also reduced by PG 901, while LD211 and γ-MSH increased its expression. Furthermore, Col3A1 expression was decreased after treatment with γ-MSH and PG 901. Overall, Mc5R activation with PG 901 induced strongest gene expression changes in H9c2 cells.

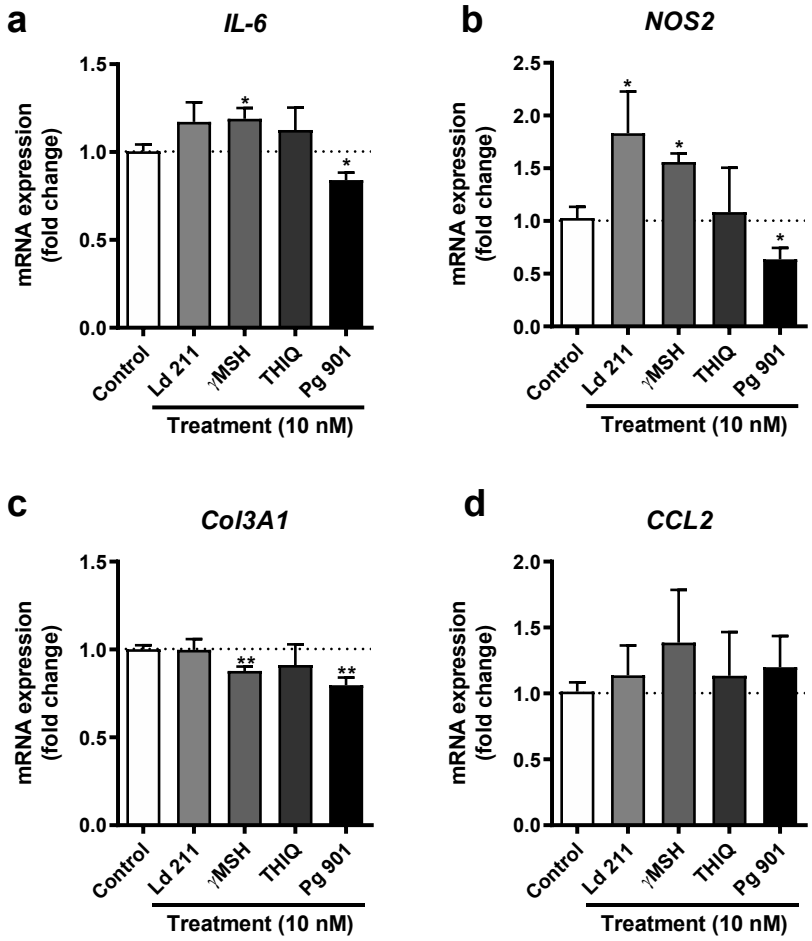

Figure 12. Compilation of results from qPCR tests performed on H9c2 cells after incubation with -8 M of different melanocortin receptor agonists for 3h. The genes assayed were IL-6 (interleukin 6), NOS2 (nitric oxide synthase 2), Col3A1 (collagen 3A1) and CCL2 (chemokine C-C motif ligand 2). One asterisk above a column denotes a P value lower than 0.05 when comparing that column to the control using a one-way ANOVA and a later Tukey multiple comparison test. Two asterisks denote a P value lower than 0.01. Data are presented as mean ± SEM, presented are 4 replicates per study condition and 6 for the controls.

2.2.4 Subtype specific melanocortin receptor agonist treatments in primary cardiomyocytes

The melanocortin receptor agonist treatments were also repeated in primary cardiomyocytes and the results can be seen in Figure 13. The effect of PG 901 on αSMA expression is stronger than that seen in H9c2 cells. Furthermore, the Mc1R agonist LD211 evoked a similar response as PG 901. The effect on NOS2 expression was negligible and did not follow the findings seen in the H9c2 cell line. The decrease in IL-6 expression caused by PG 901 is even more marked in the primary cardiomyocytes than in the H9c2 cell line, while the increase in expression caused by γ-MSH is not replicated. A decrease in IL-6 expression is also seen with LD211 treatment, an effect that was not observed in the cell line. Finally, both LD211 and PG 901 caused a decrease in Nppb expression.

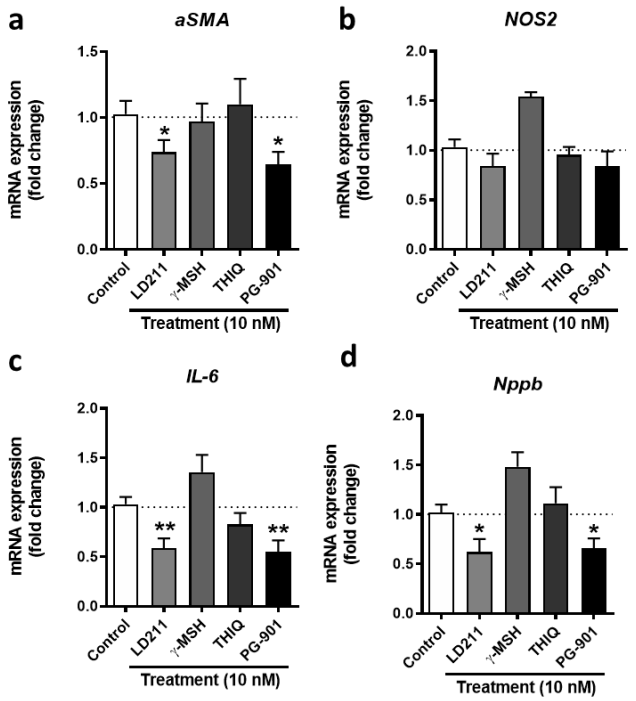

Figure 13. Compilation of results from qPCR tests performed on primary neonatal cardiomyocytes after incubation with -8 M of different melanocortin receptor agonists for 3h. The genes assayed were αSMA (alpha smooth muscle actin), NOS2 (nitric oxide synthase 2), IL-6 (interleukin 6) and Nppb (ventricular natriuretic peptide). One asterisk above a column denotes a P value lower than 0.05 when comparing that column to the control using a one-way ANOVA and a later Tukey multiple comparison test. Two asterisks denote a P value lower than 0.01. Data are presented as mean ± SEM, presented are 4 replicates per study condition and 6 for the controls.

To further study the effects of Mc1R activation seen in the previous experiments in primary cardiomyocytes, primary cardiomyocytes were further screened for concentration-responsiveness after a 3h treatment with LD211. The results are shown in Figure 14. Surprisingly, the decreases seen in αSMA, IL-6 and Nppb in the different melanocortin agonist experiment were not fully replicated in this experiment. LD211 tended to reduce αSMA expression with higher concentrations but the changes did not reach statistical significance. However, Col3A1 expression decreased after treatment with a 10 nM (-8 M) concentration of LD211.

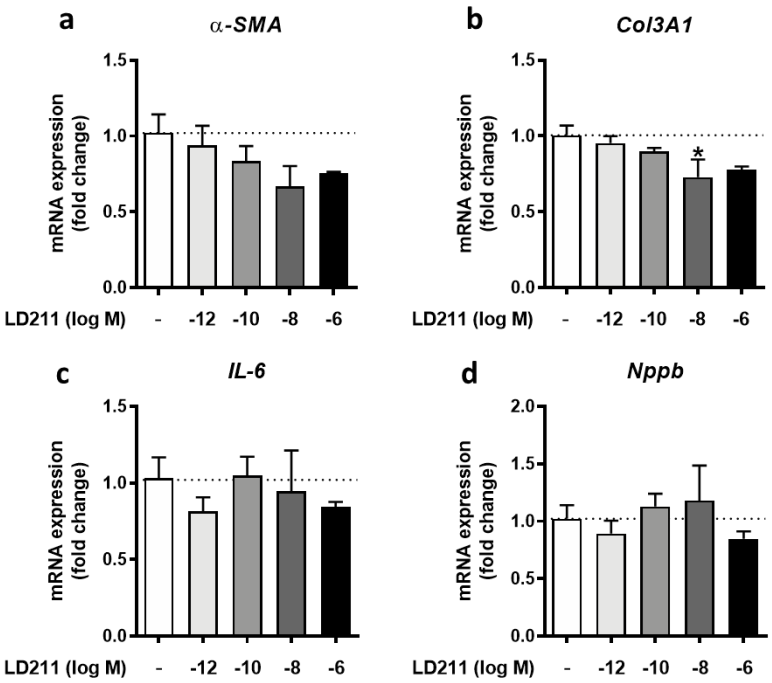

Figure 14. Compilation of results from qPCR tests performed on primary neonatal cardiomyocytes after incubation with different concentrations of the melanocortin receptor 1 agonist for 3h. The genes assayed were αSMA (alpha smooth muscle actin), Col3A1 (collagen 3A1), IL-6 (interleukin 6) and Nppb (ventricular natriuretic peptide). One asterisk above a column denotes a P value lower than 0.05 when comparing that column to the control using a one-way ANOVA and a later Tukey multiple comparison test. Data are presented as mean ± SEM, presented are 4 replicates per study condition and 6 for the controls.

A concentration series was also performed to identify what concentration of PG 901 causes the greatest effect in primary cardiomyocytes. In Figure 15, the effect of PG 901 on αSMA expression is clear as all concentrations of the Mc5R agonist decrease its expression. Similarly, the expression of IL-6 is reduced after treatment with each concentration of PG 901 used. Both of these decreases are in line with those seen in the multiple agonist experiment. Furthermore, NOS2 expression was decreased after 10 pM (-12 M) PG 901 treatment and Nppb expression after treatment with 10 nM (-8 M) concentration.

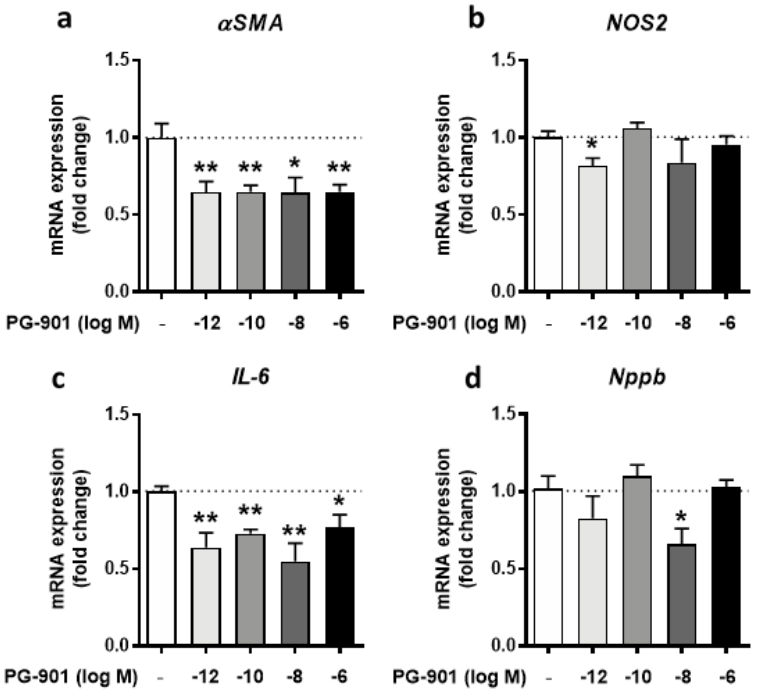

Figure 15. Compilation of results from qPCR tests performed on primary neonatal cardiomyocytes after incubation with different concentrations of the melanocortin receptor 1 agonist for 3h. The genes assayed were αSMA (Alpha smooth muscle actin), NOS2 (Nitric Oxide Synthase 2), IL-6 (Interleukin 6) and Nppb (Ventricular natriuretic peptide). One asterisk above a column denotes a P value lower than 0.05 when comparing that column to the control using a one-way ANOVA and a later Tukey multiple comparison test. Two asterisks denote a P value lower than 0.01. Data are presented as mean ± SEM, presented are 4 replicates per study condition and 6 for the controls.

2.2.4 Melanocortin receptor 5 knockdown in primary cardiomyocytes

Based on the qPCR results, PG 901 was identified to be the most potent melanocortin receptor agonist to induce gene expression changes. These results indicated that Mc5R is the most interesting melanocortin receptor subtype to continue with future research. For this reason, we investigated whether silencing of Mc5R with small interfering RNA (siRNA) could produce opposite responses to what was seen after Mc5R activation with PG 901. The results of the first experiment are summarized in Figure 16. Knockdown of MC-5R for 24 h or 48 h had no clear-cut effects on any of the genes studied. Subtle and non-significant increases were however observed in αSMA and NOS2 expression after 24 h treatment with Mc5R-specific siRNA. It was of note that Mc5R was not either significantly reduced indicating insufficient knockdown of Mc5R.

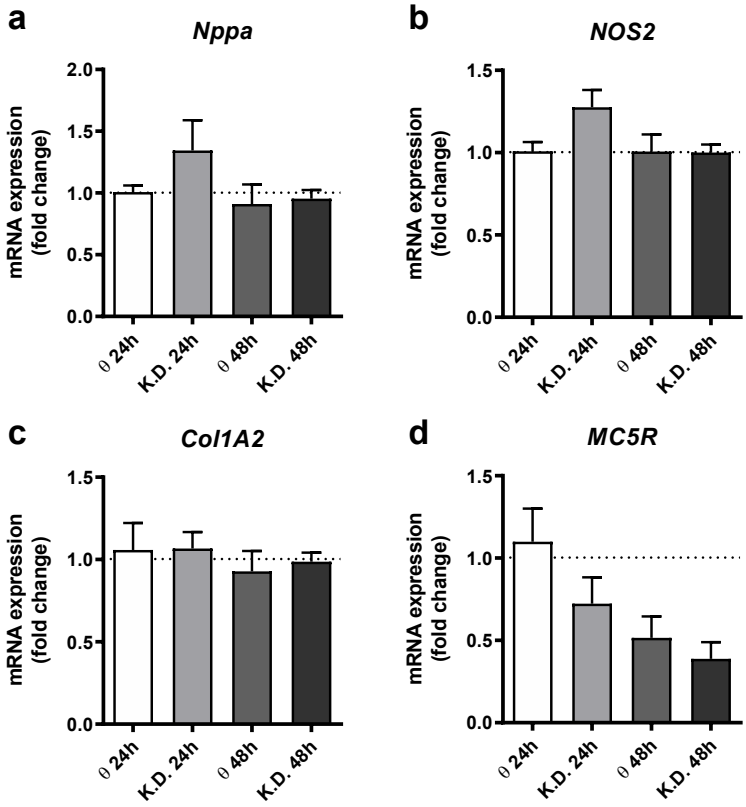

Figure 16. Compilation of results from qPCR tests performed on primary neonatal cardiomyocytes after Mc5R siRNA knockdown (K.D.) treatment for 24 and 48h. The sham knockdowns are designated by θ. The genes assayed were Nppa (atrial natriuretic peptide), NOS2 (nitric oxide synthase 2), Col1A2 (collagen 1A2) and Mc5R (melanocortin receptor 5). Data are presented as mean ± SEM, presented are 6 replicates per study condition and 6 for the controls.

Another experiment was performed with the Mc5R siRNA to test whether Mc5R cancels the effects of PG 901, the results of which can be seen in Figure 17. The Mc5R siRNA knockdown caused an increase in αSMA expression which was reverted by PG 901 treatment. This further consolidated that the Mc5R-specific siRNA was ineffective in silencing Mc5R signalling as there was still a clear response to PG 901. However, Mc5R siRNA-treated cells showed an opposite phenotype to PG 901 treated cells, pointing towards an important role for Mc5R in the regulation of cardiomyocytes.

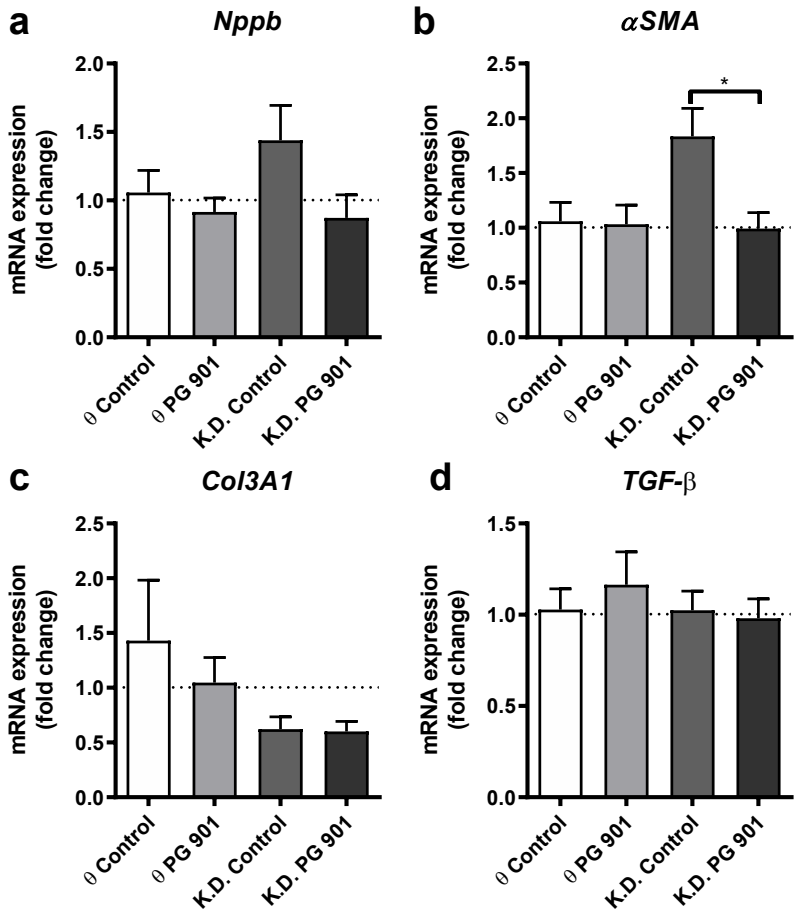

Figure 17. Compilation of results from qPCR tests performed on primary neonatal cardiomyocytes after Mc5R siRNA knockdown (K.D.) treatment for 24h and subsequent PG 901 -8M treatment for 3h. The sham knockdowns are designated by θ. The genes assayed were Nppb (ventricular natriuretic peptide), αSMA (alpha smooth muscle actin), Col3A1 (collagen 3A1) and TGF-β (transforming growth factor beta). One asterisk above a column denotes a P value lower than 0.05 when comparing that column to the control using a one-way ANOVA and a later Tukey multiple comparison test. Data are presented as mean ± SEM, presented are 6 replicates per study condition and 6 for the controls.

3. Discussion

Melanocortin signalling within cardiomyocytes has been proven to be more complex than previously known. In this study it was shown that different melanocortin receptor subtypes are present within cardiomyocytes and that their effects are sometimes antagonistic to each other, unveiling an underlying complexity previously unreported. To the knowledge of the author of this thesis only Mc5R has been confirmed as playing a role in cardiac hypertrophy(Trotta et al., 2018). The results previously presented point to the high likelihood that other melanocortin receptors may play a significant role as well. It was found that α-MSH produced decreases in expression of cardiac remodelling and hypertrophic markers (αSMA, αSKA, TGF-β and Nppb) in H9c2 cells and in primary mouse cardiomyocytes (αSMA, Col3A1 and TGF-β). These decreases were then found

to be caused by Mc5R activation in H9c2 cells and by both Mc5R and Mc1R activation in primary mice cardiomyocytes.

The attempts to study melanocortin signalling through using the phosphorylation of kinases was overall confusing. We observed some trends regarding a decrease in pJNK and pERK after PG 901 and LD211 treatment and after α-MSH, these however only appeared in some replicates, so the response was not consistent. Although not consistent overall the trends observed pointed to a decrease in pJNK and pERK which was promising as both are known to be involved in the signalling of NADPH oxidases after ischaemia, which leads to cardiac hypertrophy (Nabeebaccus et al., 2011). It is known that melanocortin receptors are G-protein-coupled receptors and have signalling dependant on cAMP, with CREB through PKA and ERK through the MAP/ERK pathway. JNK activation is driven through an increase in intracellular calcium. Despite these mediators being involved in the signalling of melanocortins, we were not able to optimize the ELISA tests to identify them consistently. Whether due to timing or sensitivity problems the exact cause of the experimental failure was not identified. All data from this first attempt at melanocortin receptor study had high variance and low replicability which could not be improved. After repeating those studies with a variety of parameters and only occasionally obtaining statistically significant results that disappeared upon replicating the study, it was decided to shelve these tests and approach the issue from a gene expression angle. The inconsistency in the significance of the results was attributed at this point to the very low amounts of protein detected and to the fact that α-MSH simultaneously activates multiple receptors that might lead to highly variable results.

The qPCR tests had much lower data dispersion so their results will be valued over those of other methods. Initially, the results obtained from the qPCRs were still not clear. There were effects on some of the genes studied but those changes in expression vanished upon repeating the experiment or the direction of the effect even changed. The first qPCR tests were performed on samples treated with α-MSH and the pleiotropic effect of the pan-agonist was thought to be a possible explanation for the inconsistent results.

To test this hypothesis, a set of experiments was carried out using melanocortin receptor subtype-specific agonists. In the rat heart cell line H9c2, PG 901 (Mc5R agonist) reduced the expression of αSMA, αSKA TGF-β, IL-6, NOS2 and Col3A1. These decreases were reproduced when the experiments were repeated with primary mouse cardiomyocytes where PG 901 produced decreases in αSMA, IL-6, Nppb and NOS2. These results show

that the activation of Mc5R has an overall protective effect on cardiac remodelling as it supresses the expression of remodelling markers (αSMA, αSKA, Nppb and Col3A1) and inflammatory markers (IL-6, TGF-β and NOS2). The effects of Mc5R activation are consistently protective against cardiac hypertrophy and show high reproducibility between models (αSMA, IL-6 and NOS2 expression was decreased in both models).

In the same multiple melanocortin receptor agonist series of experiments, LD211 increased IL-6 and NOS2 expression in H9c2 cells which was not replicated in primary cardiomyocytes. In contrast, LD211 reduced in αSMA, IL-6, Nppb and Col3A1 expression in primary cardiomyocytes. The overlap in genes affected by both Mc5R and Mc1R activation in primary cardiomyocytes warrants further research to dissect the roles of these receptor subtypes in the regulation of cardiomyocyte growth and cardiac remodelling. It was also not possible to determine if there was some contamination of fibroblasts and or endothelial cells accounting for the LD211 response in the primary cardiomyocytes or whether it was caused by a true difference between the mouse cardiomyocytes and the rat cell line. Possible causative agents for this difference would be the difference between adult and neonatal cardiomyocytes, the species difference or a possible deviation of the cell line.

The final set of experiments undertaken with the intent of studying gene expression was a Mc5R knockdown using siRNA in primary cardiomyocytes. The aim was to investigate whether silencing of Mc5R could lead to an opposite phenotype compared to cells that are activated by the Mc5R agonist PG 901. No clear-cut effects were seen in Mc5R siRNA-treated primary cardiomyocytes. The knockdown appears to not have been strong enough or long lasting-enough to significantly decrease the amount of Mc5R in the membrane of the cells and therefore affect their response to PG 901.

In conclusion, the effects of melanocortin signalling in the heart are versatile and complex. A future line of investigation that remains open is to identify and characterize the exact role of melanocortin receptors, namely Mc1R and Mc5R, in cardiac remodelling process. The results here given provide a first proof of the presence and effect of melanocortin receptors in the heart and highlight the need for further research to unravel the role of melanocortins in the heart.

Some experiments failed altogether, the results of which will be still provided in the Annex 3 as a guide for future research. Western blots attempting to analyse the protein levels directly instead of through mRNA expression failed to replicate the qPCR findings

and showed no changes in protein expression with high inter-replicate variation. While possible it is highly improbable that all the gene expression changes observed with qPCRs were not translated to proteins at all. It is more likely that the methodology used for the western blots lacked the resolution to differentiate between the small changes in protein level brought on by the tested treatments. Furthermore, Col3A1 proved a very challenging protein to work with as we were not able to isolate a clear band of it even after attempting to use a native protein gel to allow the antibody to properly bind to the correct secondary structure of the protein. Every time anti-Col3A1 labelled antibodies were used background specks appeared, likely from environmental contamination of Col3A1. Future work with the same antibody should be performed in a clean hood to minimise this contamination.

In this study, a general anti-fibrotic effect was observed for α-MSH in both cell types used and identified to be caused mostly by Mc5R activation. PG 901 caused decreases in hypertrophic genes and inflammation markers. Both αSKA and αSMA are upregulated during cardiac hypertrophy as they code for muscle proteins. Col3A1 as a building block of the extracellular matrix is known to be overexpressed during pathological cardiac remodelling. The decrease of expression of these genes forms the anti-hypertrophic effects of PG 901 found in both models.

IL-6 is a pleiotropic cytokine, but it is mostly regarded as cardioprotective due to decreased inflammation. TGF-β is generally known to be pro-inflammatory so a decrease in its expression would be cardioprotective. NOS2 produces nitric oxide upon cell stress, the accumulation of which induces cardiomyocyte apoptosis which is then followed by inflammation and scarring. The decrease of expression of these genes forms the anti-inflammatory effects of PG 901 found in both models. Finally, Nppb is hypertrophy marker so a decrease in this gene is in line with a cardioprotective effect of PG 901.

In conclusion, the results of this study point to a protective effect from cardiac hypertrophy of activation of Mc5R and to a lesser degree also Mc1R. Another study (Trotta et al., 2018) has found a potential cardioprotective effect of Mc5R activation in high-glucose induced hypertrophy which could be just one aspect of a general cardioprotective role of these receptors. If this were to be the case, there is the possibility that further research into this protective effect would end up with the development of a Mc5R agonistic treatment post-ischaemia to protect from cardiac remodelling and increase the long-term survival of those patients.

3.1 Methodological considerations

To study the potential effects of melanocortin modulation on cardiac hypertrophy it is paramount to use adequate models. To reduce the usage of test animals, the first experiments were performed in H9c2 cells. It is an established commercial rat cardiomyocyte cell line that has widely been used to model cardiac response and is especially reliable in regards to hypertrophy study and drug metabolism (Hescheler et al., 1991; Zordoky and El-Kadi, 2007; Watkins et al., 2011).

Established cell lines have many ethical, technical and financial advantages which entitles their use as experimental models over *in vivo* models. However, there might be some notable differences e.g. in gene expressions and metabolism which need to be considered. Therefore, it is valuable to verify results with primary cells and/or *in vivo* experiments. Primary neonatal cardiomyocytes are used in cardiac research over adult ones, since they are easier to culture *in vitro* while they still reproduce cardiac metabolism and cell morphology (Sreejit et al., 2008; Watkins et al., 2011). Both, rat and mouse neonatal cardiomyocytes are routinely used, but mice are easier to breed and overall cheaper to acquire.

4. Materials and methods

4.1 H9c2 cell line

A rat cardiomyocyte cell line sourced from ATCC with the identifier: H9c2(2-1) (ATCC® CRL1446™). This cell line was created by cloning an embryonic BDIX rat cardiac tissue and has been used since as a model for cardiac drug development (Kimes and Brandt, 1976). It has been characterized as a good model for cardiac metabolism and preserves many of the surface markers of an *in vivo* cardiomyocyte but lacks some of the morphological properties of primary cardiomyocyte culture; e.g. gap junctions, T tubules or organized sarcomeres along myofibrils (Hescheler et al., 1991). These cells were used at passage numbers ranging from 12 to 20. Stocks were kept in liquid nitrogen and thawed when needed. H9c2 cells were cultured in high glucose DMEM (4500mg/l, Sigma-Aldrich) supplemented with 1% Penicillin + Streptomycin and 10% foetal bovine serum (FBS). In maintenance cultures the medium was changed every third day and subcultured with dividing ratios ranging from 1:3 to 1:5 every third day or when confluency exceeded 80%. For experiments, cells were plated at 90.000 cells per 6-well plate or 50.000 cells per 12-well plate and let to grow until confluency in the wells reached between 60-70%.

4.2 Primary cardiomyocytes

Primary cardiomyocyte isolation was performed according to a revised version of Thermo Fisher's Pierce Primary cardiomyocyte isolation kit (Thermo Scientific). 0-3-day old C57Bl/6NCrl mice were euthanized, and their hearts were extracted and moved into a petri dish with ice-cold HBSS. On the dish, the hearts were cleaned with the use of tweezers and a scalpel to obtain pure myocardium. The isolated hearts were then transferred into 2 mL centrifuge tubes ranging from 500 µL to 1 mL of HBSS depending on the number of hearts (2-4). The hearts were then minced on-site at the animal facility using scissors until pieces are all smaller than 3mm in any direction. The hearts were then moved to a primary cell lab where they were digested using the kit's enzyme mixture and the final cell suspension was quantified using an automated cell counter gated between 4 and 12 µm (Judd et al., 2016) to count all cardiomyocytes and exclude other cell types. The cells were then plated to the final 500.000 cell/well concentration in 24-well plates in DMEM supplemented with 1% Penicillin + Streptomycin and 10% FBS. After 24h, the medium was refreshed and supplemented with cardiomyocyte growth supplement. The medium was refreshed again after 72 h and experiments were carried out 6-7 days post-extraction.

4.3 Elisa assays for quantifying intracellular signalling responses

ELISA assays were performed to detect the presence of phosphorylated CREB, JNK and ERK-1 using ELISA kits (DuoSet ELISA, R&D Systems). Results were normalised for total amount of protein using Thermo Fisher's Pierce BCA Protein Assay Kit (Pierce™ BCA Protein Assay Kit). These tests were performed as stated in their respective instructions while never breaking a chain of cold for the samples until they were pipetted ice-cold into the ELISA plate.

4.4 qPCR

RNA extraction was performed using either QIAzol (QIAGEN) and then purified with Zymo-Research's Quick-RNA kits (Quick-RNA Microprep for primary cardiomyocytes and Quick-RNA Miniprep for H9c2 cells). Samples were reverse-transcribed (PrimeScript RT reagent kit, Clontech) and then diluted with nuclease free water before performing a RT-PCR using a 2720 Applied Biosystems Thermal Cycler and predesigned primer and probe mix SYBR Green protocols (Kapa Biosystems) using the primer sequences given in Annex 2. Target protein expression was normalized to GADPH (H9c2

cells) or β-actin (primary cardiomyocytes), and the fold induction was calculated using the comparative ΔCt method and are presented as relative transcript levels ($2^{-\Delta\Delta Ct}$).

4.5 Melanocortin receptor agonists

To study the individual effects of activation of different melanocortin receptor subtypes and identify which melanocortin receptors are responsible for hypertrophy protecting effects the melanocortin receptor agonists LD211, γ-MSH, PG 901 and THIQ were used.

LD211 is a Mc1R-specific agonist that has been previously described in published literature (Doedens et al., 2010). In Doedens et. al. LD211 was described as compound 28. γ-MSH is a Mc3R-specific agonist, in this study D-Trp8-γ-MSH was used (Grieco et al., 2000). THIQ is a synthetic Mc4R-specific agonist (Pogozheva et al., 2005). PG 901 is a Mc5R-specific agonist with an antagonist role against Mc4R (Grieco et al., 2002).

α-MSH was used as a pan-agonist that activates melanocortin receptors 1, 3, 4 and 5 in decreasing order of affinity (Schiöth et al., 1997).

4.6 Mc5R siRNA knockdowns

To corroborate that the effects of PG 901 were caused through Mc5R activation a knockdown of the receptor gene was carried out by means of siRNA reduction of gene expression. A temporary interference in the production of Mc5R was expected to decrease the levels of the receptor present in the membranes to a level that a noticeable change in PG 901 response could be observed. The knockout was performed in accordance with Thermo Scientific's "Lipofectamine® RNAIMAX transfection protocol" using 6-well plates and using the negative control siRNA (a non-targeting siRNA) with the catalog number 4390843 and the Mc5R siRNA with catalog number 4390771 (which corresponds to the mouse Chr.18: 68337260 - 68342446 on Build GRCm38). Both siRNAs were also sourced from Thermo Scientific. Incubation under the effects of siRNA were 24 and 48h.

5. Acknowledgements

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Further experimental guidance was also provided by Keshav Thapa and James Jamal Kadiri.

This thesis as a cohesive document exists thanks to the support of Kia Katriina Peura and my family.

6. Abbreviations list

ACTH: adrenocorticotropic hormone

ANP: atrial natriuretic peptide

BNP: brain natriuretic peptide

CCL2: C-C motif chemokine Ligand 2

Col3A1: collagen 3 A 1

CREB: cAMP response element-binding protein

ERK: extracellular signal-regulated kinases

HFpEF: heart failure with preserved ejection fraction

HFrEF: heart failure with reduced ejection fraction

IL-6: interleukin 6

Mc1R: melanocortin 1 receptor

Mc2R: melanocortin 2 receptor

Mc3R: melanocortin 3 receptor

Mc4R: melanocortin 4 receptor

Mc5R: melanocortin 5 receptor

NOS2: nitric oxide synthase 2

pCREB: phosphorylated CREB

pERK: phosphorylated ERK

pJNK: phosphorylated JNK

POMC: pro-opiomelanocortin

α-MSH: alpha melanocyte stimulating hormone

αSKA: alpha skeletal actin

αSMA: alpha smooth muscle actin

γ-MSH: gamma melanocyte stimulating hormone

7. References

Abdel-Malek, Z.A. 2001. Melanocortin receptors: their functions and regulation by physiological agonists and antagonists. 58. 434–441 pp.

Abebe, T.B., E.A. Gebreyohannes, Y.G. Tefera, and T.M. Abegaz. 2016. Patients with HFpEF and HFrEF have different clinical characteristics but similar prognosis: a retrospective cohort study. *BMC Cardiovasc. Disord.* 16:232. doi:10.1186/s12872-016-0418-9.

Black, F.M., S.E. Packer, T.G. Parker, L.H. Michael, R. Roberts, R.J. Schwartz, and M.D. Schneider. 1991. The vascular smooth muscle α-actin gene is reactivated during cardiac hypertrophy provoked by load. *J. Clin. Invest.* 88:1581–1588. doi:10.1172/JCI115470.

Campbell, S.E., and L.C. Katwa. 1997. Angiotensin II stimulated expression of transforming growth factor-β1 in cardiac fibroblasts and myofibroblasts. *J. Mol. Cell. Cardiol.* 29:1947–1958. doi:10.1006/jmcc.1997.0435.

Carabello, B.A. 2002. Concentric versus eccentric remodeling. *In* Journal of Cardiac Failure. Churchill Livingstone Inc. S258–S263.

Chhajlani, V. 1996. Distribution of cDNA for melanocortin receptor subtypes in human tissues. *Biochem. Mol. Biol. Int.* 38:73–80.

Cone, R.D. 2006. Studies on the Physiological Functions of the Melanocortin System. *Endocr. Rev.* 27:736–749. doi:10.1210/er.2006-0034.

Cooray, S.N., and A.J.L. Clark. 2011. Melanocortin receptors and their accessory proteins. *Mol. Cell. Endocrinol.* 331:215–221. doi:10.1016/j.mce.2010.07.015.

Cramariuc, D., and E. Gerdts. 2016. Epidemiology of left ventricular hypertrophy in hypertension: implications for the clinic. *Expert Rev. Cardiovasc. Ther.* 14:915–926. doi:10.1080/14779072.2016.1186542.

Dobaczewski, M., W. Chen, and N.G. Frangogiannis. 2011. Transforming growth factor (TGF)-β signaling in cardiac remodeling. *J. Mol. Cell. Cardiol.* 51:600–606. doi:10.1016/j.yjmcc.2010.10.033.

Doedens, L., F. Opperer, M. Cai, J.G. Beck, M. Dedek, E. Palmer, V.J. Hruby, and H. Kessler. 2010. Multiple N -methylation of MT-II backbone amide bonds leads to

melanocortin receptor subtype hMC1R selectivity: Pharmacological and conformational studies. *J. Am. Chem. Soc.* 132:8115–8128. doi:10.1021/ja101428m.

Driesen, R.B., F.K. Verheyen, W. Debie, E. Blaauw, F.A. Babiker, R.N.M. Cornelussen, J. Ausma, M.H. Lenders, M. Borgers, C. Chaponnier, and F.C.S. Ramaekers. 2009. Re-expression of alpha skeletal actin as a marker for dedifferentiation in cardiac pathologies. *J. Cell. Mol. Med.* 13:896–908. doi:10.1111/j.1582-4934.2008.00523.x.

ELISAs and ELISA Kits: R&D Systems, ELISA Kit Manufacturer.

Engel, F.B. 2005. Cell Cycle Cardiomyocyte Proliferation: A Platform for Mammalian Cardiac Repair. doi:10.4161/cc.4.10.2081.

Feng, N., S.F. Young, G. Aguilera, E. Puricelli, D.C. Adler-Wailes, N.G. Sebring, and J.A. Yanovski. 2005. Co-occurrence of two partially inactivating polymorphisms of MC3R is associated with pediatric-onset obesity. *Diabetes*. 54:2663–7. doi:10.2337/diabetes.54.9.2663.

Fontes, J.A., N.R. Rose, and D. Čiháková. 2015. The varying faces of IL-6: From cardiac protection to cardiac failure. *Cytokine*. 74:62–68. doi:10.1016/j.cyto.2014.12.024.

Getting, S.J., C. Di Filippo, H.C. Christian, C.W. Lam, F. Rossi, M. D'Amico, and M. Perretti. 2004. MC-3 receptor and the inflammatory mechanisms activated in acute myocardial infarct. *J. Leukoc. Biol.* 76:845–853. doi:10.1189/jlb.0306175.

Girardet, C., and A.A. Butler. 2014. Neural melanocortin receptors in obesity and related metabolic disorders. *Biochim. Biophys. Acta - Mol. Basis Dis.* 1842:482–494. doi:10.1016/J.BBADIS.2013.05.004.

Gonçalves, J.P.L., D. Palmer, and M. Meldal. 2018. MC4R Agonists: Structural Overview on Antiobesity Therapeutics. *Trends Pharmacol. Sci.* 39:402–423. doi:10.1016/j.tips.2018.01.004.

Grieco, P., P.M. Balse, D. Weinberg, T. MacNeil, and V.J. Hruby. 2000. D-amino acid scan of γ-melanocyte-stimulating hormone: Importance of Trp8 on human MC3 receptor selectivity. *J. Med. Chem.* 43:4998–5002. doi:10.1021/jm000211e.

Grieco, P., G. Han, V.J. Hruby, D. Weinberg, and L.H.T. Van der Ploeg. 2002. Design and synthesis of highly potent and selective melanotropin analogues of SHU9119 modified at position 6. *Biochem. Biophys. Res. Commun.* 292:1075–1080.

doi:10.1006/bbrc.2002.6739.

Herraiz, C., J.C. Garcia-Borron, C. Jiménez-Cervantes, and C. Olivares. 2017. MC1R signaling. Intracellular partners and pathophysiological implications. *Biochim. Biophys. Acta - Mol. Basis Dis.* 1863:2448–2461. doi:10.1016/j.bbadis.2017.02.027.

Hescheler, J., R. Meyer, S. Plant, D. Krautwurst, W. Rosenthal, and G. Schultz. 1991. Morphological, biochemical, and electrophysiological characterization of a clonal cell (H9c2) line from rat heart. 69. 1476–1486 pp.

Humphreys, M.H., X.P. Ni, and D. Pearce. 2011. Cardiovascular effects of melanocortins. *Eur. J. Pharmacol.* 660:43–52. doi:10.1016/j.ejphar.2010.10.102.

Judd, J., J. Lovas, and G.N. Huang. 2016. Isolation, Culture and Transduction of Adult Mouse Cardiomyocytes. *J. Vis. Exp.* doi:10.3791/54012.

Kimes, B.W., and B.L. Brandt. 1976. Properties of a clonal muscle cell line from rat heart. *Exp. Cell Res.* 98:367–381. doi:10.1016/0014-4827(76)90447-X.

Kuusisto, J., V. Kärjä, P. Sipola, I. Kholová, K. Peuhkurinen, P. Jääskeläinen, A. Naukkarinen, S. Ylä-Herttuala, K. Punnonen, and M. Laakso. 2012. Low-grade inflammation and the phenotypic expression of myocardial fibrosis in hypertrophic cardiomyopathy. *Heart*. 98:1007–1013. doi:10.1136/heartjnl-2011-300960.

Leask, A. 2010. Potential therapeutic targets for cardiac fibrosis: TGFβ, angiotensin, endothelin, CCN2, and PDGF, partners in fibroblast activation. *Circ. Res.* 106:1675–1680. doi:10.1161/CIRCRESAHA.110.217737.

Letterio, J.J., and A.B. Roberts. 1998. REGULATION OF IMMUNE RESPONSES BY TGF-β. *Annu. Rev. Immunol.* 16:137–161. doi:10.1146/annurev.immunol.16.1.137.

Lowenstein, C.J., and E. Padalko. 2004. iNOS (NOS2) at a glance. *J. Cell Sci.* 117:2865–2867. doi:10.1242/jcs.01166.

Ma, F., Y. Li, L. Jia, Y. Han, J. Cheng, H. Li, Y. Qi, J. Du, and Y. Huang. 2012. Macrophage-Stimulated Cardiac Fibroblast Production of IL-6 Is Essential for TGF b/Smad Activation and Cardiac Fibrosis Induced by Angiotensin II. *PLoS One*. doi:10.1371/journal.pone.0035144.

Mihl, C., W.R.M. Dassen, and H. Kuipers. 2008. Cardiac remodelling: Concentric versus eccentric hypertrophy in strength and endurance athletes. *Netherlands Hear. J.*

16:129–133. doi:10.1007/BF03086131.

Mountjoy, K.G. 2010. Distribution and Function of Melanocortin Receptors within the Brain. *In* Advances in experimental medicine and biology. 29–48.

Nabeebaccus, A., M. Zhang, and A.M. Shah. 2011. NADPH oxidases and cardiac remodelling. *Heart Fail. Rev.* 16:5–12. doi:10.1007/s10741-010-9186-2.

Nagamoto, T., G. Eguchi, and D.C. Beebe. 2000. Alpha-smooth muscle actin expression in cultured lens epithelial cells. *Investig. Ophthalmol. Vis. Sci.* 41:1122–1129.

Nathan, C. 1997. Inducible Nitric Oxide Synthase: What Difference Does It Make? Perspectives Series: Nitric Oxide and Nitric Oxide Synthases Inducible Nitric Oxide Synthase: What Difference Does It Make? 100. 2417–2423 pp.

Oliver, P.M., J.E. Fox, R. Kim, H.A. Rockman, H.S. Kim, R.L. Reddick, K.N. Pandey, S.L. Milgram, O. Smithies, and N. Maeda. 1997. Hypertension, cardiac hypertrophy, and sudden death in mice lacking natriuretic peptide receptor A. *Proc. Natl. Acad. Sci. U. S. A.* 94:14730–14735. doi:10.1073/pnas.94.26.14730.

Pierce™ BCA Protein Assay Kit.

Pogozheva, I.D., B.X. Chai, A.L. Lomize, T.M. Fong, D.H. Weinberg, R.P. Nargund, M.W. Mulholland, I. Gantz, and H.I. Mosberg. 2005. Interactions of human melanocortin 4 receptor with nonpeptide and peptide agonists. *Biochemistry*. 44:11329–11341. doi:10.1021/bi0501840.

Roger, V.L. 2013. Epidemiology of heart failure. *Circ. Res.* 113:646–59. doi:10.1161/CIRCRESAHA.113.300268.

Samak, M., J. Fatullayev, A. Sabashnikov, M. Zeriouh, B. Schmack, M. Farag, A.-F. Popov, P.M. Dohmen, Y.-H. Choi, T. Wahlers, and A. Weymann. 2016. Cardiac Hypertrophy: An Introduction to Molecular and Cellular Basis. *Med. Sci. Monit. Basic Res.* 22:75–79. doi:10.12659/MSMBR.900437.

Schiöth, S., R. Muceniece, M. Larsson, and J.E.S. Wikberg. 1997. The melanocortin 1, 3, 4 or 5 receptors do not have a binding epitope for ACTH beyond the sequence of-MSH. 155. 73–78 pp.

Schultz, J.E.J., S.A. Witt, B.J. Glascock, M.L. Nieman, P.J. Reiser, S.L. Nix, T.R. Kimball, and T. Doetschman. 2002. TGF-β1 mediates the hypertrophic

cardiomyocyte growth induced by angiotensin II. *J. Clin. Invest.* 109:787–796. doi:10.1172/jci14190.

Shimizu, I., and T. Minamino. 2016. Physiological and pathological cardiac hypertrophy. *J. Mol. Cell. Cardiol.* 97:245–262. doi:10.1016/j.yjmcc.2016.06.001.

Simmonds, S.J., I. Cuijpers, S. Heymans, and E.A. V. Jones. 2020. Cellular and Molecular Differences between HFpEF and HFrEF: A Step Ahead in an Improved Pathological Understanding. *Cells*. 9:242. doi:10.3390/cells9010242.

Sreejit, P., S. Kumar, and R.S. Verma. 2008. An improved protocol for primary culture of cardiomyocyte from neonatal mice. *Vitr. Cell. Dev. Biol. - Anim.* 44:45–50. doi:10.1007/s11626-007-9079-4.

Starowicz, K., S.A. Mousa, I. Obara, A. Chocyk, R. Przewłocki, K. Wedzony, H. Machelska, and B. Przewłocka. Peripheral antinociceptive effects of MC4 receptor antagonists in a rat model of neuropathic pain - a biochemical and behavioral study. *Pharmacol. Rep.* 61:1086–95.

Tamura, N., Y. Ogawa, H. Chusho, K. Nakamura, K. Nakao, M. Suda, M. Kasahara, R. Hashimoto, G. Katsuura, M. Mukoyama, H. Itoh, Y. Saito, I. Tanaka, H. Otani, M. Katsuki, K. Nakao, and R.J. Lefkowitz. 2000. Cardiac fibrosis in mice lacking brain natriuretic peptide.

Thermo Scientific. Pierce Primary Cardiomyocyte Isolation Kit.

Thistlethwaite, D., J.A. Darling, R. Fraser, P.A. Mason, L.H. Rees, and R.A. Harkness. 1975. Familial glucocorticoid deficiency. Studies of diagnosis and pathogenesis. *Arch. Dis. Child.* 50:291–7. doi:10.1136/adc.50.4.291.

Trotta, M.C., R. Maisto, N. Alessio, A. Hermenean, M. D'Amico, and C. Di Filippo. 2018. The Melanocortin MC5R as a New Target for Treatment of High Glucose-Induced Hypertrophy of the Cardiac H9c2 Cells. *Front. Physiol.* 9:1475. doi:10.3389/fphys.2018.01475.

Umar, S., and A. Van Der Laarse. 2010. Nitric oxide and nitric oxide synthase isoforms in the normal, hypertrophic, and failing heart. *Mol. Cell. Biochem.* 333:191–201. doi:10.1007/s11010-009-0219-x.

Watkins, S.J., G.M. Borthwick, and H.M. Arthur. 2011. The H9C2 cell line and primary neonatal cardiomyocyte cells show similar hypertrophic responses in vitro. *Vitr.*

Cell. Dev. Biol. - Anim. 47:125–131. doi:10.1007/s11626-010-9368-1.

Weil, J., O. Zolk, J. Griepentrog, U. Wenzel, W.H. Zimmermann, and T. Eschenhagen. 2006. Alterations of the preproenkephalin system in cardiac hypertrophy and its role in atrioventricular conduction. *Cardiovasc. Res.* 69:412–422. doi:10.1016/j.cardiores.2005.10.016.

Yin, W., P. Zhang, J.H. Huang, Q.Y. Zhang, R. Fan, J. Li, J.J. Zhou, Y.Z. Hu, H.T. Guo, S.M. Zhang, Y.M. Wang, A.D. Kaye, C.H. Gu, J.C. Liu, L. Cheng, Q. Cui, D.H. Yi, and J.M. Pei. 2009. Stimulation of κ-opioid receptor reduces isoprenaline-induced cardiac hypertrophy and fibrosis. *Eur. J. Pharmacol.* 607:135–142. doi:10.1016/j.ejphar.2009.01.050.

Yue, T.L., J.L. Gu, C. Wang, A.D. Reith, J.C. Lee, R.C. Mirabile, R. Kreutz, Y. Wang, B. Maleeff, A.A. Parsons, and E.H. Ohlstein. 2000. Extracellular signal-regulated kinase plays an essential role in hypertrophic agonists, endothelin-1 and phenylephrine-induced cardiomyocyte hypertrophy. *J. Biol. Chem.* 275:37895–37901. doi:10.1074/jbc.M007037200.

Zordoky, B.N.M., and A.O.S. El-Kadi. 2007. H9c2 cell line is a valuable in vitro model to study the drug metabolizing enzymes in the heart. *J. Pharmacol. Toxicol. Methods*. 56:317–322. doi:10.1016/j.vascn.2007.06.001.

A. Appendices

A1: Raw experimental data

Raw data can be downloaded from:

https://drive.google.com/file/d/1REUMAxsnjHnRb8xxjyHykG4yXr_gqE76/view?usp=sharing

A2: Supplementary graphs

Supplementary graphs can be downloaded from:

https://drive.google.com/file/d/1J1c_5iBFTn12D9nR7FQ8tPchQrL-TEtf/view?usp=sharing

A3: Methods excluded from main body of thesis

A3.1 Western Blot

Western blots were carried out according to an in-house protocol previously described in published article (Rinne et al., 2017). Cells were lysed using RIPA buffer, proteins were separated by size through SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. After a blocking step with BSA, membranes were probed with primary antibodies followed by detection with HRP-linked secondary antibodies, all antibodies used can be found in Table 1. Target protein expression was normalized to correct for loading imbalances to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin depending on target protein size and potential overlap with target protein bands.

Table 1. Compilation of all antibodies used for western blotting. Target protein information can be found in the target and target organism columns. The source refers to the IgG heavy chain origin. Working dilution and providing company are also included.

Target	Target organism	Source	Dilution	Company
Col3A1	Mouse	Rabbit	1:2000	Novusbio
TGF-β1	Mouse	Rabbit	1:2000	Novusbio
αSMA	Mouse	Rabbit	1:2000	Sigma-Aldrich
αSMA	Rat	Mouse	1:2000	Biolegend
Anti-Rabbit IgG, HRP-linked	Rabbit		1:2000	Cell Signalling Technology
Anti-mouse IgG, HRP-linked	Mouse		1:2000	Cell Signalling Technology

Membranes were imaged using Sapphire Biomolecular Imager from Azure Biosystems and stripped for reprobing using Abcam's stripping protocol using the mild stripping buffer therein described.

A3.1.1 Native gel electrophoresis

For the purpose of analysing Col3A1, a native gel electrophoresis was performed according to a protocol by the Institute of Molecular Development published in 2012. The protocol was followed without significant changes. The separating gel was 5 % acrylamide, and the de-gassing was performed with an air pump set at -5 atmospheres.

A3.2 Leucine incorporation assay

To assay overall changes in protein production, tritium-labelled [^{3}H] leucine incorporation assays were performed. When the cells had reached a confluence level of 80-90% per well, the medium was changed and replaced with new medium containing tritium-labelled leucine (1-2 µCi/ml medium, Perkin Elmer). After medium change, cell were treated with melanocortin receptor agonists and allowed to grow for 24-48h. After medium aspiration and two PBS washes, proteins were precipitated using 10% tricloroacetic acid (TCA) and incubated at 4°C for 30 min. Cells were then washed twice using ice-cold MilliQ water and lysed with 0.5 M NaOH (200 µL/well). After adding NaOH into wells, plates were kept 10-20 minutes at 37°C and thereafter wells were scraped with plastic cell scrapers to ensure complete removal of cells from the wells. Cell lysates were then collected into Eppendorf tubes. 100 µL of each sample were added to 3 ml of LSC cocktail (Optiphase Supermix, Perkin Elmer) into liquid scintillation vials. Samples were then measured with Hidex 600 SL Automatic liquid scintillation counter at 5 min count time per vial.