TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 1050

MEDICA - ODONTOLOGICA

NEW PERSPECTIVES ON THE MELANOCORTINS AND THEIR CARDIOVASCULAR EFFECTS

Potential implications for the treatment of cardiovascular diseases with melanocortin analogues

by

Petteri Rinne

TURUN YLIOPISTO
UNIVERSITY OF TURKU
Turku 2012

From the Institute of Biomedicine,
Department of Pharmacology, Drug Development and Therapeutics,
University of Turku, Finland

Supervised by

Docent Eriika Savontaus, MD, PhD
Department of Pharmacology, Drug Development and Therapeutics
University of Turku

Reviewed by

Professor Michael H. Humphreys, MD
Division of Nephrology, San Francisco General Hospital
University of California San Francisco, San Francisco, USA

and

Docent Risto Kerkelä, MD, PhD
Institute of Biomedicine, Department of Pharmacology and Toxicology
University of Oulu, Oulu, Finland

Dissertation opponent

Professor Eero Mervaala, MD, PhD
Institute of Biomedicine
University of Helsinki, Helsinki, Finland

ISBN 978-951-29-5268-7 (PRINT)
ISBN 978-951-29-5269-4 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2012

Jotka tulevat suorinta tietä, saapuvat tyhjin taskuin.
Jotka ovat kolunneet kaikki polut,
tulevat säihkyvin silmin, polvet ruvella,
outoja hedelmiä hauraassa säkissään.
Niin se on, niin se on, että eksymättä et löydä perille.

- Tommy Tabermann (Veren sokeri 2008)

ABSTRACT

Petteri Rinne

New aspects on the melanocortins and their cardiovascular effects: Potential implications for the treatment of cardiovascular diseases with melanocortin analogues

Department of Pharmacology, Drug Development and Therapeutics, and Drug Discovery Graduate School, University of Turku, Finland

Annales Universitatis Turkuensis, Painosalama Oy, Turku, Finland 2012

The melanocortin peptides, including melanocyte-stimulating hormones, α-, β- and γ-MSH, are derived from the precursor peptide proopiomelanocortin and mediate their biological actions via five different melanocortin receptors, named from MC1 to MC5. Melanocortins have been implicated in the central regulation of energy balance and cardiovascular functions, but their local effects, via yet unidentified sites of action, in the vasculature, and their therapeutic potential in major vascular pathologies remain unclear. Therefore, the main aim of this thesis was to characterise the role of melanocortins in circulatory regulation, and to investigate whether targeting of the melanocortin system by pharmacological means could translate into therapeutic benefits in the treatment of cardiovascular diseases such as hypertension.

In experiments designed to elucidate the local effects of α-MSH on vascular tone, it was found that α-MSH improved blood vessel relaxation via a nitric oxide (NO)-dependent mechanism without directly contracting or relaxing blood vessels. Furthermore, α-MSH was shown to regulate the expression and function of endothelial NO synthase in cultured human endothelial cells via melanocortin 1 receptors. In keeping with the vascular protective role, pharmacological treatment of mice with α-MSH analogues displayed therapeutic efficacy in conditions associated with vascular dysfunction such as obesity. Furthermore, α-MSH analogues elicited marked diuretic and natriuretic responses, which together with their vascular effects, seemed to provide protection against sodium retention and blood pressure elevation in experimental models of hypertension.

In conclusion, the present results identify novel effects for melanocortins in the local control of vascular function, pointing to the potential future use of melanocortin analogues in the treatment of cardiovascular pathologies.

Key words: melanocortins, vasodilatation, endothelial function, nitric oxide, hypertension

TIIVISTELMÄ

Petteri Rinne

Melanokortiinien sydän- ja verisuonivaikutukset: Mahdollisuudet melanokortiinianalogien käytölle sydän- ja verisuonisairauksien hoidossa.

Farmakologia, lääkekehitys ja lääkehoito sekä Lääkekehityksen tutkijakoulu, Turun yliopisto

Annales Universitatis Turkuensis, Painosalama Oy, Turku, Finland 2012

Melanokortiinit, joihin lukeutuvat α-, β- ja γ-melanosyyttejä stimuloiva hormoni (MSH), ovat pääasiassa aivoissa ilmenevän esiasteen pro-opiomelanokortiinin pilkkoutumisiessa syntyviä, pienempiä peptidejä, jotka vaikuttavat melanokortiinireseptoreiden välityksellä elimistön fysiologisiin toimintoihin. Melanokortiinit osallistuvat merkittävälla tavalla elimistön energiatasapainon sekä sydämen ja verenkiertoelimistön keskushermostoperäiseen säätelyyn, mutta niiden paikalliset vaikutukset ja vaikutusmekanismit verenkierron säätelyssä ovat pitkälti tuntemattomia. Lisäksi, melanokortiinien mahdollisista, terapeuttisista vaikutuksista sydän- ja verisuonisairauksien hoidossa tiedetään hyvin vähän. Tämän väitöskirjatutkimuksen keskeisimpänä tavoitteena oli tutkia melanokortiinien vaikutuksia verisuonten toiminnan säätelyssä sekä arvioida löydösten merkitystä uusien hoitomahdollisuuksien kannalta.

Suonten toimintakykyä mittaavissa kokeissa havaitsimme, että α-MSH tehosti verisuonten sisäpintaa verhoavan endoteelin kykyä laajentaa verisuonia ilman, että se itse suoraan vaikutti suonten supistumistilaan. Tämä vaikutus oli yhteydessä verisuonten lisääntyneeseen typpioksidin (NO) tuottoon ja herkkyyteen NO:n verisuonia laajentavalle vaikutukselle. Viljelemällä ihmisperäisiä endoteelisoluja osoitimme melanokortiini 1 reseptoreiden välittävän α-MSH vaikutuksia NO:n tuotantoa sääteleviin tekijöihin kuten endoteliaalisen NO syntaasin määrään ja aktiivisuuteen. Näiden verisuonten toimintaa parantavien vaikutusten ansiosta, α-MSH-analogit paransivat verisuonten toimintaa kokeellisissa tautimalleissa, joihin liittyy erityisesti endoteelin toiminnan häiriö kuten lihavuuden yhteydessä. Lisäksi havaitsimme, että α-MSH:n synteettinen analogi lisää veden ja natriumin erittymistä elimistöstä sekä vaikuttaa terapeuttisesti kohonneen verenpaineen hoidossa hiirillä. Nämä löydökset laajentavat ymmärrystämme melanokortiinien vaikutuksista verenkierron säätelyssä tuoden samalla näyttöä uusista hoitomahdollisuuksista sydän- ja verisuonisairauksissa.

Avainsanat: melanokortiinit, verisuonen laajeneminen, endoteelin toiminta, typpioksidi, kohonnut verenpaine

Table of Contents

ABSTRACT **4**

TIIVISTELMÄ **5**

ABBREVIATIONS **9**

LIST OF ORIGINAL PUBLICATIONS **10**

1 INTRODUCTION **11**

2 REVIEW OF THE LITERATURE **13**

2.1 Pro-opiomelanocortin and melanocortins 13
2.2 Melanocortin receptors 15
2.2.1 MC1 receptor 15
2.2.2 MC2 receptor 16
2.2.3 MC3 receptor 17
2.2.4 MC4 receptor 17
2.2.5 MC5 receptor 17
2.2.6 Endogenous antagonists of melanocortin receptors 18
2.2.7 Pharmacokinetic and -dynamic properties of superpotent α-MSH analogues 19
2.3 Physiological roles of melanocortins 20
2.3.1 Melanocortins and regulation of energy balance 20
2.3.1.1 Overview of the central melanocortin system for sensing and orchestrating energy homeostasis 20
2.3.1.2 Peripheral metabolic signals and their interaction with the central melanocortin system 21
2.3.1.3 Integration of metabolic signals in the melanocortin system 22
2.3.1.4 Insights from monogenic syndromes of obesity 23
2.3.2 Melanocortins and the cardiovascular system 25
2.3.2.1 Hypertensive effects of melanocortins 26
2.3.2.2 Hypotensive effects of melanocortins 27
2.3.3 Melanocortins and sodium metabolism 28
2.3.3.1 γ-MSH and salt-sensitive hypertension 29
2.4 Basic principles of blood pressure regulation 30
2.4.1 Cardiovascular system 30
2.4.2 Regulation of blood pressure 31
2.4.2.1 Autonomic nervous system 33
2.4.2.2 Baroreceptor reflex 35

2.4.2.3 Autonomic imbalance and cardiovascular disease 36
2.4.3 Vascular endothelium in the control of vascular tone 36
2.4.3.1 Nitric oxide 37
2.4.3.2 Endothelial dysfunction 39

3 OBJECTIVES OF THE STUDY 41

4 MATERIALS AND METHODS 42
4.1 Animals and animal models 42
4.1.1 Ethical aspects 42
4.1.2 Pharmacologic models of acute and chronic melanocortin activation 42
4.1.3 Transgenic MSH overexpressing mouse model 42
4.2 Animal models of cardiovascular disease 43
4.2.1 Diet-induced obesity 43
4.2.2 DOCA-salt hypertension model 43
4.2.3 Pressure- and volume-overload induced by a high-sodium diet 43
4.3 Physiology 43
4.3.1 Arterial pressure measurements 43
4.3.1.1 Measurements in anesthetized mice 43
4.3.1.2 Blood pressure telemetry 44
4.3.1.3 Tail cuff measurements 44
4.3.2 Spectral analysis of heart rate and blood pressure variability 44
4.3.3 Spontaneous baroreflex sensitivity 45
4.3.4 Pharmacological testing 45
4.3.5 Doppler echocardiography 46
4.3.6 Measurements of diuresis 46
4.4 Ex vivo studies 47
4.4.1 Vascular function assessment 47
4.4.1.1 Preparation and mounting of the arteries 47
4.4.1.2 Vascular reactivity measurements 47
4.4.2 Assessment of coronary endothelial function in the isolated mouse heart 47
4.4.3 cGMP content of aortic rings 48
4.5 Biochemical analyses 48
4.5.1 Quantitative reverse-transcription PCR 48
4.5.2 Urinary catecholamine, NOx, cGMP, creatinine and electrolyte concentrations 49
4.5.3 In situ detection of vascular superoxide production 49
4.5.4 SDS-PAGE and western blotting 49
4.6 Drugs and solutions 49
4.7 Statistical methods 50

5 RESULTS 51
5.1 Hemodynamic actions and mechanisms of α-MSH analogues 51

5.2 Blood pressure regulation in transgenic MSH-OE mice 54
5.3 Cardiovascular autonomic control in transgenic MSH-OE mice 55
5.3.1 Evaluation of heart rate variability 55
5.3.2 Blood pressure and heart rate responses to adrenergic receptor blockers ..56
5.4 The effect of MSH overexpression on baroreflex function 57
5.5 Pharmacologic model of chronic melanocortin activation and autonomic balance 58
5.6 Melanocortins in the local control of vascular tone 58
5.6.1 Key observations from isometric tension recordings 59
5.6.2 The role of melanocortins in coronary flow regulation 60
5.6.3 Effects of α-MSH on the eNOS system and antioxidant enzymes in cultured human endothelial cells 61
5.7 Melanocortins and the regulation of body fluid balance 62
5.8 Therapeutic efficacy of melanocortins in hypertension 63

6 DISCUSSION 66
6.1 Methodological considerations 66
6.1.1 Genetic and pharmacologic models of chronic melanocortin activation ...66
6.1.2 Blood pressure measurement techniques 67
6.1.3 Evaluation of cardiovascular autonomic control 68
6.1.4 DOCA-salt model of hypertension 69
6.2 Acute effects of systemically administered α-MSH analogues on hemodynamics 70
6.3 Blood pressure regulation in MSH-OE mice 72
6.4 Heart rate regulation and autonomic balance in chronic models of melanocortin activation 73
6.5 The vascular protective role of α-MSH 75
6.6 Therapeutic perspectives 76

7 SUMMARY AND CONCLUSIONS 81

8 ACKNOWLEDGEMENTS 82

REFERENCES 84

ORIGINAL PUBLICATIONS 93

ABBREVIATIONS

ACh	Acetylcholine
AgRP	Agouti-related peptide
BBB	Blood-brain barrier
CFR	Coronary flow reserve
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
CO	Cardiac output
DBP	Diastolic blood pressure
DIO	Diet-induced obesity
DOCA	Deoxycorticosterone acetate
eNOS	Endothelial nitric oxide synthase
HR	Heart rate
i.c.v.	Intracerebroventricular
i.p.	Intraperitoneal
i.v.	Intravenous
MAP	Mean arterial pressure
MC	Melanocortin receptor
mRNA	Messenger ribonucleic acid
MSH	Melanocyte-stimulating hormone
MT-II	Melanotan-II
NDP-α-MSH	[Nle^4, D-Phe^7]-α-melanocyte-stimulating hormone
NO	Nitric oxide
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarius
OE	Overexpression
POMC	Pro-opiomelanocortin
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SEM	Standard error of the mean
sGC	Soluble guanylate cyclase
SV	Stroke volume

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals I-V:

I Rinne P, Harjunpää J, Scheinin M, and Savontaus E (2008). Blood pressure regulation and cardiac autonomic control in mice overexpressing α- and γ-Melanocyte Stimulating Hormone. *Peptides* **29**(11):1943-52

II Rinne P, Tikka S, Mäkelä S, Streng T, and Savontaus E (2012). Hemodynamic actions and mechanisms of systemically administered α-MSH analogs. *Peptides* **38**(1):150-8.

III Rinne P, Harjunpää J, Mäkelä S, and Savontaus E (2012). Genetic and pharmacologic mouse models of chronic melanocortin activation show enhanced baroreflex control of heart rate. *Regul Pept,* revision submitted.

IV Rinne P, Nordlund W, Heinonen I, Penttinen A-M, Saraste A, Ruohonen ST, Mäkelä S, Vähätalo L, Kaipio K, Cai M, Hruby VJ, Ruohonen S, and Savontaus E (2012). α-Melanocyte-stimulating hormone regulates vascular NO availability and protects against endothelial dysfunction. *Cardiovasc Res,* in press.

V Rinne P, Penttinen A-M, Nordlund W, Ahotupa M, and Savontaus E. α-MSH analogue attenuates blood pressure elevation and sodium retention in DOCA-salt hypertensive mice. *Manuscript submitted.*

In addition, some unpublished data are presented in this thesis.

1 INTRODUCTION

There is increasing awareness that there is a global epidemic of cardiovascular disease (CVD) encompassing a range of conditions, from hypertension to coronary heart disease, stroke, and chronic heart failure. Hypertension, characterized by chronically increased blood pressure, is one of the leading risk factors for cardiovascular complications. It is largely attributable to disturbances in the sympathetic nervous system and the renin-angiotensin system, which play a crucial role in renal sodium and water handling as well as in regulating vascular resistance. Unless adequately controlled by life-style intervention and/or medical treatment, hypertension increases morbidity and mortality for other CVD such as stroke and acute coronary syndrome, and leads frequently to end-organ damage, including cardiac hypertrophy and kidney failure. Thus, hypertension is a major threat to human health and an important public-health challenge. The World Health Organization has recently developed an action plan to tackle the global burden of these issues. This advocates key strategies for the prevention and control of noncommunicable diseases and includes hypertension and other CVD in this list. One of the objectives of the action plan is to promote research to identify new ways to treat these diseases. As a contribution to this praiseworthy objective, the specific interest of this thesis was to identify and characterise new treatment targets and modalities for CVD by investigating the cardiovascular actions and mechanisms of melanocortins.

Melanocyte-stimulating hormones (α-, β- and γ-MSH), which are derived from the precursor peptide pro-opiomelanocortin (POMC), have important roles in feeding and energy metabolism. They mediate their biological actions through a family of five related melanocortin receptors, MC1-MC5 (Chhajlani *et al.*, 1993; Chhajlani *et al.*, 1992; Gantz *et al.*, 1993a; Gantz *et al.*, 1993b; Mountjoy *et al.*, 1992). In particular, α-MSH suppresses appetite and increases energy expenditure by binding to MC4 receptors in its target neurons in the central nervous system (Schwartz *et al.*, 2000; Williams *et al.*, 2005). Owing to this favourable effect in the control of energy balance, the melanocortin system is considered as a potential target for the treatment of obesity as well as other weight disorders (Wikberg *et al.*, 2008). However, it is important to take into account the diverse roles of melanocortins as they are likely to be involved in a wide range of other physiological processes, including cardiovascular regulation and sodium metabolism. There is convincing data demonstrating that POMC-derived melanocortins, consisting of MSH peptides together with adrenocorticotropic hormone (ACTH), are involved in the central regulation of cardiovascular functions. However, the role of melanocortins in cardiovascular regulation seems to be a complex and multifaceted issue. For instance, the infusion of a highly-selective MC4-R agonist has evoked marked increases in blood pressure and heart rate in humans (Greenfield *et al.*, 2009), which has complicated the development of these types of drugs for the treatment of obesity. On the other hand, peripherally administered melanocortin receptor agonists have shown resuscitating and organ protective effects in animal models of myocardial infarct, ischemic stroke and

haemorrhagic shock, perhaps by activating central MC receptors and by triggering a vagus-nerve mediated protective pathway (Getting *et al.*, 2004; Giuliani *et al.*, 2006b; Giuliani *et al.*, 2007; Ottani *et al.*, 2010). Furthermore, melanocortins have elicited blood pressure lowering effects through brainstem melanocortin circuits (De Wildt *et al.*, 1994; Pavia *et al.*, 2003). Further studies are therefore needed to achieve a better understanding of the role of melanocortins in cardiovascular physiology and pathophysiology.

The original aim of this thesis was to gain insights into the long-term effects of melanocortins in cardiovascular regulation by studying a mouse model overexpressing α-MSH. Through series of physiological experiments on this genetic model and other pharmacologic models that mimicked melanocortin overactivity, it gradually became evident that melanocortins exerted wide-ranging effects which pointed to new implications for melanocortins in cardiovascular regulation and by which melanocortins might provide protection for the heart and the vasculature against pathophysiological processes. Having identified the novel effects for melanocortins, these hypotheses were refined and directed towards clarifying the therapeutic potential of melanocortin agonists in cardiovascular disease. This thesis focuses on highlighting the beneficial properties of melanocortins on cardiovascular health and disease. Although the translation of these results into human disease may still lie some way in the future, they provide encouragement for elucidating the potentially protective role of melanocortins in disease models that have higher relevance and validity for human disease.

2 REVIEW OF THE LITERATURE

2.1 Pro-opiomelanocortin and melanocortins

The melanocortins are a family of peptides that are cleaved from a common precursor molecule known as pro-opiomelanocortin (POMC). They were originally recognised as a factor residing in the pituitary gland that caused darkening of the skin in frogs. In the 1950s, these pituitary hormones were purified and named ACTH, α- and β-melanocyte stimulating hormones (MSH). In 1979, Nakanishi et al. published the amino acid sequence of the POMC molecule which ultimately confirmed that the melanocortin peptides were derived from this large precursor molecule (Nakanishi *et al.*, 1979). They also identified that the POMC contained yet another MSH-like peptide sequence that shared a common heptapeptide core with ACTH and the other MSH peptides. This new peptide was termed γ-MSH because of its homology to α- and β-MSH. Several other biologically active peptides were identified as cleavage products of POMC, namely β- and γ-LPH, β-endorphin and corticotropin-like intermediate peptide (CLIP) which is cleaved from ACTH (Fig. 2.1). Subsequently, three different species of γ-MSH have been identified; γ_1-, γ_2- and γ_3-MSH. γ_1-and γ_2-MSH are structurally very close to each other whereas γ_3-MSH is a longer 25-amino acid sequence (Fig. 2.2). The functional role of these different species of γ-MSH is not completely understood.

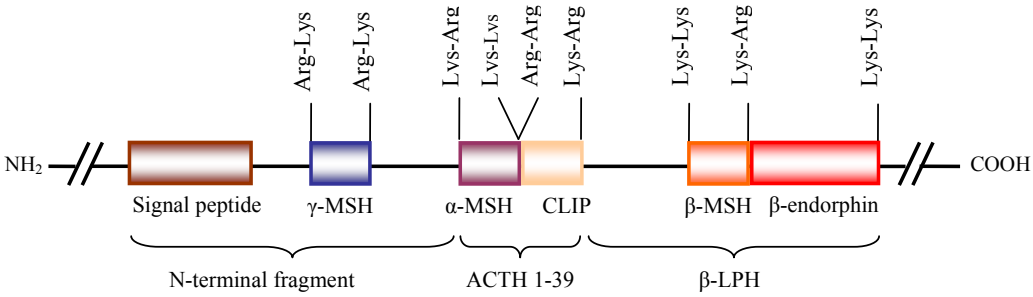

Figure 2.1. Structure of the POMC molecule showing the dibasic amino acid sites of posttranslational processing of the molecule.

POMC is predominantly expressed in the pituitary gland, particularly in the corticotrophs of the anterior lobe but also in the melanotrophs of the intermediate lobe. In addition, it is found in other parts of the CNS, including the arcuate nucleus of the hypothalamus and nucleus of the solitary tract (NTS), and in a variety of peripheral tissues such as the skin, pancreas, gastrointestinal (GI) tract, kidney and liver (Smith et al., 1988). According to the current understanding, POMC-derived peptides are released into circulation by the pituitary gland, whereas peptides in other tissues function in an autocrine or paracrine manner. Processing of POMC into biologically active components is driven by the

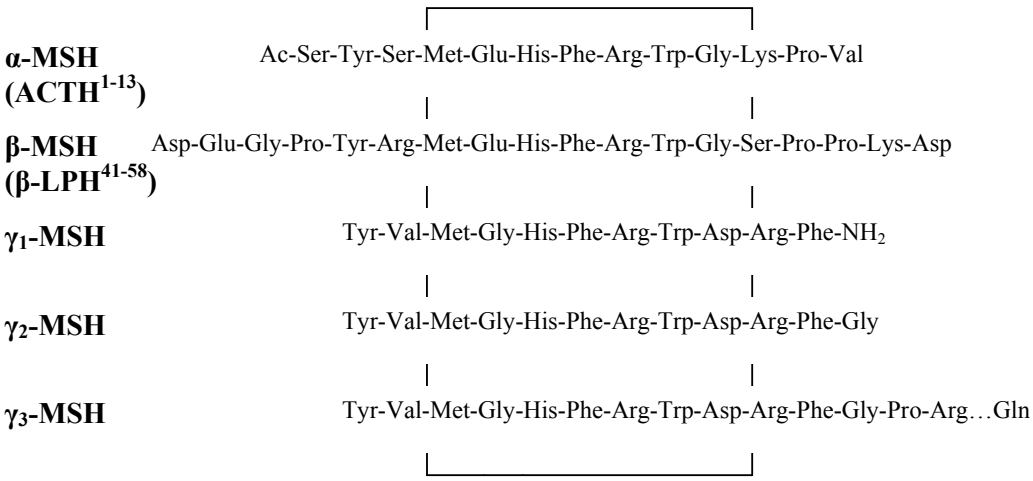

Figure 2.2. Amino acid sequences of melanocyte stimulating hormones. Scheme is modified from Humphreys, 2004.

prohormone convertases, PC1/3 and PC2 (Wardlaw, 2011). These are subtilisin-like endoproteases that cleave prohormones at adjacent pairs of basic amino acids (Lys-Lys, Lys-Arg, Arg-Arg, Arg-Lys). In the anterior pituitary, POMC is predominantly cleaved by the PC1/3 into the larger peptides, ACTH, β-LPH and an N-terminal fragment (Fig. 2.3). In the intermediate lobe and in the hypothalamus, POMC is more extensively processed: ACTH is further cleaved to produce α-MSH and CLIP, and N-terminal POMC to γ3-MSH. Processing of ACTH to yield α-MSH necessitates the activity of

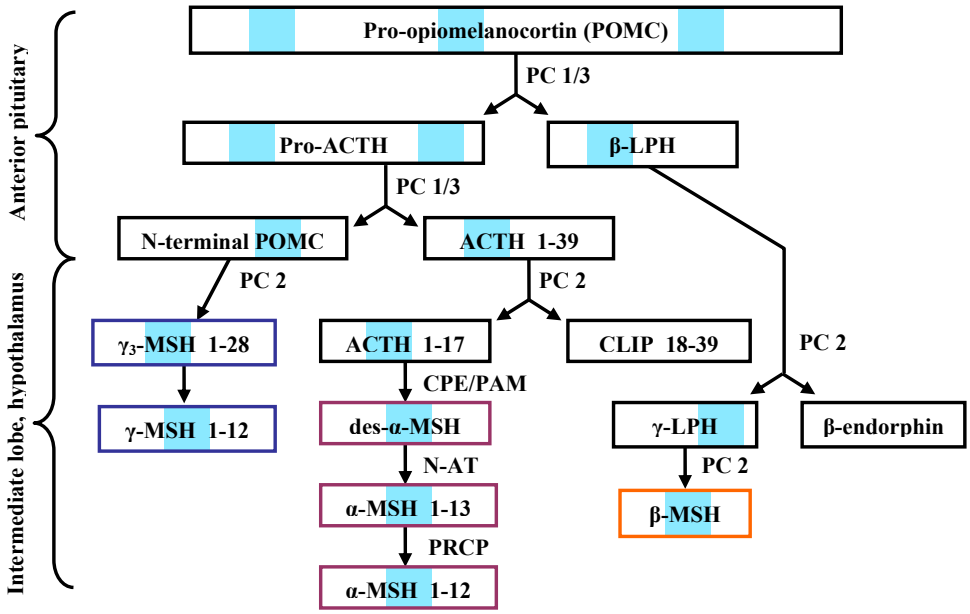

Figure 2.3. Processing of the POMC molecule. The blue shaded boxes represent the heptapeptide sequence that constitute the MSH core.

PC2 as well as two other processing enzymes, namely carboxypeptidase E (CPE) and α-amidating monooxygenase (PAM). Once produced, α-MSH undergoes a maturation process: deacetyl α-MSH is N-acetylated by a yet unidentified N-acetyltransferase (N-AT). This acetylation event stabilizes α-MSH against proteolytic degradation and prolongs its biological activity. Nevertheless, the half-life of α-MSH is very short since it is still rapidly metabolized. Recently, a new processing enzyme, prolylcarboxypeptidase (PRCP) was identified and shown to be involved in the enzymatic inactivation of α-MSH (Wallingford *et al.*, 2009).

2.2 Melanocortin receptors

The cloning of the human melanocortin receptor has paved the way to achieve a better understanding of the biological effects of melanocortins and their receptors. To date, five melanocortin receptor genes have been cloned; MC1, MC2, MC3, MC4 and MC5 receptor, corresponding to the order of their cloning (Mountjoy *et al.*, 1992). A structural comparison reveals that they have high sequence homologies ranging up to 60% identity between MC4 and MC5 receptors. The characteristics of each MC receptor subtypes are summarized in Table 2.1. All MC receptors belong to the family of G-protein coupled receptors (GPCR). They consist of a single polypeptide and share typical GPCR features, including seven α-helical transmembrane domains, an extracellular N-terminus and an intracellular C-terminus. MC receptors are, however, the smallest GPCRs known with short N- and C-termini and a small second extracellular loop, and they possess several features that distinguish them from other GPCRs. For example, they lack cysteine residues in the first and second extracellular loops. Furthermore, MC receptors are unique among GPCRs because they have their own endogenous inverse agonists, agouti-related protein (AgRP) in the CNS and agouti (also termed agouti signalling peptide; ASIP) in the periphery (Lu *et al.*, 1994; Ollmann *et al.*, 1997).

All MC receptors are coupled to adenylyl cyclase in a stimulatory fashion leading to an increase in cAMP production and activation of protein kinase A. In addition to the classical pathway involving the stimulatory G protein (Gs), MC receptors can couple to other G proteins (Gi/o, and Gq). For example, they can utilize the PI3 kinase signaling system as a second messenger. This, in turn, can activate MAP kinases including ERK1/2 and JNK (Chai *et al.*, 2007; Konda *et al.*, 1994). Furthermore, MC receptors may be subjected to regulation by phosphorylation since they have recognition sites for protein kinases A and C. However, little is known about the specific signal cascades and the eventual regulation of MC receptors by phosphorylation.

2.2.1 MC1 receptor

The MC1-R is the classic MC receptor capable of binding only α-MSH with high affinity and is predominantly expressed in peripheral tissues. It mediates the well-known melanin dispersion in melanocytes and keratinocytes of the dermis (Geschwind *et al.*,

1972; Lerner *et al.*, 1961). In cases where UV radiation evokes DNA damage in the skin, α-MSH synthesis and release is triggered which, in turn, leads to melanin synthesis and skin pigmentation (Eller *et al.*, 1996). In addition to an increase in melanin synthesis, activation of MC1 receptors switches from the synthesis of yellow-red pheomelanin to that of the more protective brown-black eumelanin, which causes darker pigmentation. The overall effect of these responses is to provide protection for the skin against the harmful effects of UV radiation.

The MC1 receptor was originally cloned from cDNA libraries and detected in melanoma cells and cells of solid melanoma tumours. The expression level of the MC1 receptor is relatively low in normal melanocytes but it becomes markedly upregulated upon transformation of melanocytes into malignant melanoma (Loir *et al.*, 1999). Previously, MC1-R signalling was considered to have melanoma-promoting activity but recent investigations have revealed that α-MSH by interacting with the MC1-R has, in fact, anti-carcinogenic and tumour-suppressive activities (Eves *et al.*, 2003; Kokot *et al.*, 2009). On the basis of these observations, it has been proposed that MC1-R could be used as a target for the diagnosis and treatment of malignant melanoma.

Following the identification of MC1 receptor in melanocytes, there are several publications describing its expression in a number of other cell types. MC1-R has a wide distribution in the immune system, including macrophages, monocytes, neutrophils, fibroblasts, microglial cells and endothelial cells (Adachi *et al.*, 1999; Bhardwaj *et al.*, 1997; Catania *et al.*, 1996; Hartmeyer *et al.*, 1997). Accordingly, there is a large body of data indicating that MC1-R has a crucial role as a mediator of immune responses. MC1-R stimulation elicits anti-inflammatory responses due to a number of concerted actions of α-MSH. It has the ability to reduce the production of pro-inflammatory cytokines while concomitantly increasing the production of anti-inflammatory cytokines (Luger *et al.*, 1997). The MC1 receptor is present also in the CNS but only in the periaqueductal gray matter of the midbrain. These central MC1 receptors might have a role in pain modulation (Mogil *et al.*, 2003; Xia *et al.*, 1995).

2.2.2 MC2 receptor

The MC2 receptor is a unique MC subtype that differs substantially from the other MC receptors in terms of its pharmacological properties (Schiöth *et al.*, 1996). MC2-R can be activated only by $ACTH_{1-39}$ and hence, it is also known as the ACTH receptor. MC2-R is expressed in high quantities in the cortex of the adrenal gland, with the highest expression levels present in the zona reticularis and fasciculate (Xia *et al.*, 1996b). Given such characteristics, it is not surprising that MC2-R has been demonstrated to control adrenocortical steroidogenesis. In addition to the adrenal gland, MC2-R seem to be present in the white adipose tissue in mice but not in humans (Boston *et al.*, 1996; Chhajlani, 1996), indicating that there are species differences in the expression profiles of the MC2 receptor.

2.2.3 MC3 receptor

The MC3 receptor is also unique among the melanocortin receptors since it is the only MC receptor that responds to stimulation with γ-MSH at physiological concentrations. Thus, γ-MSH seems to be the natural ligand for the MC3-R even though this receptor does not show apparent selectivity for γ-MSH over the other melanocortin peptides (Table 2.1). MC3 receptors are expressed in the CNS as well as in multiple tissues in the periphery, including GI tract, placenta, heart, kidney and immune cells. In the CNS, the MC3-R expression shows a rather restricted distribution with the highest densities occurring within the hypothalamus and the limbic system (Roselli-Rehfuss *et al.*, 1993). The physiological function of the MC3-R in the CNS remains enigmatic. It is an inhibitory autoreceptor on POMC neurons, since its activation inhibits the spontaneous firing of POMS neurons leading to stimulation of food intake (Cowley *et al.*, 2001). Surprisingly, it causes an obesity syndrome when deleted from the mouse genome. The MC3-R deletion in mice causes a moderate obesity syndrome characterized by subtle hyperphagia, increased adipose mass and no increase in lean body mass, suggesting that obesity in these mice results from increased energy efficiency (Butler *et al.*, 2000; Chen *et al.*, 2000). In addition to its impact on energy homeostasis, a variety of other physiological functions have been demonstrated for the MC3-R. Following the identification of its expression in blood leucocytes, MC3-R has been studied as a target for anti-inflammatory therapies (Lam *et al.*, 2004). Interestingly, its immune function seems to extend beyond peripheral mechanisms and to occur also in the CNS (Muceniece *et al.*, 2006). Furthermore, MC3-R seems to have a role in haemodynamic control (for detailed review see chapter 2.3.3).

2.2.4 MC4 receptor

The melanocortin 4 receptor is mainly expressed in the CNS where it has a very wide distribution, including the cortex, thalamus, hypothalamus, brain stem and spinal cord (Mountjoy *et al.*, 1994). Thus, its expression pattern is distinctly different and much wider than that of MC3-R. It is noteworthy that MC4-R is abundantly expressed in regions of the hypothalamus known to be involved in the regulation of energy homeostasis. Although considered as a central MC receptor, MC4-R is also found in sensory nerves in the periphery (Tanabe *et al.*, 2007). Several physiological functions have been attributed to this receptor, including sexual function and pain, but the most important role for MC4-R is certainly in the control of energy balance and cardiovascular functions (reviewed in chapters 2.3.1 and 2.3.2). After the discovery of the pivotal role in energy homeostasis, MC4-R has aroused much interest also within pharmaceutical industry as a target to treat weight disorders.

2.2.5 MC5 receptor

The MC5 receptor is widely expressed at low levels in a variety of peripheral tissues, but most distinctively in the secretory epithelia of many exocrine glands such as the adrenal, lacrimal and sebaceous glands (Chhajlani, 1996). This kind of distribution indicates that MC5-R is involved in regulating the synthesis and secretion of exocrine

gland products. The principal role of the MC5-R was initially discovered by Chen et al. who generated and studied the MC5-R knockout mouse (Chen *et al.*, 1997a). Chen made his most striking observation when performing a forced-swim test and reported that MC5-R knockout mice were much wetter than wild-type mice as they absorbed more water in their coat due to impaired water repulsion. This phenomenon was attributed to a defect in the production of sebaceous lipids. More recent data has revealed that MC5-R contributes also to pheromone secretion from tissues like such as the preputial gland, thereby affecting behaviour (Morgan *et al.*, 2006; Morgan *et al.*, 2004). As a consequence, MC5-R KO mice exhibit changes in aggression and defensive behaviour.

Table 2.1. Characteristics of the melanocortin receptors.

Receptor	Sites of expression	Principal function	Agonist profile	Endogenous antagonists
MC1	Melanocytes, keratinocytes, endothelial cells, immune system	Pigmentation, anti-inflammation	α-MSH >$ACTH_{1-39}$ >>γ-MSH	Agouti
MC2	Adrenal cortex	Steroidogenesis	$ACTH_{1-39}$	
MC3	CNS, heart, immune system	Energy homeostasis, anti-inflammation, natriuresis	γ-MSH = $ACTH_{1-39}$= α-MSH	AgRP
MC4	CNS	Energy homeostasis, blood pressure regulation, erectile function	α-MSH =$ACTH_{1-39}$ >>γ-MSH	AgRP, agouti
MC5	Exocrine cells, lymphocytes	Synthesis and secretion of exocrine gland products	α-MSH >$ACTH_{1-39}$ >γ-MSH	

2.2.6 Endogenous antagonists of melanocortin receptors

One of the most striking features of melanocortin receptors is that they have their own endogenous antagonists, agouti and AgRP. This is a unique feature among G-protein coupled receptors. Agouti and AgRP are both paracrine signalling peptides that have MC receptor subtype selectivity. In fact, they are inverse agonists because they can inhibit the activity of their respective MC receptors, even in the absence of melanocortins. Originally, the term agouti refers to a hair colour pattern seen in some mammals which is characterized by a yellow band on an otherwise dark background. Agouti is produced by the dermal papillae cells and it exerts its action via the MC1 receptor on adjacent melanocytes, resulting in the synthesis of a yellow pigment (pheomelanin) (Lu *et al.*, 1994). In rodents, agouti is expressed only in the skin but the human homologue, agouti signalling peptide (ASIP) has a wider pattern of expression, being located also in the adipose tissue and heart, and it has a functional role in the regulation of lipid metabolism (Wilson *et al.*, 1995). Following the discovery of agouti, AgRP was identified based on its sequence similarity with agouti (Ollmann *et al.*, 1997). AgRP is expressed mainly in

the arcuate nucleus of the hypothalamus where it acts as a selective antagonist of MC3 and MC4 receptors. The physiological functions of AgRP are described in chapter 2.3.2.

2.2.7 Pharmacokinetic and -dynamic properties of superpotent α-MSH analogues

[Nle^4-d-Phe^7]-α-MSH (NDP-α-MSH), also known as afamelanotide or melanotan-I, is a superpotent analogue of α-MSH that was first synthesized and characterized in 1980 (Sawyer *et al.*, 1980). Like α-MSH, it is a 13 amino acid peptide, but it has modifications in two amino acid residues. Thus, it possesses better resistance to enzymatic breakdown which prolongs its duration of action and increases its potency at MC receptors as compared with the endogenous α-MSH molecule. The half-life of subcutaneously administered NDP-α-MSH during the elimination phase is 1-2 hours in humans (Ugwu *et al.*, 1997). A shorter cyclic variant containing a lactam bridge, acetyl-Nle-[Asp-His-d-Phe-Arg-Trp-Lys]-cyclo-NH_2, referred to as melanotan-II (MT-II) has been characterized and demonstrated to be an even more stable analogue of α-MSH and thus can exert prolonged activity (Al-Obeidi *et al.*, 1989). In contrast to MT-II, NDP- α-MSH does not cross the blood-brain barrier to any significant extent and hence, it has not caused any adverse effects resulting from central melanocortin signaling (Hadley *et al.*, 2006). MT-II instead does cause more adverse effects like penile erections due to leakage into the brain (Dorr *et al.*, 1996).

NDP-α-MSH is a nonselective ligand for all melanocortin receptors except for MC2-R but the binding affinity to e.g. MC4-R is significantly higher than that of endogenous α-MSH (Sawyer *et al.*, 1980). It is the first-in-class α-MSH analogue that has been investigated in clinical studies and shown to have therapeutic efficacy in skin diseases like protoporphyria that is associated with absolute sunlight-intolerance. One pharmaceutical company Clinuvel is currently developing in Phase II and III clinical trials afamelanotide administered as subcutaneous formulations (e.g. SCENESSE®) as photoprotective drugs. The safety-data obtained from clinical trials point to a favorable safety-risk profile with minor and no drug-related serious adverse events. The most common adverse effects encountered during afamelanotide treatment have been nausea, headache and flushing (Minder, 2010).

Table 2.2. Binding affinities of endogenous and synthetic MC receptor agonists

	MC1	MC3	MC4	MC5
Endogenous agonists				
α-MSH	0.12	31	660	5700
β-MSH	1.2	13	380	14 000
γ_1-MSH	2.7	7.1	29 000	43 000
Synthetic agonists				
NDP- α-MSH	0.085	0.40	3.8	5.1
Melanotan-II	0.67	34	6.6	46

K_i values (nM) of endogenous and synthetic agonist for human melanocortin MC1, MC2, MC4 and MC5 obtained in competition with [I^{125}]-NDP- α-MSH. Modified from Wikberg *et al.*, 2000.

2.3 Physiological roles of melanocortins

Recent discoveries of genetic variants of POMC and MC receptor genes, and novel compounds acting at MC receptors have paved the way to achieving a better understanding of the melanocortin system and its interplay with other important physiological systems. There is now compelling evidence indicating that in particular the central melanocortin system plays a pivotal role in the regulation of energy homeostasis. This discovery has aroused a great deal of interest also within the pharmaceutical industry which considers that the melanocortin system may be a novel target to treat weight disorders. The current global obesity epidemic has also focused attention on this system as a promising drug target. However, it is important to note that the melanocortin system is a central element in several other physiological functions including the regulation of sexual behaviour, immune responses and the cardiovascular system. Some important properties of the melanocortin system will be reviewed in this section e.g. the regulation of energy balance, cardiovascular functions and electrolyte balance.

2.3.1 Melanocortins and regulation of energy balance

Over the past 30 years, significant advances have been made in the field of melanocortin research and it has become evident that the melanocortin system plays a fundamental role in the central regulation of energy homeostasis. The system is an essential component of the specific neurocircuitry that continuously monitors signals reflecting the energy status of the body and initiates appropriate behavioural and metabolic responses. First, the system adjusts feeding behaviour in response to acute hunger/satiety signals and long-term adipostatic signals, and translates these signals into biochemical events that ultimately determine food intake. Second, the melanocortin system regulates energy expenditure via effects on autonomic and endocrine functions. Figure 2.4 illustrates the current understanding of the melanocortin system and its role in energy homeostasis.

2.3.1.1 Overview of the central melanocortin system for sensing and orchestrating energy homeostasis

By definition, the central melanocortin system is a collection of neuronal circuits that include neurons that express neuropetide Y and AgRP (NPY/AgRP) or POMC and that originate in the arcuate nucleus of the hypothalamus (for review, see Cone, 2005). In rodents, central POMC neurons also coexist with cocaine and amphetamine-regulated transcript (CART) whereas in humans, this peptide is absent in POMC neurons but is expressed in NPY/AgRP neurons. The distribution of NPY/AgRP neurons overlaps to a large extent with those of POMC neurons. Second, the central melanocortin system includes POMC neurons that are expressed in the NTS of the brain stem and neurons that express MC3 and MC4 receptors - downstream targets of POMC and NPY/AgRP neurons. In the CNS, melanocortin peptides are agonists

of MC3-R and MC4-R, whereas AgRP serves as an inverse agonist/antagonist of these receptors. Interestingly, many MC receptors expressing neurons receive information from both agonist-expressing POMC neurons and antagonist-expressing AgRP neurons.

The POMC and AgRP/NPY neurons that constitute the central melanocortin system are located adjacent to each other in the arcuate nucleus of the hypothalamus and they convey anorexigenic and orexigenic signals, respectively. There are also reciprocal projections from hypothalamic sites to the brainstem POMC neurons. Upon activation, arcuate POMC neurons transfer information to other parts of the hypothalamus and elsewhere, and trigger a signalling cascade that eventually promotes energy expenditure and suppresses food intake. In contrast, activation of AgRP neurons inhibits melanocortin signalling, thus promoting food intake and suppressing energy expenditure. The orexigenic and anorexigenic responses are further strengthened by disinhibition of POMC and AgRP/NPY neuronal activity, respectively.

2.3.1.2 Peripheral metabolic signals and their interaction with the central melanocortin system

The adipostatic hormone leptin, which is principally produced by adipocytes, is one of the best studied peripheral factors that contribute to body weight regulation. Leptin is released into the circulation in proportion to body fat mass and exerts its effect on energy homeostasis predominantly via centrally expressed leptin receptors. Leptin directly activates POMC neurons in the hypothalamic arcuate nucleus, indicating that melanocortin signalling is downstream of leptin's adipostatic signal (Cowley *et al.*, 2001). It also upregulates POMC expression and triggers the release of POMC-derived MSH peptides and downregulates AgRP gene expression. All these effects, in parallel, create an anorexigenic signal in the brain that inhibits food intake. The classic mouse models, ob/ob and db/db mice, which display mutations in the genes encoding leptin and leptin receptor, respectively, and exhibit morbid obesity, highlight the importance of leptin signaling in body weight regulation (Halaas *et al.*, 1995; Hummel *et al.*, 1966; Ingalss *et al.*, 1950; Zhang *et al.*, 1994). Leptin also increases the activity of hypothalamic N-acetyltransferase that acetylates α-MSH and thereby, stabilizes it against enzymatic degradation (Guo *et al.*, 2004). However, although contributing to leptin signaling, the melanocortin system mediates only a subset of leptin's actions. Leptin has also other effects on energy homeostasis that are independent of the melanocortin system. Conversely, the role of the melanocortin system is not restricted to mediating the effects of leptin.

Before the discovery of leptin as the principal adipostatic hormone, it had been shown that insulin possessed some adipostatic activity. Insulin shares some key features with leptin that are a requisite for an adiposity signal: it is secreted in amounts that are related to body fat mass and it interacts with arcuate neurons that express insulin and leptin

(Niswender *et al.*, 2004). Like leptin, insulin inhibits AgRP/NPY neurons and activates POMC neurons, thus reducing food intake and promoting energy expenditure.

In addition to integrating signals from long-term adipostatic factors such as leptin and insulin, the melanocortin system plays a role in mediating feelings of hunger and satiety. Feeding behavior is tightly linked to neuronal circuits in the brainstem classically understood as the center for detection of and the response to satiety and hunger signals. POMC neurons and MC4-R expressing neurons are located especially in the dorsal vagal complex of the brainstem which consists of the area postrema, the NTS and the dorsal motor nucleus of the vagus nerve (DMV), and which seems to play a pivotal role in this control. Satiety signals from the periphery reach the brain first at the level of the NTS via vagal afferents. These vagal afferents respond to stimuli such as gut distension and contraction and gut peptides released from the stomach and duodenum. Cholecystokinin is a classic example of a gut peptide that is produced by the GI tract in response of a meal ingestion. Vagal afferents respond to cholecystokinin leading to acute inhibition of feeding (Fan *et al.*, 2004; Gibbs *et al.*, 1976). Many of the same neurons in the NTS that respond to cholecystokinin are also responsive to leptin (Ellacott *et al.*, 2006). Stimulation of brainstem MC4 receptors reduces meal size while retaining meal frequency (Zheng *et al.*, 2005). Cholecystokinin also seems to control body energy expenditure by increasing levels of uncoupling protein 1 in brown adipose tissue and thereby stimulating thermogenesis (Williams *et al.*, 2003).

In addition to mediating satiety cues, the central melanocortin system has been implicated in a direct response to acute signals of hunger (Reviewed by Cone, 2005). There is persuasive evidence for this role emerging from research on ghrelin and its impact on the melanocortin system. Ghrelin is predominantly secreted by the stomach and its level is increased during fasting and there is a marked decline in secretion after meal ingestion. Ghrelin interacts with the melanocortin system to activate orexigenic NPY/AgRP neurons in the hypothalamus. There is also evidence that ghrelin might directly inhibit POMC neuronal activity. Thus, as opposed to leptin's action, ghrelin stimulates food intake. The melanocortin system is central to ghrelin's action on food intake. The positioning of POMC and AgRP/NPY neuron bodies adjacent to the circumventricular organs; arcuate neurons to the median eminence and brainstem neurons to the area postrema, indicate that the system may be sensing also blood-borne hormones and nutrients.

2.3.1.3 Integration of metabolic signals in the melanocortin system

Integration in higher brain regions determines the central response to changes in peripheral metabolic state. The central melanocortin system is connected with the brain regions responsible for coordinating feeding behaviour and energy expenditure (autonomic and endocrine responses) that together regulate energy homeostasis to meet nutritional challenges. The POMC and AgRP/NPY neurons are located adjacent to each other in the arcuate nucleus of the hypothalamus and project in parallel to

several nuclei that are salient to the regulation of energy homeostasis, including paraventricular hypothalamic nucleus (PVH) and lateral hypothalamic area (LHA). Many other CNS peptides interact with the melanocortin system, including orexin and melanin concentrating hormone (MCH), which relay metabolic signals from the LHA to the cerebral cortex (Broberger, 2005). The cortex in turn projects back to the LHA and other feeding-regulatory areas. Furthermore, two neurotransmitter, serotonin and dopamine, seem to control the reward aspects associated with food intake. Stimulation of MC4 receptors has been shown to increase dopamine release in the nucleus accumbens which is considered as the brain region responsible for reward control of food intake (Lindblom *et al.*, 2001). Nucleus accumbens sends an inhibitory input to the LHA and thus, the LHA is positioned to integrate both homeostatic and reward-related signals in the gating of food intake.

Energy expenditure is modulated via outputs from the arcuate POMC and NPY/AgRP neurons to the PVH which controls the release of thyrotropin-releasing hormone (TRH) and corticotropin-releasing hormone (CRH). TRH and CRH then travel across the median eminence to stimulate the release of thyroid-stimulating hormone and ACTH in the anterior pituitary gland. Energy expenditure is also regulated through descending pathways from the hypothalamic and brain stem sites to autonomic preganglionic neurones. Autonomic function is coordinated via the dorsal motor nucleus of the vagus (DMV; parasympathetic) and spinal cord intermediolateral cell column (IML; sympathetic).

It has been recently shown that the central melanocortin system directly controls thermogenesis in brown adipose tissue and moreover, cellular glucose uptake, triglyceride synthesis, lipid deposition and lipid mobilization in liver, muscle and adipose tissue (Nogueiras *et al.*, 2007; Voss-Andreae *et al.*, 2007). These effects seem to be independent of feeding behaviour and precede changes in adiposity. Overall, the complexity and diversity of the melanocortin system imply that the system is central to integrating many afferent inputs with behavioural and autonomic responses that ultimately maintain energy homeostasis.

2.3.1.4 Insights from monogenic syndromes of obesity

One of the oldest known genetic models of obesity is the agouti mouse that shows ectopic overexpression of agouti protein and has a distinct metabolic phenotype characterised by hyperphagia, hyperinsulinemia, hypometabolism and increased linear growth (Wolff *et al.*, 1999). The cause of obesity in the agouti mouse was not clear until the cloning of the melanocortin receptors in the early 1990s when it was shown that the agouti protein is an antagonist of MC1 and MC4 receptors (Lu *et al.*, 1994). The finding, along with the identification of MC4-R expression in the hypothalamus, led to the hypothesis that the obesity seen in the agouti mouse may result from ectopic expression of agouti in the CNS and the consequent antagonism of the hypothalamic MC4 receptor. Development and analysis of MC4-R knockout (KO)

mouse in 1997 represents the molecular foundation for the melanocortin hypothesis as it became clear that the chronic absence of MC4-R signalling could mimic the obesity syndrome of agouti mouse (Huszar *et al.*, 1997). This concept was supported by elegant studies with melanocortin antagonists revealing that pharmacological blockade of central MC4-R stimulates also food intake and weight gain (Fan *et al.*, 1997). Following the discovery of the obesity syndrome in MC4-R KO mouse, several other mouse obesity models have been described including AgRP overexpressing and POMC deficient mouse (Graham *et al.*, 1997; Yaswen *et al.*, 1999). All these models have consolidated the importance of the melanocortin system in the maintenance of energy homeostasis.

While the unravelling of the molecular perturbations in rodent models of obesity confirmed the pivotal nature of the melanocortin system in energy homeostasis, it remained to be clarified whether the abovementioned findings could be extrapolated to humans. Through the identification of a number of monogenic obesity syndromes, the relevance of melanocortin pathways to human physiology was firmly established. One of the earliest findings and compelling evidence for the role of melanocortin system in human energy balance came from a report of two children bearing loss-of-function mutations in the POMC gene (Krude *et al.*, 1998). The clinical picture of these patients closely matched the phenotype seen in the POMC deficient mouse with hypoadrenalism due to lack of ACTH and obesity resulting from the loss of α-MSH action on MC4-R. Subsequently, the importance of MC4 receptor has been confirmed by the identification of several mutations in this gene and their association with early-onset obesity (Vaisse *et al.*, 1998; Yeo *et al.*, 1998). In fact, mutations in the MC4-R gene represent the most common monogenic cause of human obesity and seem to be responsible for up to 5% of cases of severe childhood obesity (Crowley, 2008).

Interestingly, a recent study demonstrated that obese humans with a loss-of-function mutation in the MC4-R gene had lower blood pressure and resting heart rate levels, and a lower 24-hour urinary noradrenaline excretion compared to equally overweight control subjects (Greenfield *et al.*, 2009). This is in good agreement with animal studies characterizing the cardiovascular phenotype of MC4-R KO mice (Tallam *et al.*, 2005). Thus, by combining data from animal and human studies, it has become evident that genetic perturbations in the central MC4-R signaling are associated with sympathetic nervous system activity and blood pressure regulation. Supporting pharmacological evidence for this concept will be described in the next chapter. These data provide a potential mechanistic explanation to the well-established, but poorly understood link between body weight and human hypertension. Overall, human genetics and genetically modified mouse models have provided conclusive evidence for the critical involvement of the melanocortin system in energy balance regulation, generating also further insights into the role of melanocortins in blood pressure regulation.

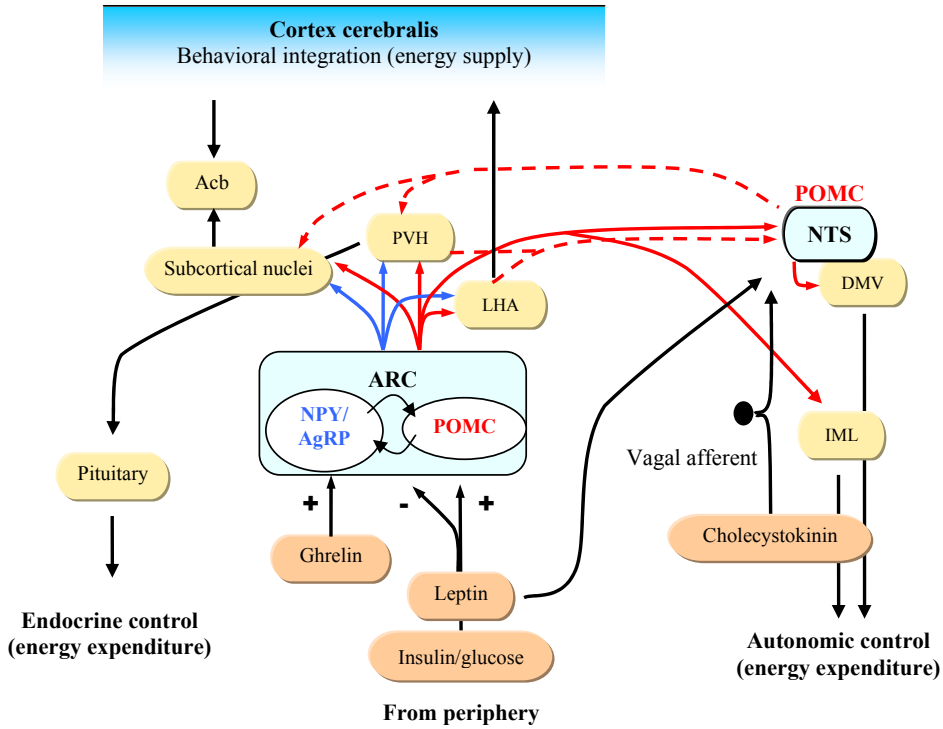

Figure 2.4. The melanocortin system and its role in energy homeostasis. Peripheral signals reflecting body energy balance and nutritional status are conveyed by leptin, insulin, glucose and ghrelin. These signals, received by the arcuate nucleus of the hypothalamus, stimulate or inhibit POMC (red arrows) and NPY/AgRP (blue arrows) neurons, which integrate them into food-intake behaviour. Leptin, for example, directly stimulates arcuate POMC neurons and concomitantly inhibits the activity of adjacent NPY/AgRP neurons, thereby causing also disinhibition of POMC neuronal activity. The arcuate POMC and NPY/AgRP neurons then project in parallel to many subcortical nuclei, lateral hypothalamus (LHA) and paraventricular hypothalamic nucleus (PVH). LHA conveys metabolic signals to the cerebral cortex and PVH to the pituitary gland, which adjusts endocrine control of energy expenditure. The connections with the nucleus accumbens (Acb) which contributes to feeding control through reward mechanisms are also indicated. Secondary projections of POMC neurons (dashed arrows) linking the hypothalamus to the brainstem also exist. POMC neurons of the nucleus tractus solitarius (NTS) receive peripheral satiety signals via vagal afferents and leptin, and release MSH peptides to activate MC4-R expressing neurons. This cascade eventually terminates food intake by evoking the sensation of satiety. Brainstem POMC neurons also modulate energy expenditure via autonomic functions. Modified from Wikberg *et al*, 2008.

2.3.2 Melanocortins and the cardiovascular system

There is increasing evidence demonstrating that the melanocortin system is crucially involved in cardiovascular regulation. The effects of melanocortins on the cardiovascular system seem to be, however, polymorphic and involve multiple signalling pathways. In the brain, the predominant extrapituitary sites of POMC expression originate in the hypothalamus and in the brainstem. These two brain areas are thought to differentially mediate the effects of melanocortins on cardiovascular homeostasis: activation of

hypothalamic melanocortin pathways elevates blood pressure whereas brain stem melanocortin signalling seems to be associated with hypotensive and bradycardic responses (Table 2.3). All the existing data imply that cardiovascular actions of melanocortins are exclusively dependent on central pathways. No peripheral site of action has been identified for MSH peptides in terms of cardiovascular actions. Furthermore, the chronic effects of melanocortins have been far less extensively studied than the effects that occur after acute administration.

2.3.2.1 Hypertensive effects of melanocortins

There is conclusive evidence that central or intravenous administration of γ-MSH causes a dose dependent, short lasting increase in blood pressure and heart rate (Gruber *et al.*, 1989). These effects are thought to be dependent on the central stimulation of the sympathetic nervous outflow, since the effects can be blocked by pithing and by adrenergic blocking drugs. In contrast to γ-MSH, intravenous injections of α-MSH or the stable analogue NDP-α-MSH have not resulted in hypertensive and tachycardic responses in rats or mice (De Wildt *et al.*, 1993; Ni *et al.*, 2006a; Van Bergen *et al.*, 1997). In the quest for an explanation for this difference between α- and γ-MSH, researchers have focused on structure-function activities and identified some essential features of MSH peptides that are needed for their cardiovascular properties (Nijsen *et al.*, 2000; Van Bergen *et al.*, 1995; Van Bergen *et al.*, 1997). A structural comparison has demonstrated that α-MSH has an N-terminal serine that is acetylated whereas γ-MSH has an N-terminal tyrosine. Furthermore, the C-termini of these peptides are distinct from each other; Lys-Pro-Val in α-MSH, Arg-Phe-NH_2 in γ_1-MSH and Arg-Phe-Gly in γ_2-MSH. The C-terminal Arg-Phe-sequence seems to be critical for the hypertensive action of γ-MSH, since α-MSH and synthetic peptides which lack this sequence do not increase blood pressure. The N-terminal shortening of γ-MSH produces also peptides that have cardiovascular activity. Apparently, the Arg-Phe-sequence needs to be near to the C-terminus, because γ_3-MSH, that has a long C-terminal extension after the Arg-Phe-sequence, is rather inactive relative to the shorter forms when injected intravenously. However, it is equipotent to the shorter forms in elevating blood pressure when injected into the cerebral ventricle (intracerebroventricularly; i.c.v.) (Ramaekers *et al.*, 2002). These observations raise the possibility that the larger size of γ_3-MSH could hinder its access through the blood-brain-barrier to a central site of action.

As γ-MSH is considered as the natural ligand for the MC3 receptor, it would be logical to postulate that this receptor is mediating the cardiovascular responses of γ-MSH. However, the truncated analogues of γ-MSH that have the ability to increase blood pressure *in vivo* exhibit no activity at the MC3 or MC4 receptors *in vitro* (Van Bergen *et al.*, 1995; Van Bergen *et al.*, 1997). Furthermore, endogenous and synthetic antagonists of these receptors do not interfere with the effects of γ-MSH to increase blood pressure. On the basis of these observations, it seemed unlikely that the MC3 receptor was involved in

mediating the cardiovascular actions of γ-MSH. The next candidate was thought to be FMRFamide-gated sodium channel. FMRFamide (Phe-Met-Arg-Phe-NH_2) was initially isolated from the clam Macrocallista nimbosa and shown to be a cardioexcitatory peptide (Price *et al.*, 1977). Subsequently, the amiloride-sensitive, FMRFamide-gated sodium channel was postulated to be the receptor responsible for mediating the cardioexcitatory responses of FMRFamide (Lingueglia *et al.*, 1995). Activation of this channel leads to a rapid inward sodium current, an effect that can be blocked by amiloride (Lingueglia *et al.*, 2006). There is good evidence for the involvement of this receptor in the action of γ-MSH e.g. from studies with benzamil, an analogue of amiloride. Pretreatment with benzamil was able to block the increase in blood pressure seen after central administration of γ-MSH (Ni *et al.*, 2006a). This finding suggests that there is an interaction between the FMRFamide-gated sodium channel and γ-MSH. Further support for this report emerged recently from studies with MC3-R and MC4-R deficient mice (Ni *et al.*, 2006a). Blood pressure increased equally in both knock-out models and their wild-type littermates after γ-MSH injection verifying that neither MC3 nor MC4 receptor is responsible for these responses. In conclusion, the hypertensive and tachycardic actions of γ-MSH take place through FMRFamide-gated sodium channels instead of any known melanocortin receptor.

Despite the lack of cardiovascular effects after intravenous injections, α-MSH has been shown to increase blood pressure and heart rate when administered into the cerebroventricular system (Ni *et al.*, 2006a). This effect is dependent on central stimulation of sympathetic nervous outflow and appears to be exerted through MC4 receptors (Matsumura *et al.*, 2002). Direct support for this concept was obtained from findings in MC4-R deficient mice. The injection of α-MSH into the lateral cerebral ventricle caused a marked increase in blood pressure in wild-type mice while MC4-R KO mice displayed no response to the same treatment (Ni *et al.*, 2006a). Supporting the concept for MC4-R based mechanism, an elegant human study recently demonstrated that subcutaneous infusion of a synthetic peptide agonist highly selective for MC4-R in 28 overweight or obese volunteers caused significant increases in both systolic and diastolic blood pressures (Greenfield *et al.*, 2009).

2.3.2.2 Hypotensive effects of melanocortins

In contrast to hypothalamic actions, accumulating data imply that increased melanocortin signalling within the brainstem leads to bradycardia and hypotension, thus opening a completely new and fascinating avenue of investigation. These opposing responses are thought to be dependent on activation of brainstem MC4 receptors (Chitravanshi *et al.*, 2009; Li *et al.*, 1996; Tai *et al.*, 2007). Hypotensive and bradycardic effects have been observed after microinjections of α-MSH directed into the NTS. In addition, microinjection of α-MSH into the nucleus ambiguous (nAmb) causes a dose-dependent decrease in heart rate without affecting blood pressure levels. The nAmb is the predominant source of parasympathetic innervation to the heart and MC4 receptor

expression has been identified in this nucleus. It has been shown that nAmb neurons can be activated by α-MSH, an effect that is seemingly dependent on MC4 receptors. It elicits bradycardia via an increase in cardiac vagal activity, because the bradycardic response to α-MSH can be abolished by bilateral vagotomy.

Table 2.3. Heart rate and blood pressure responses to acute administration of α- and γ-MSH.

Peptide	Route/site of administration					
	Systemic (i.v.)		Central (i.c.v.)		Brainstem	
	BP	HR	BP	HR	BP	HR
α-MSH	↔	↔	↑	↑	↓	↓
γ-MSH	↑	↑	↑	↑	↓	↓

2.3.3 Melanocortins and sodium metabolism

Early work demonstrated that acute intraperitoneal administration of α-MSH or β-MSH could induce natriuresis in rats and hamsters (Hradec *et al.*, 1979; Orias *et al.*, 1972). These peptides caused in a dose-dependent manner significant increases in urinary sodium and potassium excretion without producing any change in urine output (Orias *et al.*, 1972). If anything, the effect on urine output seemed to be antidiuretic. Subsequently, γ-MSH was also shown be natriuretic in the rat (Lymangrover *et al.*, 1985). Based on the finding made by Lin et al. that infusion of γ-MSH directly into the renal artery caused no change in sodium excretion from the contralateral kidney (Lin *et al.*, 1987), it was postulated that natriuresis by MSH peptides occurred directly through an intrarenal mechanism.

A previous report, elucidating the melanocortin receptor profile in the kidney, provided evidence that MC3-R, MC4-R, and MC5-R were expressed in rat renal cortex and medulla (Ni *et al.*, 2006b). This data along with the finding that γ-MSH-induced natriuresis was reversible by selective MC3/4-R (SHU9119) and MC3-R (SHU9005) antagonists support the hypothesis that natriuresis occurs, at least in part, through renal MC3 receptor (Humphreys, 2004; Ni *et al.*, 1998). Although recent publications (Kathpalia *et al.*, 2011; Lindskog *et al.*, 2010), reporting a lack of expression for MC3-R in the kidney, have questioned this possibility, a study by Cope et al confirmed the earlier finding and demonstrated that MC3-R protein is expressed in the rat kidney and that the expression of MC3-R is highly upregulated in rats fed with a high-sodium diet (Cope *et al.*, 2012). Furthermore, they showed that γ-MSH treatment stimulates cAMP formation in cultured inner medullary collecting duct cells. The increase in intracellular cAMP was prevented by SHU9119. Similar findings were presented by Ni et al in 2006 (Ni *et al.*, 2006b). Controversy still remains concerning the role of renal nerves in the natriuretic response, since renal denervation was previously reported to prevent the γ-MSH-evoked natriuresis, but Cope et al did not observe such an effect. Taken together, these two studies

provide compelling evidence that functional MC receptors are present in the kidney and that they are responsible for mediating the natriuretic action of MSH peptides. However, the exact mechanism and site of action of MSH peptide-induced natriuresis are still unknown.

Further research demonstrated that γ-MSH has a physiological role in body sodium metabolism. γ-MSH participates in the reflex natriuresis that occurs after acute unilateral nephrectomy (AUN). AUN induces an increase in both sodium and potassium excretion from the remaining kidney through an adaptive mechanism that is dependent on pituitary function (Lin *et al.*, 1985). Lin et al. also demonstrated that AUN is followed by an increase in plasma γ-MSH concentration and that this increase is an essential link between the AUN and natriuresis since an antibody specific to γ-MSH was able to block the induction of natriuresis after experimental AUN (Lin *et al.*, 1987). These findings inspired subsequent studies to investigate the possibility that γ-MSH could contribute to sodium handling under more general circumstances. Indeed, dietary sodium excess affects pituitary POMC processing in a preferential fashion in rats (Mayan *et al.*, 1996). High-sodium diet upregulates the expression of γ-MSH in the neurointermediate lobe of the pituitary and increases secretion of the peptide into the circulation, a response that is not encountered for α-MSH. A more recent study has shown that the plasma γ-MSH concentration tends to rise also in mice when exposed to high-sodium diet (Ni *et al.*, 2003).

2.3.3.1 γ-MSH and salt-sensitive hypertension

Studies characterising genetic mouse models of γ-MSH deficiency and resistance have provided supportive evidence for the importance of the γ-MSH system in sodium metabolism. First, targeted deletion of PC2 gene, a mouse model of γ-MSH deficiency, leads to increased susceptibility to salt-sensitive hypertension (Humphreys, 2004; Ni *et al.*, 2003). PC2 knockout mice exhibit markedly increased blood pressure while ingesting high sodium diet. Second, the blood pressure elevation in MC3-R KO mice after exposure to high-sodium diet parallels the findings in PC2 KO mice, indicating that γ-MSH resistance leads similarly to the development of a hypertensive state. The salt-sensitive hypertension of PC2 KO mice can be reversed by intravenous administration of γ-MSH but not in MC3-R KO mice. Thus, the normalization of blood pressure requires integrity of the MC3 receptor. Furthermore, antihypertensive effect of γ-MSH in PC2 KO mice occurs probably through a central site of action, because low doses of γ-MSH lowered blood pressure readily when given into the cerebroventricular system whereas the same dose as an intravenous injection was ineffective in that regard. Administration of γ-MSH increased concomitantly the rate of urinary sodium excretion as blood pressure was falling but the rapidity of the antihypertensive effect argues more strongly for a central mechanism rather than for direct signalling pathways to affect renal sodium handling. The concomitant natriuresis could still contribute to the maintenance of normal blood pressure by improving the overall regulation of body fluid balance.

The crucial role for the MC3 receptor as a mediator of γ-MSH-induced natriuresis can be associated with the finding that mice deficient in this receptor do not develop this natriuretic response. As mentioned earlier, natriuresis evoked by γ-MSH and other MSH peptides occurs via an interaction with renal nerves, since denervated kidneys do not respond to MSH peptides in the same ways as intact kidneys. Finally, it is noteworthy emphasizing that the nature of γ-MSH injections is like a double-edged sword: γ-MSH is natriuretic and lowers blood pressure in hypertensive PC2 KO mice at low picomolar concentrations whereas at higher concentrations, the natriuretic effect is lost and the peptide is more likely to increase blood pressure. The latter outcome probably reflects a pharmacological effect rather than a physiological response. In conclusion, MSH peptides induce natriuresis and seem to have a significant role in fluid and electrolyte homeostasis, since in particular the γ-MSH-system responds to homeostatic disturbances such as dietary sodium excess and thus it contributes thereby to the maintenance of normal blood pressure.

2.4 Basic principles of blood pressure regulation

2.4.1 Cardiovascular system

The main components of the cardiovascular system are the heart and blood vessels which enable the transport and exchange of nutrients, gases, hormones and other factors between blood and surrounding tissues, thus contributing to the maintenance of homeostasis. Blood vessels constitute the circulation which can be divided into the systemic and pulmonary circulation. With regards to the systemic circulation, the left ventricle ejects blood into the aorta which branches into the major distributing arteries and they, in turn, into successively smaller arteries and arterioles until they become capillaries. Venules collect blood from the capillaries and merge together to form veins. Blood from veins enters either the superior or inferior vena cava which return the blood into the right atrium of the heart. (Guyton *et al.*, 2006)

The cardiac cycle, the cardiac events that occur between two heartbeats, consists of a period of relaxation (diastole) and a period of contraction (systole). During diastole, the heart is filled with blood, which is pushed forward to the aorta during systole. Because of this pulsatile mode of action, two pressure levels can be detected: systolic and diastolic pressure. The aorta distributes blood to the arterial system but more importantly minimizes the pulsatile pressure. Aortic compliance determines, in part, the rise in aortic pressure from its diastolic to systolic value and thereby the dampening of arterial pressure. The small arteries and arterioles represent the primary vessels that regulate arterial blood pressure as well as blood flow within each organ, and are therefore referred to as resistance arteries. These resistance vessels are highly innervated by autonomic nerves and actively respond to various stimuli by constricting or dilating. In experimental pharmacology, mesenteric arteries (e.g. from mouse or rat) are widely used to model the function of human resistance arteries. Capillaries are the

smallest vessels within the microcirculation and primarily responsible for the exchange of oxygen, carbon dioxide, water, electrolytes, proteins and other products between the plasma and the interstitial tissues. Capillaries are devoid of smooth muscle and incapable of regulating capillary pressure. Instead resistance arteries and venules ensure that the mean capillary pressure remains within optimal range. As venules merge together to form veins, the primary function of the vessels becomes the regulation of regional blood volume, hence veins are referred to as capacitance vessels. Venous volume and venous pressure are regulated through changes in venous tone, the balance between constriction and dilatation. These changes eventually affect also cardiac output, which is defined in the next paragraph.

Table 2.4. The relative sizes, functions and pressure levels of different blood vessels in humans.

Vessel	Diameter (mm)	Function	Mean pressure (mmHg)
Aorta	25	Pulse dampening, distribution	~95
Arteries	0.2-4.0	Distribution, resistance	80-90
Arterioles	0.01-0.2	Resistance	30-80
Capillaries	0.006-0.010	Exchange	15-30
Venules	0.01-0.2	Exchange, capacitance	5-15
Veins	0.2-5.0	Capacitance	5-10
Vena cava	35	Collection of venous blood	3-8

Blood pressure is a function of cardiac output (CO) and peripheral resistance. Cardiac output, in turn, is the volume of blood pumped into the aorta per minute and is dependent on three variables: end-diastolic volume, myocardial contractility and heart rate. End-diastolic volume is the volume of blood in the ventricular chamber at the end of diastole and is determined by venous pressure. Myocardial contractility describes the relative ability of the heart to eject a stroke volume (SV) at a given afterload (arterial pressure) and preload (end-diastolic volume). The interdependence and definitions of these variables are as follows:

$$\text{CO (ml/min)} = \text{heart rate (beats/min)} \times \text{stroke volume (ml/beat)}$$

$$\text{Arterial pressure} = \text{Cardiac Output (CO)} \times \text{Total peripheral resistance (TPR)}$$

2.4.2 Regulation of blood pressure

“Why does the body need to regulate blood pressure?” is the fundamental question for setting up this thematic entity. The overall goal of cardiovascular regulation is to ensure an adequate blood flow through each organ. The basis for achieving this goal is the regulation of arterial blood pressure. In general, adequate blood flow to each organ is assured by the phenomenon known as autoregulation: each organ and each tissue of the body has the ability to control its own blood flow. Autoregulation is clearly associated

with the metabolic needs of tissues. As the metabolism of the tissue increases, the local blood vessels dilate commensurately to this metabolic requirement allowing the improved supply of nutrients necessary for the increment of metabolism. Furthermore, increased blood flow to the tissue facilitates the transport of the end-products of the metabolism away from the tissues. Adjustments in the regional blood flow to match oxygen and nutrient supply with cellular demands are probably the most important function of blood pressure regulation. Second, blood pressure control is needed to prevent the blood flow requirements of one tissue from interfering with the blood flow to other tissues. Exercise training, for instance, presents one of the greatest challenges to arterial pressure control i.e. serious consequences would occur unless the secondary control function was operating properly. Exercise causes a tremendous vasodilatation in the muscles that is compensated by reduced blood flow to the gastrointestinal tract to maintain normal blood pressure level. Without blood pressure control, exercise would cause a significant drop in blood pressure and thereby reduce the blood flow to the brain. (Guyton, 1980)

Another benefit of blood pressure regulation is that it allows the pressure level to adapt to prevailing conditions and to various bodily needs. For instance, during sleep, the blood pressure level decreases, thus conserving the energy of the heart, whereas during exercise, blood pressure can increase to a significant extent to ensure better blood flow to the exercising muscles. Finally, long-term control of blood pressure ensures that the pressure level does not reach morbidly high levels which could be damaging to the circulation itself. The minimum average pressure that is required for maintaining adequate blood flow through each organ is determined by several factors related to the physical structure of the body. First, hydrostatic pressure sets a condition for the minimum pressure level. To force blood up to the brain and to ensure also that all the other tissues are receiving an adequate blood flow under any circumstances, a normal arterial pressure of 100 mmHg in humans is near to the minimum safe value. Second, the capacity of blood flow control and adaptability to various physiological demands determines the minimum value of blood pressure. If the blood pressure level was significantly below 100 mmHg, the blood vessels would have to be constantly dilated to ensure a sufficient blood flow to match the basal needs of oxygen and nutrients of all tissues. In that situation, when the metabolic activity of the tissues needs to increase, as happens in the muscles during exercise, there would be no capacity for further dilatation to allow an extra supply of oxygen and nutrients. Hence, the essence of blood pressure regulation is to keep the pressure level high enough so that there is capacity to allow better blood flow to tissues when there are increased needs without the risk of significant hypotension and compromised blood flow to other tissues. On the other hand, blood pressure control is needed to prevent the blood pressure from rising to such a level that would be damaging to the blood vessels and the heart.

In principle, the two major challenges in arterial pressure control are to assure a steady baseline level of blood pressure over the long-term and to provide appropriate short-

term changes in the circulation and cardiac function to maintain a safe pressure level. To fulfill these two requirements, the body has developed a hierarchy of pressure control systems based on the time scale in which they operate. Starting from the most rapidly acting systems – the nervous system mediates cardiovascular reflexes that are initiated almost instantaneously after any deviation from normal pressure level. Typical examples of these reflexes are chemoreceptor and baroreceptor reflexes, the latter of which is reviewed in more detail in chapter 2.4.2.2. However, the nervous system is considered to buffer the blood pressure rather than to participate in the long-term regulation. Next, the control systems that operate with intermediate rapidity include e.g. capillary fluid shift between the blood vessels and interstitial fluids and humoral factors involving the renin-angiotensin-system (RAS) and vasopressin. Like the nervous system, these intermediate control systems provide pressure buffering mechanisms, but also significantly contribute to the long-term control of blood pressure. Although the role of the RAS in blood pressure regulation is not reviewed in detail in this thesis, it is important to note that it is a major regulatory system involved in the control of body fluid and sodium balance, and vascular tone. Disturbances in the RAS are strongly linked to systemic hypertension. Furthermore, pharmacological targeting of this system is one of the most effective ways to treat hypertensive patients, highlighting the clinical significance of the RAS in blood pressure regulation. Finally, the functions of the RAS lend support to the concept that the long-term regulation of arterial pressure is closely related to the control of kidney function and body fluid volumes.

2.4.2.1 Autonomic nervous system

The autonomic nervous system is one of the main regulatory mechanisms controlling circulatory homeostasis. It can be divided physiologically and anatomically into parasympathetic (vagal) and sympathetic divisions, which often act in a reciprocal manner. However, rather than being strictly opposites of each other, the relationship between the two divisions is considered as cooperative and integrated activities. The efferent autonomic outflow to cardiovascular end-organs is regulated and modulated by an input from the afferent receptors and control from higher brain centers, and ultimately by central integration of these signals in the medulla. The output is further modulated by local and humoral factors.

Organization of the autonomic innervation

The neural control of the circulation is operated via the parasympathetic neurons that innervate mainly the heart and via sympathetic efferents that innervate blood vessels, the heart, the kidneys and the adrenal medulla. The sympathetic efferents have a dominant role in blood pressure regulation. The background activity of the autonomic nervous system is monitored by a core network that resides in several parts of the NTS, rostral ventrolateral medulla (RVLM), the spinal cord and the hypothalamus. The majority of the sensory input from the peripheral receptors terminates in the NTS. A group of adrenergic neurons in the RVLM innervates and controls the sympathetic neurons located

in the intermediolateral column of the thoracolumbar spinal cord. The parasympathetic nervous system originates from medial medullary sites (nucleus ambiguous, NTS, and dorsal motor nucleus) and is modulated by the impulses from the hypothalamus, which also modulate sympathetic outflow (Guyenet, 2006; Spyer, 1994).

In the peripheral part of the autonomic nervous system, the common path of innervation is conventionally viewed as a two-neuron chain consisting of a preganglionic and a postganglionic neuron. Sympathetic ganglia are located in a paravertebral chain and the parasympathetic ganglia near to the effector end-organs. In both sympathetic and parasympathetic ganglia, synaptic transmission is cholinergic: excitation of the preganglionic neuron stimulates release of acetylcholine into the synaptic cleft, which in turn stimulates cholinergic nicotinic receptors on the postganglionic neuron. In the parasympathetic nervous system, the postganglionic neurons are also cholinergic but synaptic transmission is mediated by muscarinic receptors on the effector organs. At most sympathetic postganglionic neurons, noradrenaline serves as a neurotransmitter which activates adrenergic receptors on the sympathetic effector organs. There are some exceptions, e.g. sweat glands and certain blood vessels are innervated by sympathetic nerves but the transmission is cholinergic. Furthermore, the adrenal medulla is innervated directly by preganglionic sympathetic neurons and this gland releases adrenaline and noradrenaline into circulation upon activation. These catecholamines differ in their selectivity towards different adrenergic receptors: noradrenaline has higher selectivity for α- than β-receptors, whereas adrenaline has somewhat higher selectivity for β- than α-receptors.

Sympathetic nervous system

Under physiological conditions, adrenergic influences on the heart are regulated predominantly by the β_1-receptors. Stimulation of cardiac β_1-receptors increases heart rate, conduction velocity in the atrioventricular node and in the ventricles, and ventricular contractility, thus causing an increase in cardiac output. With the exception of the capillaries, nearly all vascular beds receive a sympathetic innervation. In the periphery, blood vessels express both α- and β_2-receptors. Stimulation of vascular α_1-receptors causes vasoconstriction by triggering calcium influx in the vascular smooth muscle cells. Stimulation of β_2-receptors causes instead vascular smooth muscle relaxation and thereby vasodilatation. Most of the adrenergic influences on vascular tone are mediated via vascular smooth muscle cells, but the endothelium plays also a role as a mediator of adrenergic effects. The vasomotor tone, a partial state of vasoconstriction, is maintained by tonic activity of the α-adrenergic sympathetic vasoconstrictor system and the changes in this system are one of the principal mechanisms determining peripheral resistance.

Parasympathetic nervous system

The parasympathetic nervous system contributes to the regulation of heart function and to a lesser extent to the control of vascular tone. In a normal and healthy heart,

resting heart rate is governed by the parasympathetic input to the heart. ACh released by the vagal postganglionic nerves exerts its action through cardiac muscarinic receptors which are predominantly of M_2-subtype. Increase in cardiac vagal activity decreases heart rate, atrioventricular conductivity and slightly depresses myocardial contractility. It may also induce coronary vasodilatation. In terms of vascular autonomic control, only the cranial, visceral and genitourinary vascular beds receive any significant parasympathetic innervation. However, muscarinic receptors (M_3-subtype) are abundantly expressed in endothelial cells and vascular smooth muscle cells. In the presence of healthy endothelium, exogenous ACh evokes vasodilatation by stimulation of endothelial muscarinic receptors. Endothelial dysfunction (e.g. in atherosclerosis and hypertension) disrupts this signaling and reverses the ACh-stimulated effect: stimulation of muscarinic receptors in the smooth muscle cells becomes dominant and vasoconstriction ensues.

2.4.2.2 Baroreceptor reflex

The baroreceptor reflex contributes substantially to the short-term regulation of sympathetic tone, heart rate and blood pressure. The feedback system that adjusts the circulation and blood pressure level in response to changes in the blood pressure level is initiated by arterial pressure sensors called baroreceptors. These receptors can be found in low quantities in the wall of almost all large arteries, but the most important baroreceptors are located in the aortic arch and in the carotid sinus. They respond to stretching of the arterial wall so that when blood pressure rises, the increase in passive wall tension activates baroreceptors. This leads to increased firing rate of the baroreceptors which then transmits signals through the glossopharyngeal (IX cranial nerve) and vagus (X cranial nerve) nerves to the NTS located in the medulla of the brain stem. The NTS, in turn, modulates the activity of sympathetic and parasympathetic neurons which rapidly affects the autonomic control of the heart and blood vessels. Any abrupt increase in blood pressure is translated into stimulation of parasympathetic activity and inhibition of sympathetic activity. As a consequence, peripheral resistance (dilatation of arteries and veins) and cardiac output (negative chronotropy and inotropy) decrease. Conversely, baroreceptor firing is reduced if the blood pressure suddenly declines, which leads to disinhibition of sympathetic activity in the NTS. Under normal physiological conditions, there is a tonic inhibition of sympathetic outflow from the medulla due to baroreceptor firing. Thus, a decrease in baroreceptor firing removes this brake on sympathetic activity triggering vasoconstriction, tachycardia and positive inotropy. The baroreceptor reflex is most sensitive and effective in the normal operating range of blood pressure. Therefore, even small changes in blood pressure around its "set point" are readily detected by baroreceptors in order to maintain blood pressure at the normal level. This "set point" is reset, for instance, during exercise explaining how blood pressure remains elevated under certain circumstances. It is important to note that this baroreceptor resetting can occur also in chronic settings as baroreceptors tend to adapt to long-term changes in blood pressure. This is what happens in chronic hypertension (Guyton *et al.*, 2006). The baroreflex was long viewed as short-term regulator but there

is currently a shift in the paradigm of baroreceptor-mediated blood pressure regulation. Recent findings from animal and human studies indicate that baroreceptors are involved in long-term blood pressure control.

2.4.2.3 Autonomic imbalance and cardiovascular disease

There is a large body of evidence to indicate that autonomic imbalance, characterized by a hyperactive sympathetic system and a hypoactive parasympathetic system, is strongly associated with some forms of CVD such as hypertension and ischemic heart disease. Autonomic imbalance often occurs before these disease states have been established and predicts cardiovascular morbidity and mortality. However, the causal role of autonomic imbalance in the development of CVD remains a subject of debate. Furthermore, the actual source of autonomic imbalance is not known with certainty. Rather than being a result of a single defect, the putative causes are complex, involving decreased baroreflex function, defects in central autonomic processing and an interplay with abnormalities in the renin-angiotensin system, NO-cGMP-pathway and redox balance. (Danson *et al.*, 2009)

In most cases of hypertension, the sympathetic control is heightened with respect to normotensive controls, while parasympathetic tone at rest is diminished. Such abnormalities are found in young normotensive individuals with a genetic predisposition towards hypertension before the onset of the disease (Davrath *et al.*, 2003). Epidemiological data indicate that vagal impairment is an independent predictor of cardiovascular and all-cause mortality (Fox *et al.*, 2007). In CVD, vagal activity decreases and heart rate becomes less regulated by vagal activity. Thus, resting heart rate and other measurers of vagal activity such as heart rate variability and baroreflex function can be used to assess autonomic imbalance and the risk for CVD.

2.4.3 Vascular endothelium in the control of vascular tone

Endothelial cells are differentiated epithelial cells that form a thin, single layer of cells called the endothelium, which lines the inner surface of the entire vascular and lymphatic trees. In an adult human, endothelium has a large surface area and constitutes a total volume that is comparable to that of the liver. Endothelium was long viewed as an inert semipermeable barrier only involved in the exchange of substances between blood and surrounding tissues. Transport of plasma proteins and electrolytes across the endothelium involves two different routes: paracellular transport through interendothelial junctions and transcellular caveolae-mediated vesicular transport. Interendothelial junctions contribute to the maintenance of the endothelial barrier function. However, electron microscopic studies of the vessel wall, physiological studies demonstrating the interaction between lymphocytes and numerous subsequent studies paved the way for the current view of the endothelium as a dynamic organ that has a wide array of secretory, synthetic, metabolic and immunologic functions. In terms of vascular homeostasis, endothelium has a crucial role in maintaining the balance between vasodilatation and vasoconstriction as well as in

regulating the proliferation and migration of smooth muscle cells and governing platelet function (Cines *et al.*, 1998).

The discovery of endothelium-dependent responses in the control of vascular tone and understanding of its importance in maintaining cardiovascular health are groundbreaking events in vascular physiology. It became gradually evident that endothelial cells respond to a wide variety of substances e.g. agents released by autonomic nerves and platelets, circulating hormones, cytokines and drugs as well as to physical and chemical stimuli (e.g. shear stress and pH), whereupon the endothelial cells release factors that modulate vascular tone and permeability. The role of the endothelium as a major regulator of vascular tone was consolidated after the discovery of endothelium-derived NO and its vasodilating property. However, the release of NO is not the only mechanism to evoke endothelium-dependent changes in vascular tone. In addition to NO, endothelial cells release prostacyclin (PGI_2), endothelial hyperpolarizing factors (EDHF), which cause smooth muscle cell relaxation and vasodilatation (Busse *et al.*, 2002). In fact, endothelial cells do not release only potent relaxing factors but also endothelin-1 (ET), the most potent vasoconstrictor identified to date, and other vasoconstrictors such as thromboxane A_2 (TXA_2) and platelet-activating factor (PAF) (Levin, 1995). These diverse vasoactive factors are not stored in intracellular vesicles but are regulated either via their rapid metabolism or at the level of gene transcription. Endothelial cells also directly communicate with vascular smooth muscle cells via myoendothelial gap junctions that allow the conduction of electronic tone (e.g. EDHF-mediated responses) and also the transfer of ions and small molecules such as calcium and cyclic nucleotides.

2.4.3.1 Nitric oxide

The seminal observation of Robert Furchgott demonstrated that the endothelium is required for the vasodilator response to acetylcholine since endothelium-denuded arteries failed to exhibit this response (Furchgott *et al.*, 1980). This experiment has profoundly deepened our understanding of the local control of vascular tone. Early bioassay studies demonstrated that endothelial cells release vasoactive substance(s) which was termed endothelium-derived relaxing factor (EDRF). The original EDRF was shown to diffuse readily from the endothelium to the underlying smooth muscle layer to stimulate soluble guanylyl cyclase, thus increasing the production of cGMP (Arnold *et al.*, 1977; Ignarro *et al.*, 1986). This chain of events eventually causes vascular smooth muscle relaxation and vasodilatation by decreasing the intracellular Ca^{2+} content. EDRF was shown to have a short biological half-life and to be scavenged rapidly by superoxide anions. Shortly later, the original EDRF entity was identified as nitric oxide (NO) (Palmer *et al.*, 1987). In 1998, Ignarro, Furchgott and Murad received a Nobel Prize for their elegant studies that highlighted the physiological importance of NO.

After the identification NO as the EDRF, the enzymes responsible for NO production were subsequently discovered and named in the order in which they were discovered:

NOS I (neuronal NOS, nNOS), NOS II (inducible NOS, iNOS) and NOS III (endothelial NOS, eNOS) (Bredt *et al.*, 1990; Förstermann *et al.*, 1991; Stuehr *et al.*, 1991). Another classification of NOS isoforms is based on the cell types from which they were originally purified, cloned and characterized: nNOS from cerebellum, iNOS from macrophages and eNOS from endothelial cells. However, it has since become clear that the different NOS isoforms have a much wider tissue distribution than was originally appreciated; eNOS, for instance, has been identified in cardiac myocytes, red blood cells, platelets and brain (hippocampus) as well as in renal and lung epithelium (Balligand *et al.*, 1993; Dinerman *et al.*, 1994; Kleinbongard *et al.*, 2006; Sase *et al.*, 1995; Shaul *et al.*, 1994). Nevertheless, in the vasculature under normal physiological conditions, eNOS is the main source of NO.

nNOS and eNOS are constitutively expressed and regulated by Ca^{2+}-calmodulin (CaM) and by phosphorylation, whereas iNOS is not generally expressed in unstimulated cells, and is activated independently of CaM during inflammatory defense reactions. Although constitutively expressed, eNOS expression levels are regulated by numerous physical and chemical stimuli, one of the most important factors being the shear stress generated by the viscous drag of blood flowing over the endothelial cell surface. In addition to being transcriptionally regulated, eNOS activity and NO production are modulated by multiple posttranslational mechanisms and signaling pathways. (Fleming *et al.*, 2003)

All NOS isoforms utilize L-arginine as the substrate and require the same cofactors: reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and 5,6,7,8-tetrahydrobiopterin (BH_4). NO synthases are multi-domain enzymes consisting of an N-terminal oxygenase domain that binds heme, L-arginine and BH_4 and a C-terminal reductase domain with binding sites for NAPDH, FMN, FAD and CaM. All isoforms are synthesized as monomers, but their normal function requires dimerization of the enzyme. During the synthesis of NO, NADPH-derived electrons pass to flavins and further to the heme which uses these co-factors to bind and activate O_2. NO is thereafter catalyzed from L-arginine in a stepwise synthesis involving first a reaction of hydroxylation and then an oxidation step. As mentioned earlier, the Ca^{2+}-calmodulin -complex is essential for activation of eNOS. In terms of posttranslational mechanisms, eNOS can be phosphorylated on its serine, threonine, and tyrosine residues, but the most important sites seem to be Ser^{1177} in the reductase domain and Thr^{495} within the CaM-binding domain. eNOS activity is strongly enhanced by phosphorylation of Ser^{1177}-residue which occurs in response to various stimuli such as shear stress, bradykinin and insulin. The phosphorylation can be mediated in several ways e.g. by protein kinase B (Akt), protein kinase A (PKA) or calmodulin-dependent kinase II (CaMKII). The kinases involved in the process vary with stimuli; shear stress, for instance, elicits phosphorylation of Ser^{1177} through PKA. In contrast, phosphorylation on Thr^{495} decreases eNOS activity. (Fleming *et al.*, 2003)

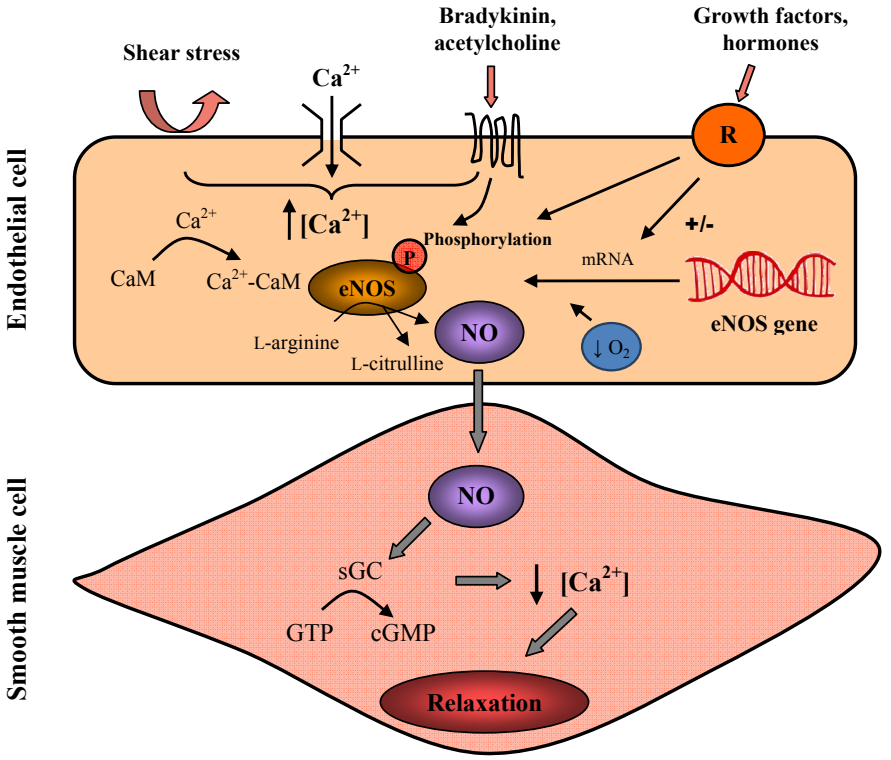

Figure 2.5. Current scheme for NO-dependent vascular relaxation. Shear stress of flowing blood on the surface of vascular endothelium causes calcium influx and thereby increases the intracellular calcium concentration (flow-dependent NO formation). Second, endothelial receptors for a variety of ligands (e.g. bradykinin and acetylcholine) stimulate calcium release and subsequent NO production (receptor-stimulated NO formation). Calcium-activated calmodulin (Ca^{2+}-CaM) then promotes the activity of endothelial nitric oxide synthase (eNOS, type III). The transcription of eNOS enzyme is regulated by hormones and growth factors. Hypoxia ($\downarrow O_2$) is also an important factor affecting eNOS expression. eNOS activity is modulated post-translationally by phosphorylation through stimulation of specific receptors on the endothelial surface (R). NO is produced through conversion of l-arginine by the enzyme eNOS. NO then diffuses across the smooth muscle cells where it activates soluble guanylyl cyclase (sGC), with a resulting increase in cGMP. cGMP induces smooth muscle relaxation by multiple mechanisms; e.g. by inhibiting calcium entry into the cell, thus reducing intracellular calcium concentrations.

2.4.3.2 Endothelial dysfunction

In most arterial diseases as well as in other cardiovascular diseases states, endothelium undergoes functional and structural alterations thus losing its protective function in vascular homeostasis. The loss of normal endothelial function is often referred to as endothelial dysfunction (Félétou *et al.*, 2006). A common denominator in systemic hypertension, for example, is endothelial dysfunction, which involves reduced production, decreased bioavailability, and/or impaired cellular effects of NO. Endothelial dysfunction is further manifested by an enhanced propensity to produce endothelium-

derived contracting factors such as ET-1 and by reduced sensitivity of vascular smooth muscle cells to endothelium-derived vasodilators. Depending on the pathology, vascular bed and the presence of other risk factors such as diabetes and obesity, the mechanisms underlying endothelial dysfunction may be multifactorial and considerably different from each other.

Commonly, in hypertensive vessels and in other situations where endothelial dysfunction is a pathogenic hallmark, the expression of eNOS enzyme is paradoxically increased, indicating that the ability of eNOS to produce NO has become compromised and/or the bioavailability of NO has declined. The former can be attributed to a phenomenon known as eNOS uncoupling whereby the electron transfer chain is disturbed and the enzyme becomes a source of superoxide anions instead of NO (Xia *et al.*, 1996a). Reduced NO bioavailability can arise from attenuated production but also from increased breakdown of NO by reactive oxygen species (ROS). Thus, oxidative stress, conditions involving chronically elevated ROS levels, is one of the main mechanisms contributing to endothelial dysfunction (Touyz *et al.*, 2011). Vascular ROS production is increased in different experimental models of hypertension, supporting a role for oxidative stress in hypertension, but the clinical evidence still remains controversial. It is unclear whether oxidative stress is a cause or a consequence of hypertension.

Finally, it is worth mentioning that in healthy arteries, the endothelium serves as non-adhesive and anti-thrombotic surface thereby providing vascular protection. Since endothelium-derived vasodilator substances exert anti-thrombotic and -proliferative actions and contribute significantly to the protective role of endothelium in vascular homeostasis, an impairment of endothelium-dependent vasodilatation is often accompanied by dysregulation of endothelial-blood cell-interaction which may lead to inflammation, vascular lesions and thrombosis. The structure of the vascular wall may also be altered as a consequence of reduced availability of NO and other vasodilators.

3 OBJECTIVES OF THE STUDY

The current studies were undertaken to investigate acute and long-term effects of melanocortins on blood pressure regulation. Additionally, in light of the undefined role of melanocortins in circulatory regulation and cardiovascular pathologies, the main objectives of the present study were:

1) to investigate whether systemic administration of α-MSH analogues elicits hemodynamic effects in anesthetized or conscious mice

2) to examine how a long-term increase in melanocortin activity can affect blood pressure regulation

3) to assess autonomic nervous system regulation and baroreflex function in genetic and pharmacologic mouse models of chronic melanocortin activation

4) to identify whether melanocortins contribute to the local control of blood vessel tone, and if so, by which mechanisms

5) to evaluate whether drug treatment targeted at the melanocortin system has any therapeutic value in experimental hypertension

4 MATERIALS AND METHODS

4.1 Animals and animal models

All experiments were performed on adult male mice. The mice were kept in the Central Animal Laboratory, University of Turku, and were housed at constant temperature (21 ± 2 °C) and humidity (50 ± 5 %), and on a 12 h light/dark cycle (lights on at 6 a.m.) with free access to water and food.

4.1.1 Ethical aspects

Experiments were planned with care to minimize the number of animals needed and to ensure that pain and suffering of the animals were kept to a minimum. Euthanasia was carried out according to general guidelines. All experiments were approved by the national Animal Experiment Board in Finland and conducted in accordance with the European Communities Council Directive.

4.1.2 Pharmacologic models of acute and chronic melanocortin activation

To study acute effects of melanocortins, the nonselective melanocortin agonist NDP-α-MSH (0.001-0.3 mg/kg) or MT-II (0.001-0.3 mg/kg) was given to adult male C57Bl/6N mice as a single intraperitoneal (i.p.) injection. In anesthetized mice, NDP-α-MSH and MT-II were administered i.v. at escalating doses (0.1-100 nmol/kg). To pharmacologically mimic chronic melanocortin activation, adult male C57Bl/6N mice were treated with NDP-α-MSH or MT-II (0.3 mg/kg/day, i.p.) for 7-21 days. In all experiments, control mice were injected with an equal volume of saline.

4.1.3 Transgenic MSH overexpressing mouse model

Transgenic MSH overexpressing (MSH-OE) mice were studied as a chronic model of increased melanocortin activity. MSH-OE mice were previously generated to overexpress N-terminal POMC under the control of the CMV promoter (Savontaus *et al.*, 2004). The transgene contained part of the 5'UTR, the signal sequence, the sorting sequence, g_3-MSH, the joining peptide, and α-MSH including the C-terminal glycine necessary for amidation. Overexpression of mature MSH peptides was shown to be limited to tissues that normally process POMC. Transgenic mice were originally produced on a C57BL/6 X CBA genetic background, and backcrossed to C57BL/6J- A^{wJ} for 6-8 generations to produce the mice used in this study. All experiments were performed on adult male wild-type and transgene homozygous MSH-OE mice.

4.2 Animal models of cardiovascular disease

4.2.1 Diet-induced obesity

To study subchronic melanocortin effects in diet-induced obese (DIO) animals, a cohort (*n=16*) of male 8-week-old C57Bl/6N mice was placed on a Western style diet (Research Diets Inc.). After 9 weeks on the diet, the mice were allocated into groups for the pharmacologic intervention and treated by daily i.p. injections with saline or MT-II (0.3 mg/kg) for 3 weeks. To determine body composition, DIO mice were subjected to quantitative NMR scanning (EchoMRI-700, Echo Medical Systems). Glucose tolerance tests were performed after a 4 hour fast. Blood glucose concentrations were measured before and 20, 40, 60, and 90 minutes after an i.p. glucose injection (1 g/kg).

4.2.2 DOCA-salt hypertension model

To investigate the effects of melanocortins in hypertensive animals, 4-month-old male C57Bl/6N mice were used in the deoxycorticostrenoacetate plus salt (DOCA-salt) model of hypertension. A slow-release (21-day) 50 mg DOCA pellet (Innovative Research of America) was implanted through a mid-scapular incision under isoflurane anesthesia. DOCA-salt animals received 1% NaCl in drinking water starting with the first day of DOCA-treatment. Control animals received normal drinking water. After 7 days of DOCA-treatment, control and DOCA-salt mice were randomized to receive daily injections of either saline or NDP-α-MSH (0.3 mg/kg, i.p.) for the following 14 days.

4.2.3 Pressure- and volume-overload induced by a high-sodium diet

To study whether transgenic MSH overexpression could have beneficial effects in terms of cardiovascular adaptability to a hemodynamic challenge, 12-month-old WT and MSH-OE mice were subjected to a state of dietary sodium surfeit. After baseline measurements of blood pressure and heart rate by a tail-cuff method, mice were placed on a high-salt diet (8 % NaCl, Research Diets Inc., USA) for 4 weeks to induce volume- and pressure-overload.

4.3 Physiology

4.3.1 Arterial pressure measurements

4.3.1.1 Measurements in anesthetized mice

Mice were anesthetized with either isoflurane (induction 4 %, maintenance 2 %) or a combination of chloral hydrate (300 mg/kg) and ketamine (100 mg/kg), and kept on a heating pad to maintain normothermia. A polyethylene catheter (PE-10, Clay-Adams, Il, USA) was inserted into the left carotid artery for the measurement of mean arterial pressure (MAP) and heart rate (HR) using a liquid pressure transducer (emka Technologies,

VA, USA) attached to 4-channel amplifier (emka Technologies, VA, USA). Data was acquired and analyzed using PowerLab and Chart5 software (ADInstruments, CO, USA). The femoral vein was cannulated to allow the administration of test compounds. After completion of preparative surgery, mice received a subcutaneous injection of normal saline (0.9% NaCl, 10 µl/g body weight) to replace surgical fluid losses. After recording baseline MAP and HR, mice were injected i.v. with increasing doses of NDP-α-MSH ranging from 10^{-10} to 10^{-7} mol/kg body weight in a volume of 1µl/g body weight. MAP and HR were recorded for the subsequent 10 min, after which a higher dose of NDP-α-MSH was administered. Similar experiments were carried out with MT-II.

4.3.1.2 Blood pressure telemetry

Mouse BP transmitters (TA11PA-C10, Data Sciences International, St. Paul, MN) were used to directly measure arterial pressure and heart rate in conscious mice. The mice were anesthetized with isoflurane and kept on a heated table throughout the surgery and during recovery to maintain body temperature. Mice were shaved and a ventral midline incision was made to access the left carotid artery. The catheter was inserted into the artery and the transmitter probe was positioned subcutaneously on the right flank of the animal. Buprenorphine (0.1 mg/kg s.c, Temgesic®, Schering-Plough, Belgium) was used for perioperative and postoperative analgesia. Mice were allowed 10-14 days of recovery from surgery before any measurements. Data were sampled every 1 or 5 minutes for 10 s with a sampling rate of 1000 Hz (Dataquest A.R.T. Software Version 3.1). In the more detailed evaluation of cardiovascular functions (4.3.2 - 4.3.4), blood pressure waveforms were stored and analyzed on a beat-by-beat basis.

4.3.1.3 Tail cuff measurements

In studies III, IV and V, systolic arterial pressure and heart rate were measured in conscious mice by a noninvasive tail-cuff method (TSE Systems, Bad Homburg, Germany). Mice were trained for at least 2 consecutive days before the actual data collection. After the training sessions, measurements were performed in a randomized order at the beginning of the experiment to assess the basal level of blood pressure and thereafter twice a week to follow blood pressure changes in response to a high sodium diet (Study III) or DOCA-salt (Study V). In each recording session before the data acquisition, mice were placed on a heated chamber (35 °C) and allowed to settle for at least 5 min to make the pulsations of the tail artery detectable. After obtaining at least seven valid readings in a measurement series of ten readings, the average of the valid readings was regarded as the SBP. If no valid readings were obtained, the measurement was stopped and repeated later.

4.3.2 Spectral analysis of heart rate and blood pressure variability

To evaluate autonomic cardiovascular control in the frequency domain, power spectral densities were computed by applying fast-Fourier transformation (FFT) (Baudrie *et al.*, 2007; Chen *et al.*, 2005; de Boer *et al.*, 1985; Gross *et al.*, 2002). Data series for systolic

blood pressure (SBP) and pulse-interval (PI) were linearly interpolated (20 Hz) and FFT was thereafter calculated on a 1,024-point series. Frequency band cutoffs were set at 2.5–5 Hz for high frequency (HF), 0.15–0.7 Hz for low frequency (LF), and 0–0.15 for very LF (VLF) as well as 0–5.0 Hz for total power. Integrals of the amplitude spectrum were calculated and they were used to define the spectral powers of the different frequency bands. Spectral powers of the LF and HF zones were normalized to account for differences in total power. Normalized LF and HF were calculated with the following method: LF_{nu} = LF / (Total power − VLF) * 100 and HF_{nu} = HF / (Total power − VLF) * 100.

4.3.3 Spontaneous baroreflex sensitivity

The baroreceptor heart rate reflex was investigated using the sequence method described in detail by Bertinieri et al (Bertinieri *et al.*, 1988). For this purpose, blood pressure signals were recorded on a beat-by-beat basis for 30 minutes (9 -10 *am*). Up sequences of 3 or more beats, with a delay of either 0 (Study I) or 3 (Study III) beats were analyzed. Up sequences were defined as baroreflex operations in which the SBP increases paralleled the pulse interval (PI) lengthenings. In study I, sequences with at least 0.5 mmHg SAP changes and 5 ms PI changes were analyzed with linear regression and were included in the analysis only if the correlation coefficient was > 0.80. In study III, no thresholds for the coefficient of correlation, SBP and PI changes were used as recommended by Laude et al (Laude *et al.*, 2008; Laude *et al.*, 2009). Spontaneous baroreflex sensitivity (BRS) was assessed as the slope (ms/mmHg) of the linear regression lines between SBP and PI values. BRS for each mouse was calculated as the mean value of all valid slopes obtained. The proportion of valid baroreflex sequences with respect to the total number of SBP ramps was applied as an index of baroreflex effectiveness (Laude *et al.*, 2008). In addition, the mean proportion of valid baroreflex sequences in the recordings was computed using the following equation: Baroreflex power (%) = (Number of heart beats in valid baroreflex sequences) / (Total number of heart beats) * 100 (Penttilä *et al.*, 2001).

4.3.4 Pharmacological testing

In study II, hemodynamic effects of peripherally administered α-MSH analogues, NDP-α-MSH and MT-II, were tested in conscious mice. Mice were implanted with telemetric devices and received i.p. injections of NDP-α-MSH or MT-II (0.01-0.3 mg/kg). Saline served as a vehicle control. The treatments sessions were conducted during the morning hours and included a control 30-min recording and a 120-min recording after drug injection. To test the effects of MC3/4-R antagonism, mice were pretreated with SHU9119 (1 mg/kg, i.p.) given 15 minutes prior to MT-II administration. The contribution of cardiac sympathetic activity to the hemodynamic effects of MT-II was examined by administering the selective β_1-adrenoceptor blocker metoprolol (4 or 8 mg/kg, i.p.). Fifteen minutes after this injection, mice were subjected to i.p. injections of MT-II. Furthermore, hexamethonium bromide (25 mg/kg, i.p.) was used to test the

effects of ganglionic blockade on MT-II-induced hemodynamic responses. Based on the relatively short duration of action of hexamethonium, the treatments were reversed and hexamethonium was administered 60 min after saline/MT-II. Pre-treatment values were compared with the lowest values occurring within 20 min after the hexamethonium administration.

To evaluate autonomic control of blood pressure and heart rate, mice were randomly assigned (Latin square-based block design) to one of the following treatment sessions: saline (10 ml/kg), muscarinic blockade by atropine (2 mg/kg), β_1-adrenergic blockade by metoprolol (4 mg/kg) or α_1-adrenergic blockade with prazosin (1 mg/kg) (da Costa-Goncalves *et al.*, 2008; Gross *et al.*, 2005; Janssen *et al.*, 2000). All substances were given i.p.

The treatments sessions were conducted during the morning hours (between 9 and 11 *am*) and separated by at least 24 h. Each session included a control 30-min recording and a 60-90-min recording after drug injection. A separate session with an i.p.-injection of saline was conducted to validate the recovery period needed to eliminate the stress-induced BP and HR changes associated with handling and injection. The mean values from 45 to 60-90 min after drug injection were used to analyze the respective drug responses.

4.3.5 Doppler echocardiography

In the assessment of coronary flow reserve (CFR), mice were randomly assigned to a transthoracic echocardiography (TTE) procedure. TTE measurements were performed using an Acuson Sequoia system (Siemens Acuson, Mountain View, CA) equipped with a 15-MHz linear transducer (15L8). Before the TTE examination, mice were anesthetized with 1.5 % isoflurane, shaved and kept on a heated table to maintain normothermia (Saraste *et al.*, 2008; Saraste *et al.*, 2006). Doppler measurements of flow velocities in the middle left coronary artery (LCA) were performed. The diastolic peak and mean flow velocities in the LCA were recorded in baseline (1.5 % isoflurane) and during elevated hyperaemic flow (2.5 % isoflurane) (Hartley *et al.*, 2008; Hartley *et al.*, 2007). CFR was calculated as the ratio of hyperaemic to baseline velocity in the LCA. All measurements were done off-line so that the observers were blinded to genotype. All parameters were averaged from three consecutive cardiac cycles.

4.3.6 Measurements of diuresis

Acute diuresis, occurring within the first 4 hours after the injection, was evaluated based on a change in body weight. Diuretic effect was also assessed by collecting and measuring 24-h urine output. To evaluate the dependence of the diuretic responses on MC receptor activation, mice were pretreated with the selective MC3/4-R antagonist SHU9119 (1 mg/kg, i.p.) given 30 minutes prior to NDP-α-MSH (0.3 mg/kg) administration.

4.4 Ex vivo studies

4.4.1 Vascular function assessment

4.4.1.1 Preparation and mounting of the arteries

Mouse thoracic aortae and small mesenteric arteries (80-100 μm in diameter), and porcine coronary arteries were excised rapidly and placed in ice-cold oxygenated Krebs solution. Arterial rings (2 mm in length) were mounted in a microvessel myograph (Danish Myograph Technologies, Aarhus, Denmark) for isometric tension recording. After mounting, the vessels were equilibrated for half an hour in a Krebs solution aerated with 95 % O_2 and 5 % CO_2 and heated to 37°C. The passive wall tension-internal circumference relationship was then determined and the internal circumference for each vessel was set to 0.9 * L_{100}, where L_{100} is the internal circumference the vessel would maintain under a transmural pressure of 100 mmHg (Delaey *et al.*, 2002; Peiper *et al.*, 1973). After a 45 min equilibration period, the arteries were contracted repeatedly with 62 mM KCl until maximal and reproducible contractions were obtained.

4.4.1.2 Vascular reactivity measurements

Vascular reactivity of isolated arteries was studied by adding vasoactive agents cumulatively into the myograph chambers. The rings were equilibrated for 20-30 min between different experiments and washed at least 3 times with Krebs solution during this period. To study vasoconstrictor responses, concentration-response curves to phenylephrine (PE) were constructed in the absence and presence of a nitric oxide synthase inhibitor (L-NNA, 100 μM), which was applied to organ chambers 30 min prior the start of a recording. Cumulative doses of either acetylcholine (ACh) or sodium nitroprusside (SNP) were added to the organ bath to invoke endothelium-dependent or endothelium-independent relaxation, respectively. Vasorelaxation was studied in arterial rings precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or phenylephrine. The extent of precontraction was adjusted to 50-70% of the reference contraction to 62 mM KCl (Matoba *et al.*, 2000). The contribution of vasodilator NO to ACh-induced relaxation was determined by the inhibitory effect of L-NNA (100 μM). Data were collected and analyzed using PowerLab and Chart5 softwares (ADI Instruments, Colorado Springs, CO).

4.4.2 Assessment of coronary endothelial function in the isolated mouse heart

Mice were anesthetized with pentobarbital sodium (50-100 mg/kg) and administered intravenous heparin (0.2 ml of 1000 IU/ml). Thereafter, the heart was isolated, washed in ice-cold saline and mounted according to the Langendorff method. The heart was retrogradely perfused through the aorta at constant pressure (60 mmHg) with modified Krebs bicarbonate buffer equilibrated with 95% O_2 + 5% CO_2 at 37°C. No electric pacing was applied to the heart. Hearts were allowed to equilibrate for 30-min before the onset of

drug applications. Coronary vasodilator responses to acetylcholine and bradykinin were assessed in the isolated heart. Vasoactive drugs were administered as a bolus injection just above the aortic cannula and coronary flow rate was monitored by timed collection of the outflow perfusate. Vasodilatation was expressed as increase in coronary flow after bolus injection. After the experiments, hearts were weighed and coronary vasodilator responses were expressed in ml/min/g of wet tissue weight.

4.4.3 cGMP content of aortic rings

Aortic segments were equilibrated (37°C, 30 min) in Krebs-Henseleit buffer and then stimulated with NDP-α-MSH (1 μM) for 60 min. The tissue samples were homogenized in 5% trichloroacetic acid (TCA). TCA was extracted using water-saturated ether prior to assaying the samples for cGMP content with an enzyme immunoassay (Cayman Chemicals). The cGMP levels of equilibrated, untreated rings were taken as the control.

4.5 Biochemical analyses

4.5.1 Quantitative reverse-transcription PCR

Total RNA from cell culture was extracted (Qiagen) and reverse-transcribed (Applied Biosystems). Quantitative RT-PCR was performed using SYBR Green protocols (Kapa Biosystems) on Applied Biosystems 7300 Real-Time PCR system. Primers were designed using Oligomer's and Roche's primer design programs and the primer set specificity was verified with a dissociation curve analysis. Primer sequences are given in Table 4.1. mRNA expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the comparative ΔCt method ($2^{-\Delta\Delta Ct}$) and are presented as relative transcript levels.

Table 4.1. Quantitative RT-PCR primers.

Gene name Accession number	**5'-3' primer sequence**	**Amplicon size**
NOS3 NM_000603.4	Forward: GCATCCCTACTCCCACCAG Reverse: TTCTTCACACGAGGGAACTTG	92
GPX1 NM_000581.2	Forward: CCAGTCGGTGTATGCCTTCTC Reverse: GAGGGACGCCACATTCTCG	106
CAT NM_001752.2	Forward: CTCGTGGGTTTGCAGTGAAAT Reverse: TCAGGACGTAGGCTCCAGAAG	187
SOD2 NM_000636.2	Forward: CTGGACAAACCTCAGCCCTA Reverse: TGATGGCTTCCAGCAACTC	62
GAPDH NM_002046.3	Forward: GAGTCCACTGGCGTCTTCAC Reverse: TTCACACCCATGACGAACAT	119

4.5.2 Urinary catecholamine, NOx, cGMP, creatinine and electrolyte concentrations

24-h urine samples were collected in metabolic cages. Catecholamines were extracted with activated alumina and analyzed with HPLC and coulometric electrochemical detection. Urinary nitrite plus nitrate (NO_x), cGMP and creatinine concentrations were determined with commercially available colorimetric assay kits (Cayman Chemicals, Ann Arbor, Michigan, USA). Sodium and potassium concentrations were measured by flame photometry.

4.5.3 In situ detection of vascular superoxide production

Frozen mouse aortae were cut into 10 μm thick transverse sections and placed on a glass slide. Dihydroethidium (5 μM, Molecular Probes) was topically applied onto each section and incubated for 30 min at 37 °C to reveal the presence of superoxide anions ($O_2^{\bullet -}$) by fluorescence microscopy.

4.5.4 SDS-PAGE and western blotting

Cell culture and tissue samples were lysed in RIPA buffer supplemented with protease and phosphate inhibitors cocktail. Aliquots (30 μg) of total protein were added to fivefold Laemmli buffer (0.25 mol/l Tris-HCl, pH 6.8, 10% SDS, 50% glycerol, and 12.5% β-mercaptoethanol) and size-fractioned by electrophoresis on 6-10 % SDS-polyacrylamide gels and transferred onto nitrocellulose membranes by electroblotting. Membranes were probed overnight at 4 °C for eNOS, phospho(Ser1177)-eNOS, Mn-SOD (BD Biosciences, San Jose, California, USA) or actin (Sigma-Aldrich Co.). Membranes were further incubated with peroxidase-conjugated secondary antibodies (Sigma-Aldrich Co.), and analyzed using enhanced chemiluminescence autoradiography (Pierce, Rockford, IL). The results for total protein expression were normalized to actin to correct for loading.

4.6 Drugs and solutions

For *in vivo* studies, all drugs except prazosin were dissolved in a 0.9 % NaCl (10 μl/g body weight). Prazosin was dissolved in a 10 % glucose solution containing 5 % PEG. L-NNA and $PGF_{2\alpha}$ were dissolved in 90% ethanol, and other drugs used in *ex vivo* experiments were dissolved in distilled water. The selective MC1 antagonist MSG606 was synthesized in the laboratory of Prof. Hruby as previously described (Juni *et al.*, 2010). All other drugs except $PGF_{2\alpha}$ (Cayman Chemicals) were purchased from Sigma-Aldrich. The composition of Krebs solution was as follows: 119 M NaCl, 4.6 M KCl, 15 M $NaHCO_3$, 1.2 M NaH_2PO_4, 2.5 M $CaCl_2$, 1.2 M $MgCl_2$ and 5.5 M glucose.

4.7 Statistical methods

All statistical analyses were performed using GraphPad Prism (versions 4.0 and 5.0, GraphPad Software, San Diego, CA) and SAS Enterprise Guide 3.0 (SAS Institute Inc, USA) programs. Two-tailed P values of less than 0.05 were considered statistically significant. All data are expressed as mean ± SEM. The following statistical analyses were used in the original articles:

I Hemodynamic parameters and locomotor activity were evaluated using ANOVA for repeated measures. Activity values were transformed logarithmically to adjust for normal distribution. Nonparametric t-test (Mann-Whitney U-test) was used to analyze heart rate and blood pressure variability.

II Dose-response analyses and comparisons between three experimental groups were made with one-way ANOVA followed by Dunnett's or Bonferroni *post hoc* tests. Time-effect relationships of administered compounds were evaluated using ANOVA for repeated measures. Two-way ANOVA was used to analyze the effects of two independent factors.

III Genotype differences in circadian BP and HR rhythms were evaluated using ANOVA for repeated measures. Differences between experimental groups were evaluated using the Student's t test, one-way or two-way ANOVA followed by Bonferroni *post hoc* tests.

IV Differences between two groups were analyzed by unpaired Student's t test and comparisons between three or more groups by one-way ANOVA followed by Bonferroni *post hoc* tests. For two independent factors, two-way ANOVA was used. Concentration-response curves were generated using nonlinear regression and E_{max} and EC_{50} values were compared using the extra sum-of-squares *F* test.

V Dose-responses were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Comparisons between three or more groups were made by one-way ANOVA followed by Bonferroni *post hoc* tests. Two-way ANOVA was used for two independent factors, two-way ANOVA.

5 RESULTS

5.1 Hemodynamic actions and mechanisms of α-MSH analogues

In the study examining the acute effects of peripherally administered α-MSH analogues on systemic hemodynamics, BP and HR responses to i.p. injections of NDP-α-MSH and MT-II were monitored in anesthetized and conscious mice. Under isoflurane anesthesia, NDP-α-MSH and MT-II at increasing doses caused a small decline in blood pressure (data not shown). Heart rate was unaffected by NDP-α-MSH or MT-II administration. In contrast to the effects observed under isoflurane anesthesia, NDP-α-MSH and MT-II caused moderate elevations in MAP and HR in mice anesthetized with ketamine and chloral hydrate. These findings highlight that the anesthetic regimens determine the hemodynamic responses to α-MSH analogues. In conscious mice, the stress response associated with handling and injection caused immediate and short-lasting increases in MAP and HR, but furthermore, NDP-α-MSH and MT-II treatments increased both MAP and HR in a dose-dependent manner (data not shown). Figure 5.1. illustrates the time course of changes in MAP and HR following the i.p. injection (0.3 mg/kg) of MT-II. Treatment with NDP-α-MSH or MT-II caused a significant increase in MAP, but the hypertensive effect seemed to vanish within 120 minutes after the injection. The tachycardic effect of α-MSH analogues lasted until the end of the monitored period.

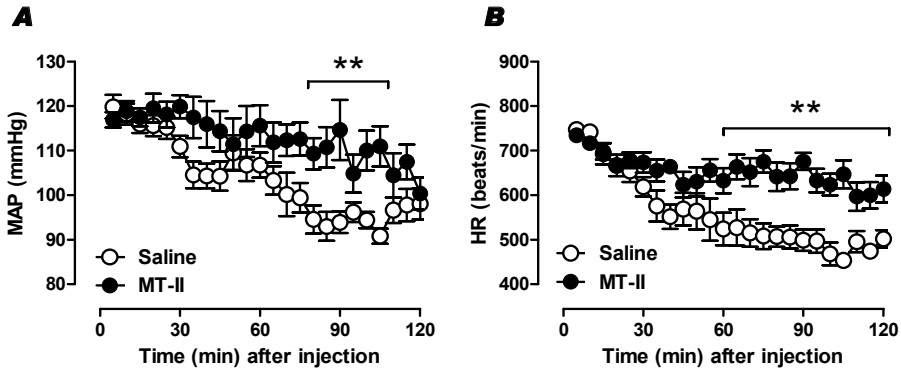

Figure 5.1. Intraperitoneal injection of MT-II elicits transient increases in blood pressure and heart rate. (**A** and **B**) Time-effect relationship for the changes of MAP and HR after intraperitoneal (i.p.) injection of MT-II (0.3 mg/kg). ** $P < 0.01$ versus control saline-treated mice. n = 8-10 per group in each graph. Values are mean ± S.E.M. Figure is modified from original communication II.

To test the dependence of the hemodynamic effects of MT-II on MC3/4-R activation, mice were pretreated with the selective MC3/4-R antagonist SHU9119 (1 mg/kg, i.p.) prior to MT-II treatment. Figure 5.2. shows the time-effect relationships and comparisons

of MAP and HR between control mice, saline pretreated mice and SHU9119 pretreated mice. Pretreatment with SHU9119 significantly attenuated the rise in MAP and HR evoked by MT-II (Fig. 5.2).

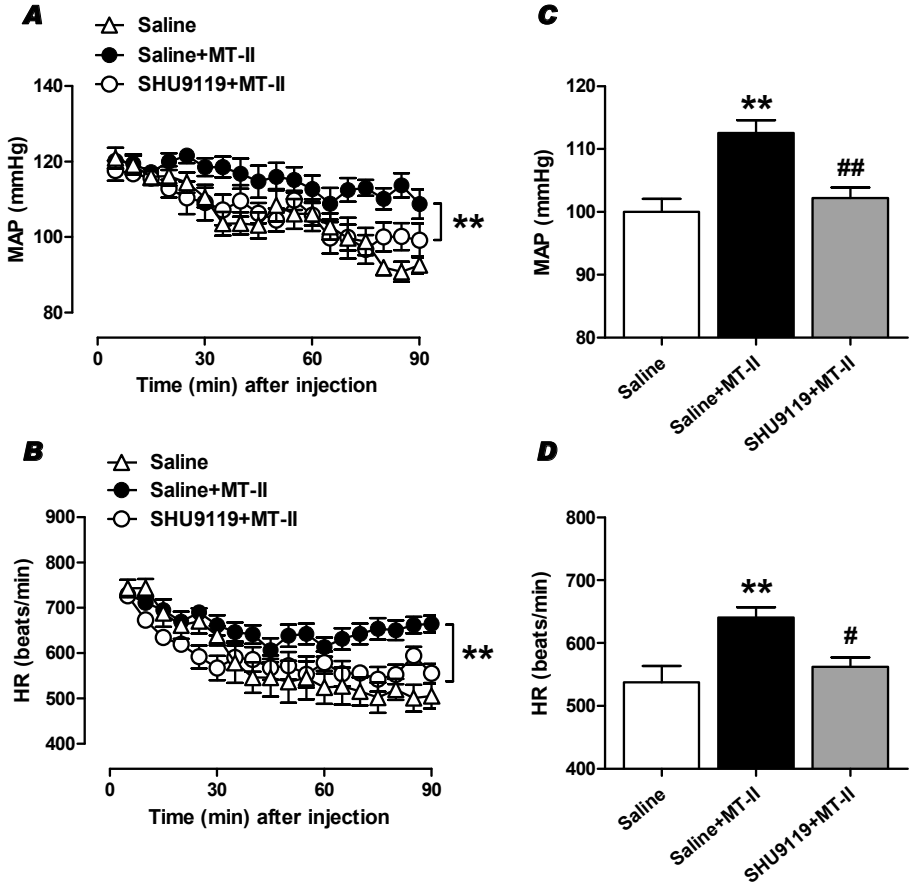

Figure 5.2. The MC3/4 receptor antagonist SHU9119 blocks the pressor and tachycardic effects of MT-II administration. (**A** and **B**) Time-effect relationship for the changes of MAP and HR after MT-II (0.3 mg/kg, i.p.) administration. Mice were pretreated with either saline or SHU9119 (1 mg/kg, i.p.) 15 min prior to MT-II administration. (**C** and **D**) Mean values of MAP and HR averaged from 45th to 90th min after MT-II injection. $^{**}P < 0.01$ versus saline, # $P < 0.05$ and ## $P < 0.01$ versus saline+MT-II.n = 8-10 per group in each graph. Values are mean ± S.E.M. Figure is from original communication II.

To test whether the MT-II evoked increase in HR could be attributed to enhanced cardiac sympathetic activity, mice were pretreated with the selective β_1-receptor antagonist metoprolol and administered saline or MT-II (0.3 mg/kg, i.p.) 15 minutes later. Metoprolol abolished the increase in blood pressure after MT-II treatment, but did not completely block the HR response to MT-II (data not shown).

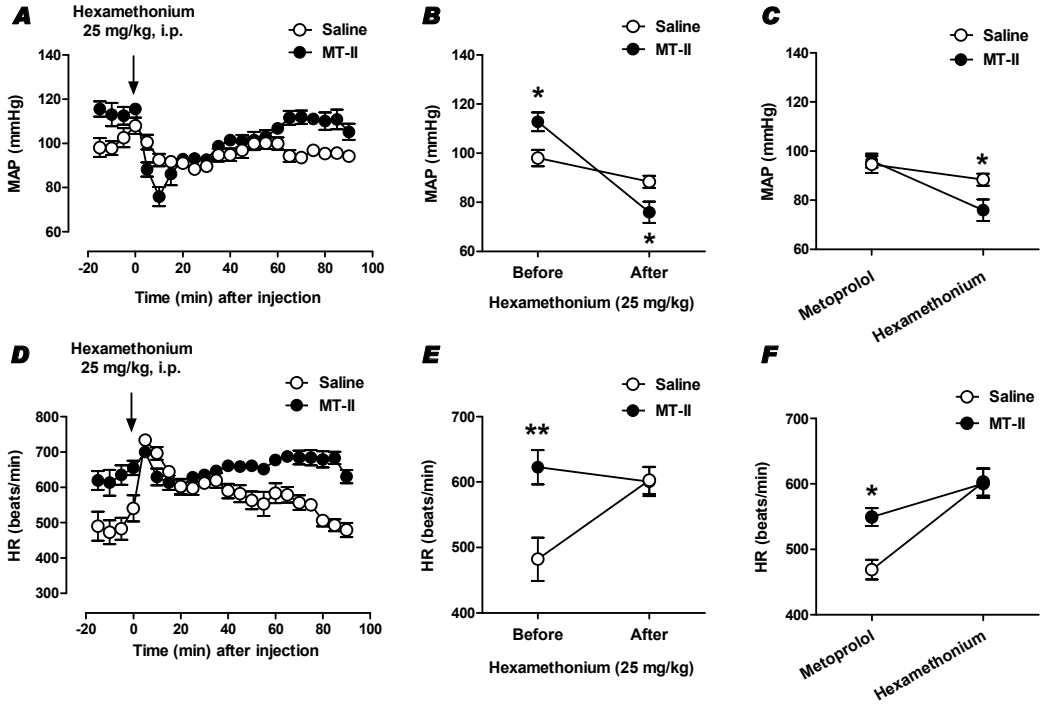

Figure 5.3. Effects of the ganglionic blocker hexamethonium on blood pressure and heart rate in MT-II-treated mice. (**A**) Time course of MAP before and after inhibition of ganglionic transmission by hexamethonium (25 mg/kg i.p.) in saline- and MT-II-treated mice. Saline or MT-II was given 60 min before hexamethonium. (**B**) Averaged peak response to ganglionic blockade. Time-effect relationship (**D**) and averaged peak response (**E**) of HR to hexamethonium administration. (**C** and **F**) Comparison of the MAP and HR levels after metoprolol (8 mg/kg) and hexamethonium (25 mg/kg) treatments. * $P < 0.05$ and ** $P < 0.01$ versus saline-treated mice. Figure is from original communication II.

Furthermore, mice were treated with the ganglionic blocker hexamethonium (25 mg/kg, i.p.) to eliminate the influence of the sympathetic and parasympathetic nervous systems on hemodynamics. Mice treated with MT-II showed a pronounced drop in MAP after hexamethonium treatment, suggesting that the intrinsic blood pressure level is lower in these mice compared to control saline-treated mice (Fig. 5.3A and B). Ganglionic blockade with hexamethonium elevated HR only in control mice and thus, abolished the initial difference in HR between saline and MT-II-treated mice (Fig. 5.3D and E). Furthermore, HR level was markedly higher in saline-treated mice after the hexamethonium treatment compared to the level observed after the administration of metoprolol (Fig. 5.3F), revealing the significant contribution of the parasympathetic component in the regulation of resting heart rate.

5.2 Blood pressure regulation in transgenic MSH-OE mice

To study the cardiovascular effects of chronic melanocortin activation under post-surgical stress and in physiological baseline conditions, transgenic MSH-OE mice were studied at 3 and 6 months of age by radiotelemetry and the observed phenotype was assessed against age-matched wild type (WT) mice. Interestingly, both young and aged MSH-OE mice showed lower daytime locomotor activity soon after the implantation surgery (Fig. 5.4, C and F). This finding was associated with a similar decrease in MAP, which was more pronounced in young MSH-OE mice (Fig. 5.4A, unpublished data). The restoration of circadian blood pressure and HR rhythm occurred within 5 days after the surgery in both age groups and strains of mice.

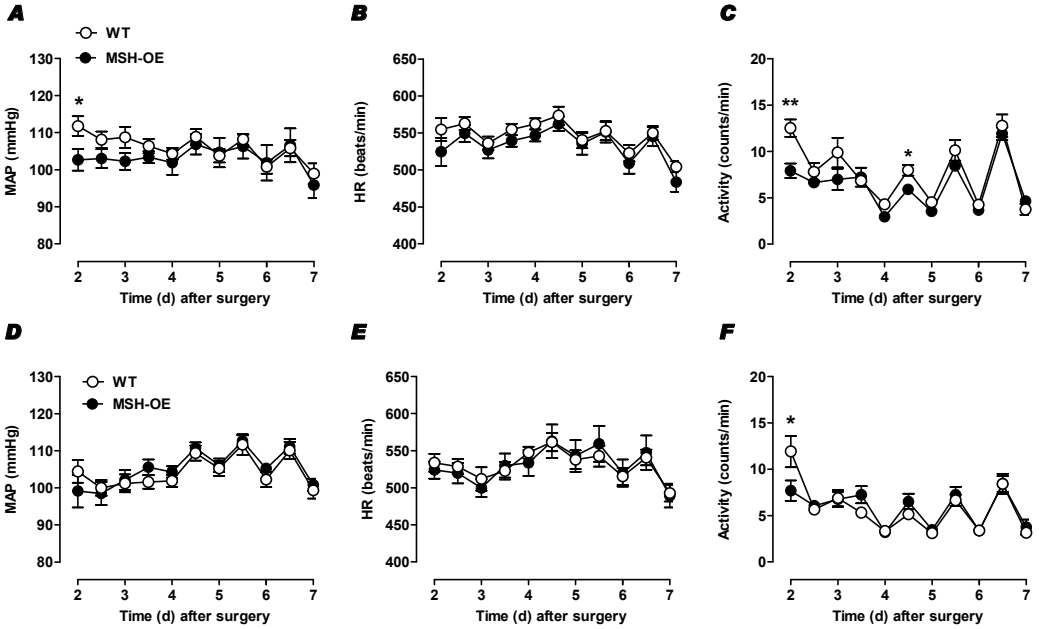

Figure 5.4. Telemetric measurements during the recovery period. Recovery from (**A**-**F**) Restoration of circadian rhythm in MAP, heart rate and locomotor activity after the implantation surgery for radiotelemetry in 3-month-old (**A**-**C**) and 6-month-old (**D**-**F**) MSH-OE mice. Data represent 12-hour mean values of light (day) and dark (night) periods. * $P < 0.05$; ** $P < 0.01$ vs. WT mice.

Baseline values of hemodynamic parameters and locomotor activity were analyzed between days 10 and 12 after the surgery. The general pattern of circadian rhythm in MAP and HR was similar between the two strains of mice (Fig. 5.5). Comparison between the different age groups reveals that MAP had slightly increased (Fig. 5.5, A and D) and nighttime locomotor activity (Fig. 5.5, C and F) decreased with age. HR was slightly but significantly lower in young MSH-OE mice compared to age-matched WT mice (Fig. 5.5B). A similar trend was observed in aged MSH-OE mice (Fig. 5.5E). No

differences were noted in MAP and locomotor activity of MSH-OE mice compared to age-matched WT mice.

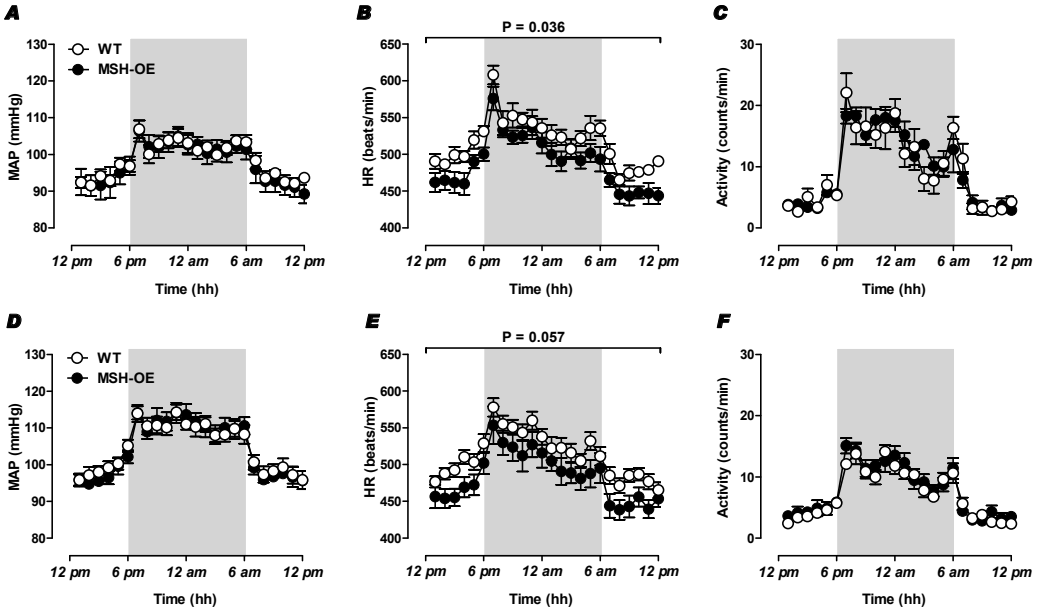

Figure 5.5. **Reduced basal heart rate in young and aged MSH-OE mice.** Circadian rhythm of mean arterial pressure (**A** and **D**), heart rate (**B** and **E**) and physical activity (**C** and **F**) in 3-month-old (upper panel) and 6-month-old (lower panel) WT and MSH-OE mice. The data is averaged from day 10 to day 12 after implantation of the blood pressure transmitter. The dark period of the day between 6 pm and 6 am is marked in gray. Exact P-values for the genotype effect (ANOVA for repeated measures) are shown in the graphs. Values are mean ± SEM, *n* = 7-12 per group in each graph. Figure is modified from original communication III.

5.3 Cardiovascular autonomic control in transgenic MSH-OE mice

5.3.1 Evaluation of heart rate variability

One way to evaluate cardiac vagal activity in MSH-OE mice is to conduct a frequency-domain based analysis of heart rate variability. Fig. 5.6A illustrates the distribution of the spectral powers of heart rate variability over the range 0 to 5 Hz, and also the different spectral components used in the analysis. The overall heart rate variability was not different between the WT (986 ± 160 ms^2) and MSH-OE (1 082 ± 174 ms^2) mice. However, when the power spectra were subdivided into different spectral components and the power values were normalized, MSH-OE mice had a tendency (P = 0.11) towards increased LF power accompanied by significantly decreased HF power (Fig. 5.6, B and C). Additionally, the LF/HF ratio was increased in MSH-OE mice (Fig. 5.6D), suggesting an enhancement in cardiac vagal tone.

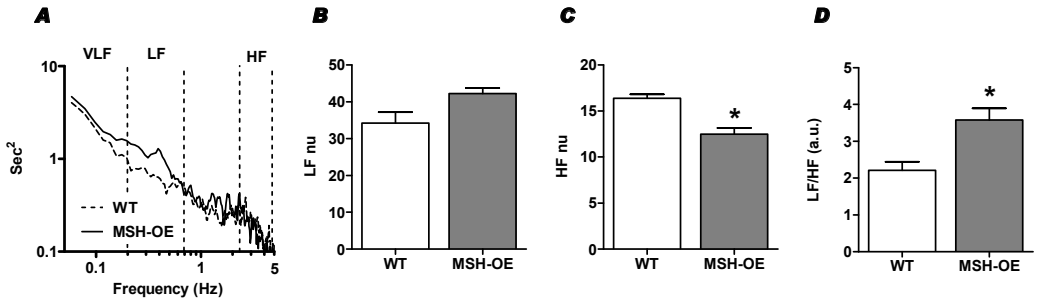

Figure 5.6. **Heart rate variability in WT and MSH-OE mice**. (**A**). Power spectra were subdivided into 3 frequency ranges as indicated by dotted lines: VLF; very low frequency (0-0.15 Hz), LF; low frequency (0.15-0.7 Hz) and high frequency (2.5-5.0 Hz). MSH-OE mice tended to have increased LF power (**B**), accompanied by reduced HF power (**C**). Further, the LF/HF ratio of HRV was increased in MSH-OE mice (**D**). * $P < 0.05$ between WT and MSH-OE mice. Figure is modified from original communication I.

5.3.2 Blood pressure and heart rate responses to adrenergic receptor blockers

To further evaluate autonomic control of HR and BP, cardiovascular responses to autonomic blocking agents were monitored by radiotelemetry. First, MAP and HR responses to the stress caused by handling and i.p. injection were investigated by administering saline to the mice. This treatment revealed that 3-month-old MSH-OE mice were able to restore their HR more rapidly than the age-matched WT mice (Fig. 5.7A). However, forty-five minutes after the injection of saline, MAP and HR had returned to a comparable level with baseline values in both groups of mice. Figures 5.7 B and C illustrate the changes in MAP and HR of 3-month-old WT and MSH-OE mice after receptor blockade with atropine, metoprolol, prazosin, and after combined blockade with atropine and metoprolol. There were no inter-genotype differences in changes of MAP in any of the treatments. However, significant differences ($P < 0.001$ by 2-way ANOVA) were noted in HR changes between WT and MSH-OE mice.

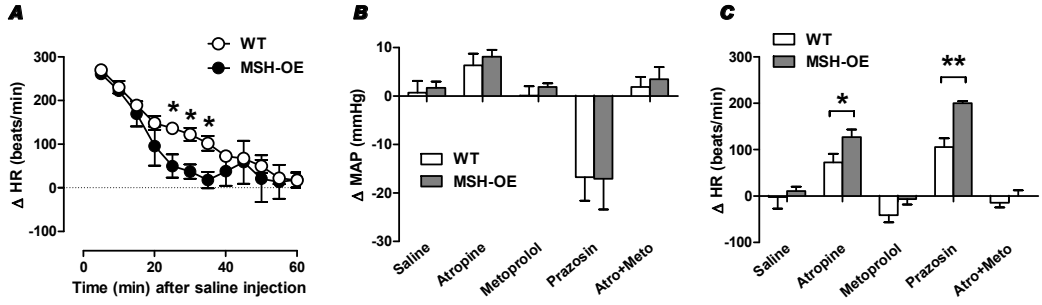

Figure 5.7. **Increased cardiac vagal activity in MSH-OE mice**. (**A**) Change in heart rate of 3-month-old WT and MSH-OE mice after control injection with saline. (**B** and **C**) Changes in MAP and HR of 3-month-old WT and MSH-OE mice after receptor blockade with atropine, metoprolol, prazosin, and after combined blockade with atropine and metoprolol. n = 7-12 per group in each graph. * $P < 0.05$, ** $P < 0.01$ versus WT mice. Figure is modified from original communication III.

After muscarinic blockade with atropine, MAP increased similarly in both groups, but the increase in HR was higher in MSH-OE mice, pointing to enhanced vagal tone. Metoprolol did not affect MAP but lowered HR below baseline values more appreciably in WT mice than in MSH-OE mice. The HR changes after combined application with atropine and metoprolol were minor and comparable between the genotypes, revealing that the intrinsic level of HR is very close to the pre-treatment, baseline values in both groups of mice (unpublished data). Blockade of α_1-adrenergic receptors by prazosin decreased MAP in both groups. However, the compensatory increase in HR after prazosin was more prominent in MSH-OE mice, suggesting enhanced baroreflex control of HR. In summary, vagal input to the heart was augmented in MSH-OE mice. Furthermore, normalization of stress-induced HR increase was more effective in MSH-OE mice than in WT mice.

5.4 The effect of MSH overexpression on baroreflex function

To further evaluate the impact of MSH overexpression on baroreflex function, spontaneous baroreflex sensitivity (BRS), effectiveness and power were calculated by the sequence technique (Fig. 5.8). There were no statistically significant differences in BRS between the genotypes. However, MSH-OE mice displayed significantly increased baroreflex effectiveness, indicating that blood pressure elevations are more likely to be accompanied by subsequent changes in heart rate in the expected direction. Also the baroreflex power was significantly increased in MSH-OE mice, suggesting that MSH overexpression had increased the number of baroreflex operations. These findings suggest that MSH overexpression reinforces the relative contribution of the baroreflex to heart rate regulation.

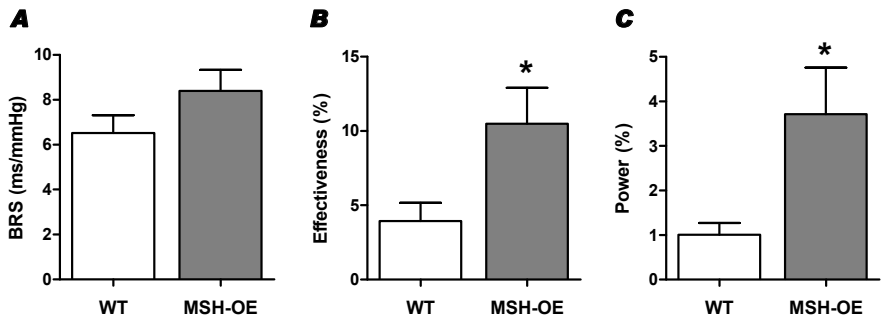

Figure 5.8. **MSH overexpression improves baroreflex control of heart rate.** Baroreflex sensitivity (**A**), effectiveness (**B**) and power (**C**) calculated by the sequence technique for up-slopes of systolic blood pressure. * $P < 0.05$ versus WT mice. Figure is modified from original communication I.

5.5 Pharmacologic model of chronic melanocortin activation and autonomic balance

Cardiovascular responses to control saline injection, atropine, metoprolol and prazosin were evaluated in mice treated with MT-II for 7 days. Repeated and chronic exposure to MT-II did not change the typical stress-associated responses in MAP and HR evoked by a control saline injection (data not shown). Furthermore, MT-II treated mice showed no alterations in MAP responses to atropine, metoprolol or prazosin (Fig. 5.9A). In terms of HR changes, MT-II-treated mice tended to show a stronger response to atropine and a weaker response to metoprolol (Fig. 5.9B), but these changes did not reach statistical significance ($P = 0.06$). MT-II-treated mice showed also a stronger compensatory increase in HR in response to vasodilatation induced by the α_1-adrenergic receptor antagonist prazosin (Fig. 5.9B).

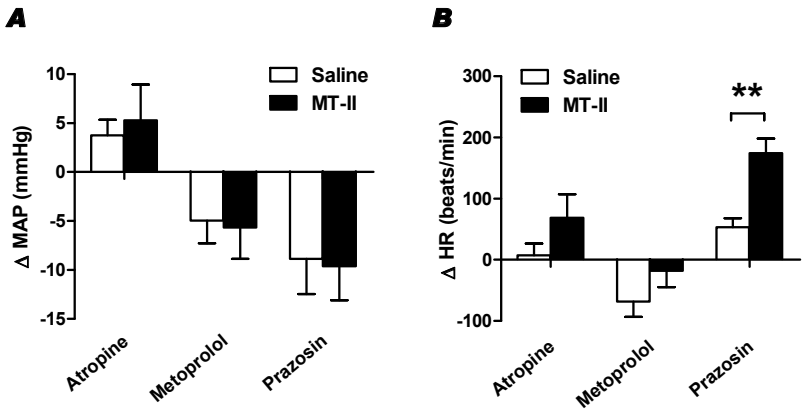

Fig. 5.9. **Chronic MT-II treatment enhances the HR response to prazosin.** (**A** and **B**) Changes in MAP and HR of saline- and MT-II-treated mice after receptor blockade with atropine, metoprolol and prazosin. Values were averaged from 45th to 60th min after the injection. Comparisons were made using 2-way ANOVA followed by Bonferroni *post hoc* tests. n = 6-7 mice per group. ** P < 0.01 versus saline-treated mice. Data are presented as means ± S.E.M. Figure is modified from original communication III.

5.6 Melanocortins in the local control of vascular tone

No study had ever addressed the direct effects of melanocortins on blood vessel tone. This spurred the interest to investigate whether melanocortins could affect vascular functions through a peripheral site of action. Moreover, several papers reported that endothelial cells both in humans and rodents express MC1 receptors and seem to be capable of processing POMC into biologically active α-MSH (Hartmeyer *et al.*, 1997; Lindskog *et al.*, 2010; Scholzen *et al.*, 1999). Given the evident expression of MC1-R in the endothelium, it was hypothesised that α-MSH by acting through the endothelial MC1-R might have a role as a regulator of NO-cGMP-pathway.

5.6.1 Key observations from isometric tension recordings

To examine whether α-MSH could directly modulate the vascular tone of isolated arteries, NDP-α-MSH was applied to arteries and changes in vascular tone were monitored by wire myography. First, the addition of α-MSH to preconstricted mouse aortae or mesenteric arteries caused no vasorelaxation or vasoconstriction (Fig. 5.10A). Likewise, porcine coronary arteries showed no direct response to α-MSH (unpublished data). Next, to determine whether α-MSH could modify endothelial function, isolated arteries were pretreated with α-MSH (1 μM) for 60 min and the responses to endothelium-dependent vasodilators were assessed. *In vitro* pretreatment with α-MSH significantly enhanced the aortic responses to ACh (Fig. 5.10B). These responses were confined to the larger arteries as the same pretreatment failed to improve ACh-responses in resistance arteries (Fig. 5.10C).

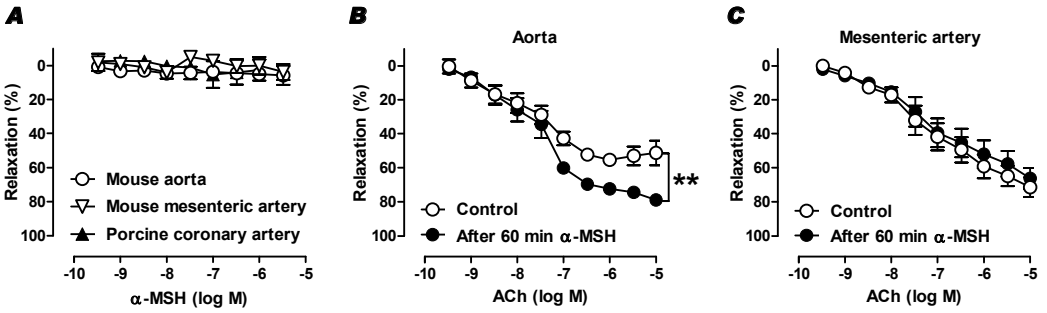

Figure 5.10. α-MSH improves endothelium-dependent vasodilatation. (**A**) α-MSH does not cause direct effects on blood vessel tone of selected vessel preparations (**B, C**) Endothelium-dependent relaxations of mouse aorta and mesenteric artery in control preparations and in preparations pretreated with 1 μM α-MSH for 60 min. ** $P < 0.01$ vs. control. Figure is modified from original communication IV.

To examine whether the same effect could be reproduced by an *in vivo* treatment, 10-month-old mice and diet-induced obese (DIO) C57Bl/6N mice were subjected to acute (single administration) and subchronic (daily injections for 3 weeks) treatment with potent analogues of α-MSH; NDP-α-MSH and MT-II (0.3 mg/kg, i.p.), respectively. Since it was desired to examine a chronic model of increased melanocortin activity, 5-month-old MSH-OE mice were studied.

Of note, acute and subchronic melanocortin treatments improved relaxation responses to ACh in the aorta of 10-month-old mice and DIO-mice (Fig. 5.11, A and B). In MSH-OE mice, no distinct increments in the maximum responses to ACh were noted (Fig. 5.11C), probably because the endothelial function was well preserved in the WT control animals. NOS inhibition with L-NNA significantly attenuated relaxation responses to ACh and eliminated influence of melanocortin treatments on the initial ACh-responses, implying that increased NO bioavailability contributes to the melanocortin-evoked effects on endothelial function. In contrast to findings in aortic preparations, melanocortins were ineffective in enhancing ACh-responses in small mesenteric arteries (data not shown).

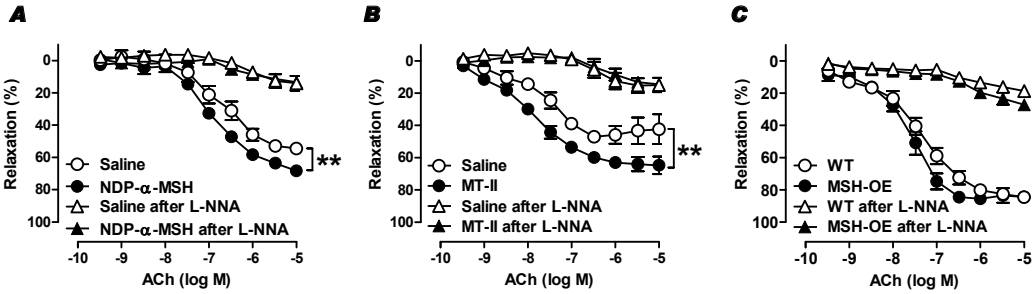

Figure 5.11. **Increased melanocortin activity enhances endothelium-dependent vasodilatation.** Endothelium-dependent relaxation to acetylcholine (ACh) and the effect of NOS blockade on corresponding responses in the aorta of NDP-α-MSH-treated 10-month-old mice (**A**), MT-II-treated DIO mice (**B**) and MSH-OE mice (**C**). E_{max} responses were estimated by nonlinear regression and compared using the extra sum-of-squares F test, where * $P < 0.05$ and ** $P < 0.01$ versus control mice. Figure is modified from original communication IV.

In addition to improving endothelium-dependent relaxation in the aorta, chronic MT-II treatment and MSH-OE increased endothelium-independent vasodilatation evoked by SNP. This effect was apparent in the aorta and small mesenteric arteries alike, indicating improved signaling downstream of NO, regardless of the vessel size (data not shown). Taken together, the data indicate that melanocortins improve vascular function by strengthening signaling through the NO-cGMP-pathway.

5.6.2 The role of melanocortins in coronary flow regulation

To determine whether similar effects existed also in an intact coronary circulation, coronary vascular function was assessed in the isolated and Langendorff -perfused hearts from saline and NDP-α-MSH treated mice. Vasodilator responses to endothelium-dependent agonists were studied by measuring increases in coronary outflow. Responses to ACh were comparable between the groups while bradykinin induced significantly greater vasorelaxation and consequent increase in total coronary flow in the hearts of NDP-α-MSH treated mice (Fig. 5.12A and B). This observation could be attributed to increased NO production because in mice, responses to bradykinin in the integrated coronary bed are predominantly NO-dependent (Gödecke *et al.*, 2002).

Furthermore, *in vivo* effects of increased melanocortin activity on coronary vascular function were studied by applying Doppler echocardiography. Isoflurane, known as a potent coronary vasodilator, was used here to assess coronary flow reserve (CFR), the ratio of hyperaemic to baseline flow velocity. Coronary flow velocities were measured in young (3-mo) and aged (6-mo) mice anesthetised with low (1.5 %) and high (2.5 %) levels of isoflurane to generate baseline and elevated hyperaemic coronary flows, respectively. CFR was significantly increased in young and aged MSH-OE mice compared to age-matched WT mice (Fig. 5.12C).

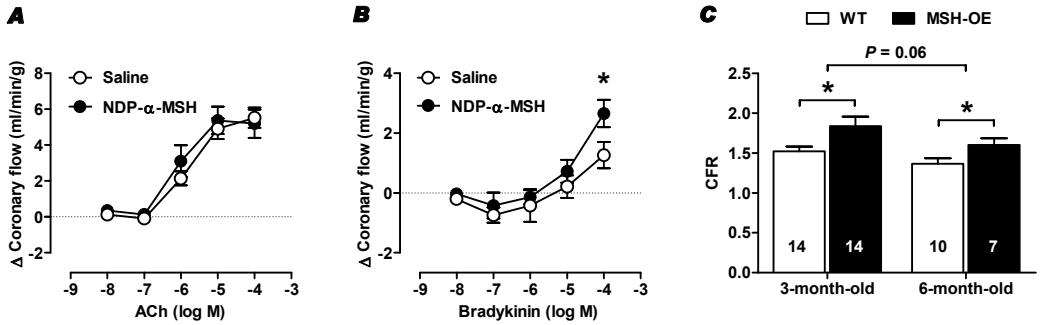

Figure 5.12. Melanocortins improve coronary flow regulation. Changes in coronary flow response to increasing doses of ACh (**A**) and bradykinin (**B**) in saline- and NDP-α-MSH-treated hearts (n=8 per group), * P<0.05 vs. saline-treated mice. (**C**) Coronary flow reserve (the ratio of hyperemic to baseline flow velocity) of WT and MSH-OE mice. * P<0.05 versus age-matched WT mice. Figure is modified from original communication IV.

5.6.3 Effects of α-MSH on the eNOS system and antioxidant enzymes in cultured human endothelial cells

In an attempt to investigate the mechanism by which α-MSH improves endothelial function, cultured primary (HUVEC) and transformed (EA.hy926) endothelial cells were stimulated with α-MSH and the effects on eNOS expression and phosphorylation at Ser^{1177} were examined. Stimulation with NDP-α-MSH for 4h elicited a subtle increase in eNOS mRNA expression, which then seemed to be downregulated after 18h stimulation (Fig. 5.13A). These changes in mRNA expression were translated into a tendency (P = 0.07) of increased eNOS protein levels after 18h treatment with 1 μM NDP-α-MSH (Fig. 5.13B). Furthermore, NDP-α-MSH stimulated eNOS phosphorylation at Ser^{1177} in a

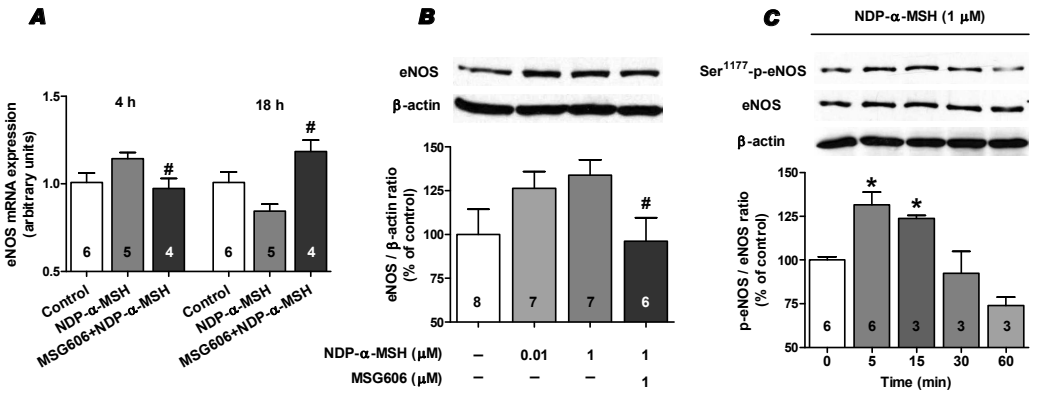

Figure 5.13. α-MSH regulates eNOS expression and phosphorylation in human endothelial cells. (**A**) Primary HUVECs were stimulated with α-MSH for 4h and 18h and analyzed for eNOS mRNA expression (**B**) Transformed endothelial cells (EA.hy926) were treated with α-MSH for 18h and analyzed thereafter for eNOS protein levels by western blotting. (**C**) EA.hy926 cells were stimulated with α-MSH (1 μM) for the indicated times and quantified for the degree of phosphorylation of eNOS on Ser^{1177} by western blotting. * P<0.05 versus control, # P<0.05 versus NDP-α-MSH. Figure is modified from original communication IV.

time-dependent manner (Fig 5.13C). Pretreatment with the selective MC1-R antagonist MSG606 abolished the stimulatory effects of α-MSH on eNOS expression (Fig. 5.13B) and phosphorylation (data not shown).

Furthermore, the effects of α-MSH on mRNA expression of the antioxidant enzymes catalase, glutathione peroxidase (GPx1) and mitochondrial superoxide dismutase (Mn-SOD) were investigated in HUVECs. NDP-α-MSH selectively increased the expression of Mn-SOD (data not shown), an enzyme responsible for the neutralization of superoxide anions to oxygen and hydrogen peroxide. Taken together, the results demonstrate that α-MSH, by binding to endothelial MC1-R, regulates the eNOS system at the transcriptional and posttranslational levels, and increases Mn-SOD expression. All of these effects, separately and in concert with each other, are likely to enhance the local availability of NO in blood vessels, supporting the findings from vascular function measurements.

5.7 Melanocortins and the regulation of body fluid balance

To investigate the diuretic and natriuretic actions of NDP-α-MSH, NDP-α-MSH was administered as an i.p. injection (0.3 mg/kg, i.p.) to chow-fed C57Bl/6N mice. Thereafter, body weight, 24-hour urine and electrolyte output were monitored and compared to saline-treated controls. NDP-α-MSH induced a marked diuretic effect as evidenced by a change in body weight. This response occurred in a dose-dependent manner (data not shown). Pretreatment with the MC3/R receptor antagonist SHU9119 prevented the NDP-α-MSH-induced diuresis. Supporting these findings, 24-h urine volume was also

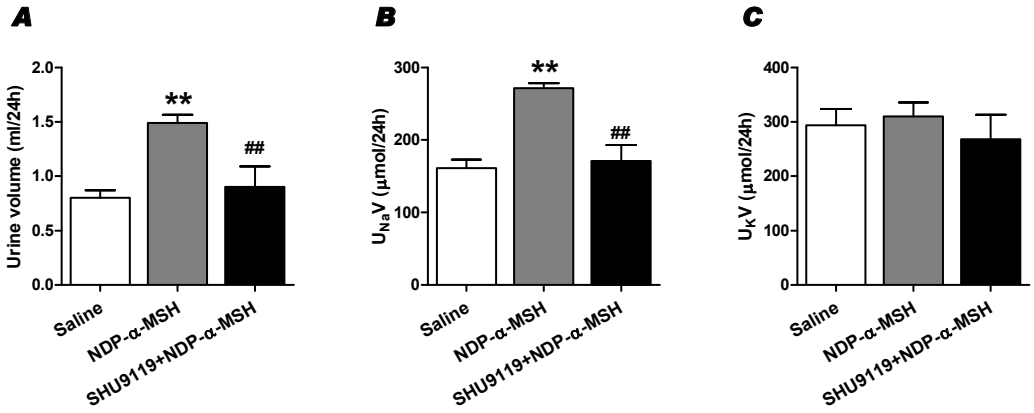

Figure 5.14. α-MSH analogues elicit significant diuretic and natriuretic responses. (**A, B**) NDP-α-MSH (0.3 mg/kg) induced marked increases in 24-h urine volume and sodium excretion ($U_{Na}V$) that were blocked by SHU9119 (1 mg/kg, 30-min before NDP-α-MSH). (**C**) NDP-α-MSH and SHU9119 had no effect on urinary potassium excretion. Drugs were given as a single i.p. injection just prior to starting urine collection in metabolic cages. Antagonists (1 mg/kg, i.p) were given 30 minutes prior to administration of NDP-α-MSH. All experiments were performed on normal chow-fed C57Bl/6N mice. ** $P<0.01$ versus saline group, ## $P<0.01$ versus NDP-α-MSH/MT-II. Figure is modified from original communication V.

increased by NDP-α-MSH administration (Fig. 5.14A). Furthermore, NDP-α-MSH markedly increased urinary excretion of sodium (Fig. 5.14B), indicating natriuretic activity. NDP-α-MSH administration did not induce kaliuresis despite the profound diuresis and natriuresis (Fig. 5.14C). Pretreatment with SHU9119 abolished the NDP-α-MSH-induced diuresis and natriuresis (Fig. 5.14).

5.8 Therapeutic efficacy of melanocortins in hypertension

It was decided to determine whether chronic treatment with NDP-α-MSH has any therapeutic efficacy in systemic hypertension and therefore 4-month-old C57Bl/6N mice were used in the deoxycorticosterone acetate plus NaCl (DOCA-salt) model of hypertension and treated with NDP-α-MSH (0.3 mg/kg/d, i.p.) starting on the 7th day after the beginning of the DOCA-salt treatment. Notably, NDP-α-MSH -treatment was able to restrain the progression of hypertension in DOCA-salt mice without affecting blood pressure levels of normotensive control animals (Fig 5.15A). This effect was observed in two separate cohorts of mice with two measurement techniques; radiotelemetry and tail cuff plethysmography. Interestingly, NDP-α-MSH-treated mice showed a normal dip in 12-h day-time pressure values after 21 days on DOCA-salt and thus, they displayed an increased circadian amplitude compared to saline-treated mice suggesting that NDP-α-MSH could prevent the disruption of circadian blood pressure rhythm after DOCA-salt-treatment (data not shown).

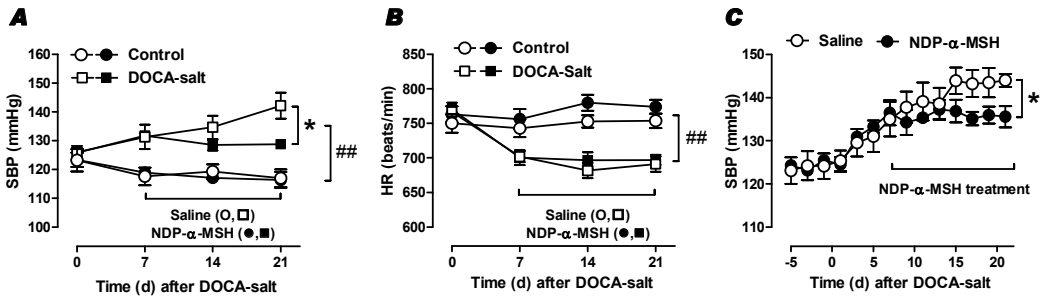

Figure 5.15. Hemodynamic effects of NDP-α-MSH-treatment on DOCA-salt induced hypertension. Mice were subcutaneously implanted with a DOCA pellet and received 1% NaCl in drinking water. Daily treatment with NDP-α-MSH (0.6 mg/kg, s.c.) was started 7 d after the beginning DOCA-salt treatment. (**A, B**) Systolic blood pressure and heart rate changes in control and DOCA-salt mice measured by tail cuff-method, n = 6-7 per group. (**C**) Systolic blood pressure measured by radiotelemetry, n = 4-5 per group. * P<0.05 versus saline-treated mice, ## P < 0.01 versus DOCA-salt mice. Figure is modified from original communication V.

Table 5.1 describes the effects of NDP-α-MSH-treatment on electrolyte balance in control and DOCA-salt mice. Water intake and urine volume were markedly increased after the DOCA-salt challenge, but NDP-α-MSH caused no significant effects on these

parameters. However, NDP-α-MSH-treatment increased urinary sodium excretion in control and DOCA-salt mice. Owing to the enhanced sodium excretion, NDP-α-MSH-treated mice seemed to be somewhat protected from DOCA-salt induced hypernatremia. DOCA-salt challenge was also associated with hypokalemia caused by excess potassium loss into urine, but NDP-α-MSH-treatment was not able to prevent the disruption of potassium homeostasis.

Table 5.1. Effects of NDP-α-MSH treatment on body fluid and electrolyte balance and organ weights in control and DOCA-salt mice.

Parameter	Units	Control		DOCA-salt	
		Saline *n =7*	NDP-α-MSH *n =7*	Saline *n = 12*	NDP-α-MSH *n = 11*
Body weight	g	35.0 ± 1.8	34.4 ± 1.6	34.3 ± 0.6	34.5 ± 0.9
Serum electrolytes					
Na^+	mmol/l	162 ± 5	162 ± 6	182 ± 5 ^{A}	166 ± 6 ^{B}
K^+	mmol/l	10.5 ± 0.4	10.2 ± 0.6	6.4 ± 0.3^{A}	6.6 ± 0.3^{A}
Renal function					
Water intake	ml/24h	5.4 ± 0.3	6.0 ± 0.3	18.3 ± 3.1^{A}	25.2 ± 3.9^{A}
Urine volume	ml/24h	1.44 ± 0.31	1.94 ± 0.16	12.3 ± 2.3^{A}	18.8 ± 3.3^{A}
$U_{Crea}V$	mg/24h	1.04 ± 0.16	1.20 ± 0.15	1.06 ± 0.06	1.09 ± 0.08
$U_{Na}V$	mmol/24h	0.29 ± 0.05	0.39 ± 0.03^{B}	2.19 ± 0.35^{A}	3.30 ± 0.3^{A,B}
$U_{K}V$	mmol/24h	0.53 ± 0.06	0.53 ± 0.08	1.10 ± 0.07^{A}	1.30 ± 0.09^{A}

All measurements are means ± SEM. $U_{Crea}V$, urinary creatinine excretion; $U_{Na}V$, urinary Na^+ excretion; $U_{K}V$, urinary K^+ excretion; BW, body weight. Two-way effects by ANOVA: ^{A}, $P < 0.05$ vs. control mice; ^{B}, $P < 0.05$ vs. saline-treated mice. Table is modified from original communication V.

Exposure to DOCA-salt increased urinary excretion of NO metabolites and decreased cGMP excretion (Fig. 5.16, A and B). Despite the lack of effect on NO levels, NDP-α-MSH-treatment increased urinary excretion of cGMP both in control and DOCA-salt mice (Fig. 5.16B), indicating improved signaling through the NO-cGMP-pathway. Urinary 8-isoprostane excretion, a marker of oxidative stress, was significantly increased by DOCA-salt-treatment but it was unaffected by NDP-α-MSH-treatment (Fig. 5.16C).

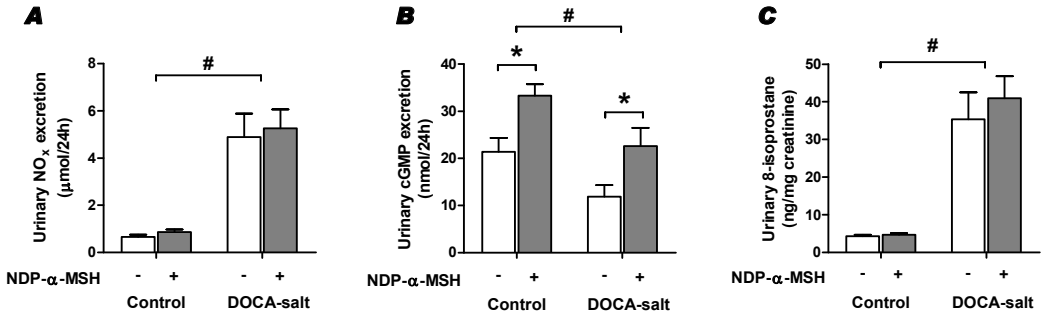

Figure 5.16. Melanocortin treatment strengthens the integrity of the NO-cGMP-pathway. Urinary excretion of NO metabolites (**A**), cGMP (**B**) and 8-isprostane (**C**) in control and DOCA-salt mice. * P<0.05 versus saline-treated mice, # P<0.01 versus control mice. Figure is from original communication V

Vascular ROS production was increased in DOCA-salt mice but it was unaffected by NDP-α-MSH treatment (data not shown).

6 DISCUSSION

6.1 Methodological considerations

6.1.1 Genetic and pharmacologic models of chronic melanocortin activation

The present studies employed both genetic and pharmacologic approaches to generate mouse models that represent universal overactivity of the melanocortin system. To pharmacologically mimic universal melanocortin activation, potent and stable analogues of α-MSH were administered as a single injection or by daily injections for 1-3 weeks and transgenic MSH-OE mice were studied as a genetic model of chronic melanocortin activation.

Two different analogues of α-MSH were used in the present studies: NDP-α-MSH and melanotan-II. These are widely used analogues in melanocortin research and well-characterized in terms of their receptor-binding properties and pharmacokinetic profiles (for review, see chapter 2.2.6). This pre-existing knowledge helped us to plan experiments so that they would meet certain prerequisites. First, NDP-α-MSH and MT-II were known to have relatively high binding affinities for all melanocortin receptor subtypes (except for MC2-R). Second, it was essential to control for the acute pressor and tachycardic actions as far as it was possible. The decision to use NDP-α-MSH instead of MT-II in DOCA-salt mice was based on the observations that NDP-α-MSH caused a milder pressor effect in the acute phase. Furthermore, since MT-II is presumed to cross blood-brain-barrier more readily than NDP-α-MSH, it was hoped to minimize centrally-mediated melanocortin effects. Beneficial blood pressure control was achieved by NDP-α-MSH-treatment at moderate dosing regimen, but the full therapeutic efficacy of melanocortin treatment in this disease model might be underestimated due to the short biological half-life of NDP-α-MSH. By choosing a longer acting analogue, the blood pressure lowering effect might have been more prominent. To obtain an extended treatment effect for the once-daily injections, it was decided to administer the MT-II-analogue to DIO mice.

The characterization of the MSH-OE mouse model revealed new interesting insights into the long-term regulation of cardiovascular functions by the melanocortins. However, the exact mechanism by which MSH overexpression leads to alterations in cardiovascular functions cannot be concluded from these studies. One of the unsettled issues is the contribution of two distinct melanocortins and their signaling pathways since both α- and γ-MSH have been shown to produce cardiovascular effects. Despite the fact that the transgenic approach used here does not provide the basis for making definitive statements about the involvement of different melanocortin receptors and brain areas, one could argue that a universal increase in melanocortin signalling can produce wide-ranging and potentially beneficial effects without conditional and selective targeting. This approach can be an asset since it is very challenging to implement a treatment strategy with a

localized agonist-effect. The possibility that both α- and γ-MSH may have contributed to the cardiovascular phenotype of MSH-OE mice is, however, a considerable limitation. Future studies will be crucial in defining the link between different melanocortin signalling pathways and the observed cardiovascular phenotypes.

6.1.2 Blood pressure measurement techniques

Two widely used and well-established blood pressure measurement techniques were used in this thesis: tail-cuff method and radiotelemetry. The advantages and limitations of these techniques are reviewed in this chapter.

The measurement of systolic blood pressure by tail-cuff sphygmomanometry has been a standard technique for the long-term evaluation of blood pressure in rats and mice and utilized with a variety of experimental designs. Although the tail-cuff method provides a simple and straightforward estimate of arterial pressure, it suffers some major limitations in terms of its usefulness (Kurtz *et al.*, 2005; Lorenz, 2002). Most important of these limitations is the stress to which the animals are subjected. Animals need to be immobilized for several minutes in order to obtain accurate readings of blood pressure, since movement of animals causes artifacts that disturbs the detection of pressure signal and can thereby lead to false readings. Although acclimatization of the animals to the measurement protocol can mitigate this problem, it must be appreciated that these blood pressure measurements are taken under some degree of anxiety and stress. This can be justified and illustrated by the high heart rates measured by tail cuff: over 700 beats/min compared to 500 beats/min which is a typical heart rate level in resting mice. Unlike the rat, the stress level in mice remains elevated even after several weeks of training (Gross *et al.*, 2003). However, it seems evident that blood pressure levels are less affected by this type of stress. In addition, measurements of tail-cuff pressure can be highly variable from reading to reading. It is therefore important to take multiple readings to compensate for this variability and moreover, to exclude those readings that do not conform to pre-established criteria. Finally, it should be acknowledged that the tail-cuff pressure is only a representative value of a single time point and does not provide information, for instance, about circadian changes in blood pressure, pulse pressure or diastolic pressure. Despite these limitations, the tail-cuff method can represent a valuable approach for monitoring changes in blood pressure over extended period of time when used with appropriate caution.

Over the past decade, radiotelemetry has become the gold standard for the accurate evaluation of blood pressure in rodents as it enables long-term recordings of blood pressure and heart rate as well as other cardiovascular parameters in conscious, unrestrained animals. Today, through advances in surgical approaches and considerable miniaturization of the implantable devices, this technique provides significant benefits over other methodologies. The most commonly applied surgical approach, which utilizes carotid artery cannulation and subcutaneous placement of the transmitter body,

is less stressful in terms of its invasiveness and is characterized by a more rapid recovery as compared to the previously used approaches. Nevertheless, it is important to take into account when planning experiments that full recovery from anesthesia and surgery occurs within approximately 5-7 days as indicated by the return of normal circadian rhythms in hemodynamic parameters and physical activity. In conclusion, the usefulness and applicability of the radiotelemetric techniques are their diversity and versatility since they permit round-the-clock recordings of blood pressure without disturbance to the animal and also more sophisticated analyses of cardiovascular functions such as the evaluation of heart rate variability and baroreflex function.

6.1.3 Evaluation of cardiovascular autonomic control

One of the objectives of this thesis was to evaluate autonomic nervous system status and particularly, the vagal input to heart rate regulation in MSH-OE mice. This objective was technically challenging to accomplish because parasympathetic activity is difficult to measure directly. This is attributable to several factors including the anatomical location of the vagus nerve, the scarcity of parasympathetic nerves in the periphery and the rapid removal of ACh from blood. However, it can be indirectly evaluated based on the heart rate responses to vagus nerve stimulation and blockade (e.g. atropine) or on physiological observations such as resting heart rate and heart rate recovery from exercise. The more sophisticated measurements are also indirect and noninvasive such as evaluation of heart rate variability (HRV) and baroreflex sensitivity (BRS). These are validated measures of parasympathetic activity but they are not interchangeable. Furthermore, they are complicated by interpretation, particularly in animal studies. Therefore, BRS and HRV were measured in parallel in the present studies and complemented with other parameters (resting heart rate, heart rate recovery from handling and atropine response) to evaluate cardiac vagal activity.

Heart rate is principally determined by the intrinsic firing rate of the automatic pacemaker cells of the sinoatrial node and the modulating influences of the sympathetic and parasympathetic nervous systems (Levy *et al.*, 1969). Changes in autonomic nervous system activity produce changes in sinus rate over time, and these fluctuations have been termed heart rate variability (Sayers, 1973). There is a temporal difference between the sympathetic and parasympathetic control of heart rate and based on this difference, HRV analysis can be utilized to evaluate the relative balance of the autonomic nervous system. Following the onset of vagal stimulation, the latency of the sinus node response is very rapid occurring within one second while the sympathetic regulation is distinctly slower (Borst *et al.*, 1983; Koizumi *et al.*, 1985; Levy *et al.*, 1970). Accordingly, changes in cardiac vagal activity contribute mainly to the high frequency beat-to-beat fluctuations in heart rate, whereas sympathetic activity affects HRV at lower frequencies (Akselrod *et al.*, 1981; Malliani *et al.*, 1991). However, vagal activity modulates HRV at all frequencies and thus, there is no explicit lower frequency limit for the vagal activity (Pomeranz *et al.*, 1985; Taylor *et al.*, 1998). Therefore, the vagal effects cannot be strictly separated

from the sympathetic influences. This constitutes a considerable limitation of the conventional power spectrum analysis of HRV as a tool to assess changes in sympathetic cardiac regulation. Furthermore, the frequency limits and the relative contributions of autonomic divisions to high and low frequency HRV vary substantially between humans and animals (Baudrie *et al.*, 2007; Stauss, 2007; Thireau *et al.*, 2008). This has to be taken into account when analyzing and interpreting data from animal experiments.

In humans, the ratio of spectral powers (LF/HF) has been used as an index of relative autonomic balance, a higher ratio indicating increased sympathetic tone. However, the interpretation of the LF/HF-ratio is very different in animal studies. In mice, muscarinergic blockade by atropine reduces the overall variability as well as the spectral power of the LF range (Janssen *et al.*, 2000; Janssen *et al.*, 2002; Japundzic *et al.*, 1990). On the other hand, β_1-adrenoceptor blockade has either no effect or even enhances LF oscillations. Since autonomic blockers do not influence heart rate variability at frequencies over 3 Hz, the fast heart rate fluctuations are believed to be mainly determined by respiratory movements. Therefore, based on these observations, the LF/HF ratio does not seem to be a reliable estimate of autonomic balance in mice, because HF oscillations are mainly induced by mechanical factors and atropine reduces rather than increases LF power.

The analysis of spontaneous BRS calculated by the sequence method provides an additional technique to evaluate the vagally mediated rapid regulation of heart rate, as an extension and complement to the already discussed analysis of HRV (Bertinieri *et al.*, 1988). Measurement of BRS provides important, yet only limited information on arterial baroreflex function; for instance, BRS does not give direct information on the vascular component of the baroreflex and on autonomic function in other parts of the heart than the sinoatrial and atroventricular node. Despite these limitations, it is a feasible and reliable method to assess the vagal component of the heart rate regulation, since baroreflex operations are predominantly under the influence of the vagus nerve. This can be evidenced by the finding in humans and mice that muscarinic blockade by atropine attenuates baroreflex function. Altogether, analyses of HRV and BRS need to be interpreted with appropriate caution, but they can provide important information that complements other aspects and measures of cardiac vagal activity.

6.1.4 DOCA-salt model of hypertension

Experimental hypertension can be modeled using a variety of methods. In particular, the DOCA-salt model of hypertension is widely used and well characterized in both rats and mice. This was also the model selected here, because it is a relatively cheap and straightforward technique to employ, and it recapitulates important features of human hypertension.

DOCA-salt hypertension is induced by administration of the mineralocorticoid (deoxycorticosterone acetate, DOCA) and a high-salt diet via drinking water, thus it is called DOCA plus salt. Progression of blood pressure in this model is often enhanced

by performing uninephroctomy to the animals, but this approach was not employed here. DOCA-salt-induced hypertension is thought to occur in several stages. In the early phase (days) of DOCA-treatment, sodium is retained at the expense of potassium. This is followed by a second phase (weeks) during which sodium homeostasis is rebalanced but potassium becomes chronically depleted. There is an initial increase in sodium and water intake that, for the most part, is compensated by an increase in sodium and water excretion. Likewise, in terms of blood pressure changes, there is recent evidence demonstrating that DOCA-salt hypertension develops in stages. An abrupt increase in arterial pressure occurs during the first few days, followed by a delayed, slower rise in arterial pressure over the next few weeks, leading to sustained hypertension. This is in good agreement with the present observations from the blood pressure development in DOCA-salt mice. (Yemane *et al.*, 2010)

The pathogenesis of DOCA-salt-induced hypertension is not fully understood (Schenk *et al.*, 1992; Yemane *et al.*, 2010). However, it appears that this form of hypertension is dependent on neural and hormonal pressure mechanisms, including altered renin-angiotensin and vasopressin system activity, elevated sympathetic drive and attenuated baroreflex function. Furthermore, DOCA-salt hypertension induces the characteristic changes in vascular reactivity such as impaired endothelium-dependent vasodilatation and enhanced vasoconstrictor responses (Shirasaki *et al.*, 1988; Somers *et al.*, 2000; Van de Voorde *et al.*, 1986). The endothelial dysfunction present in these mice is susceptible to therapeutic interventions that aim at restoring vascular homeostasis, thereby reducing elevated vascular tone and blood pressure.

6.2 Acute effects of systemically administered α-MSH analogues on hemodynamics

It was demonstrated that NDP-α-MSH and MT-II produce acute elevations in blood pressure and heart rate pressor after systemic injections in conscious mice. Furthermore, these findings shed new light on the mechanisms whereby systemically administered α-MSH analogues affect hemodynamics.

Hemodynamic effects of systemically administered α-MSH have been previously investigated in rats and mice, but mainly in the anesthetized state (Hill *et al.*, 2002; Li *et al.*, 1996; Ni *et al.*, 2006a). These studies demonstrated that systemic administration of α-MSH has no effect on hemodynamics, while i.c.v. injections of the same peptide significantly elevate blood pressure and heart rate. In contrast, the present findings demonstrate that the potent analogues of α-MSH, NDP-α-MSH and MT-II, elicit a marked increase in heart rate accompanied by a modest elevation in blood pressure following their systemic administration to conscious mice. The opposite effects on hemodynamics between α-MSH and its analogues could be attributed to a difference in their biological half-life and ability to active the centrally located MC receptors i.e. MC3 and MC4 receptors. NDP-α-MSH and MT-II are less susceptible to proteolytic degradation than

α-MSH, which could ensure sufficient access to the relevant MC-R expression sites in the brain after systemic administration. However, the use of anesthesia in previous studies limits the comparability of these findings in mice and therefore, definite conclusions on the causes of the differing effects between α-MSH and its analogues cannot be drawn.

The selective MC3/4 receptor antagonist SHU9119 was used to dissect the underlying mechanisms and showed that the pressor and tachycardic actions of MT-II could be blocked with this antagonist. Since the MC4 receptor has been shown to be the receptor responsible for mediating the hemodynamic effects of centrally administered α-MSH (Dunbar *et al.*, 2000; Matsumura *et al.*, 2002; Ni *et al.*, 2006a), it is likely that this particular receptor subtype mediates the hemodynamic actions of systemically administered MT-II. Furthermore, supporting the concept of MC4-R-based mechanism, a recent clinical study demonstrated that subcutaneous infusion of a MC4-R preferring synthetic agonist evoked significant increases in both systolic and diastolic blood pressures (Greenfield *et al.*, 2009).

Several lines of evidence indicate that α- and γ-MSH similarly increase heart rate and blood pressure when administered into the cerebroventricular system. These responses are mediated via MC4-R and FMRF-gated sodium channels, respectively, and reflect central sympathoexcitation, because pharmacologic blockade of adrenergic receptors or pithing prevents the pressor and cardioaccelerator actions (Callahan *et al.*, 1985; Gruber *et al.*, 1989; Ni *et al.*, 2006a; Van Bergen *et al.*, 1998). Furthermore, central administration of α-MSH has been shown to increase renal sympathetic nerve activity (Matsumura *et al.*, 2002). However, the relative importance of cardiac accelerator or vasomotor nerves in the α-MSH-evoked sympathoexcitation and the consequent blood pressure increase has remained elusive. Based on the finding that α-MSH analogues caused a more pronounced tachycardic effect, it was decided to investigate whether the pressor effect is dependent on increased cardiac sympathetic activity. Indeed, these findings indicate that β_1-receptor blockade eliminates the difference in blood pressure between control and MT-II treated mice, suggesting that the pressor effect might result from the cardioaccelerator effect. However, further research needed to assess whether α-MSH analogues also enhance contractility of the heart and stroke volume. If they proved to elicit a positive inotropic effect in combination with the positive chronotropic effect, the dependence of the pressor effect primarily on cardiac effects would be more plausible.

The finding that β_1-receptor blockade did not completely eliminate the cardioaccelerator properties of MT-II implies that there is a yet unidentified mechanism that contributes to the tachycardic action of α-MSH analogues in addition to sympathoexcitation. Assuming that vagal tone dominates autonomic control of resting heart rate in mice (Just *et al.*, 2000), it is likely that withdrawal of vagal tone contributes to the tachycardic action of α-MSH analogues. Supporting this concept was the finding that the intrinsic heart rate level observed after ganglionic blockade with hexamethonium was higher than the resting heart rate. Hexamethonium treatment also abolished the difference in heart

rate between control and MT-II-treated mice, suggesting that the tachycardic action of α-MSH analogues is partly attributable to inhibition of vagal nerve activity. In addition, the pressor effect of α-MSH analogues might be counterbalanced by a hypotensive effect, since the intrinsic blood pressure level after the blockade of ganglionic transmission was lower in MT-II-treated mice. This might explain why the tachycardic action of α-MSH analogues was more prominent than the pressor effect. Taking into account the fact that melanocortins are natriuretic (Humphreys *et al.*, 2011), the opposing effect could be caused by volume-dependent hypotension, but further research will be needed to clarify this possibility.

In summary, the findings demonstrate that systemically administered α-MSH analogues are hemodynamically active and significantly elevate blood pressure and heart rate in conscious mice. The effects are mediated via activation of MC3/4 receptors and partly via the enhancement of cardiac sympathetic activity, but other mechanisms in addition to the sympathoexcitation seem to be involved. In view of the fact that α-MSH analogues have been used in rodents, for instance, to investigate the therapeutic value of melanocortins in myocardial infarct (Bazzani *et al.*, 2001; Giuliani *et al.*, 2006a), it is important to take into account the hemodynamic actions of these compounds and the consequent increase in cardiac workload when using them via systemic routes of administration.

6.3 Blood pressure regulation in MSH-OE mice

One of the main objectives of this thesis was to elucidate, through cardiovascular phenotyping of the MSH-OE mouse model, how a long-term increase in melanocortin signalling would affect blood pressure regulation. Under physiological conditions, MSH-OE mice had normal blood pressure in comparison with age-matched WT mice indicating that a universal increase in melanocortin signaling does not affect long-term regulation of blood pressure. Furthermore, 12-month-old MSH-OE did not differ from WT-mice in terms of their basal BP levels or BP responses to a high-sodium-diet. However, telemetric recordings from the recovery period revealed one interesting finding. The profile of blood pressure tracing indicated that young MSH-OE mice had significantly lower MAP soon after the surgery and it paralleled the features of locomotor activity tracings. A similar decrease in locomotor activity appeared also in aged MSH-OE mice but the difference in MAP was not as significant as in young mice. The explanation for these findings remains obscure. Altered tolerance against stress-induced hypertension might be attributable to nociceptive modulation since melanocortins have been demonstrated to produce analgesia in persistent pain models (Chen *et al.*, 2006; Zvejniece *et al.*, 2006). In the present study, mice were given buprenorphine for postoperative (days 0-2 after surgery) pain relief which has probably produced synergistic analgesia and more profound sedation together with melanocortin activation. Further studies will be needed to clarify the mechanism behind the altered physical behaviour and blood pressure regulation under post-surgical stress in MSH-OE mice.

Centrally mediated effects of melanocortins on the cardiovascular system seem to be complex and involve multiple signalling pathways. In the brain, the predominant sites of POMC expression originate in the hypothalamus and in the brainstem (Fan *et al.*, 2004; Jacobowitz *et al.*, 1978; Palkovits *et al.*, 1987; Watson *et al.*, 1978; Zheng *et al.*, 2005). These two brain areas are thought to differentially mediate the acute effects of melanocortins on cardiovascular homeostasis. According to current understanding, activation of hypothalamic melanocortin pathways leads to sympathetic activation and an increase in blood pressure and heart rate (Haynes *et al.*, 1999; Kuo *et al.*, 2004; Kuo *et al.*, 2003; Ni *et al.*, 2006a; Versteeg *et al.*, 1998). In contrast, increased melanocortin signalling within the brainstem has reported to elicit bradycardia and hypotension, i.e. responses that are likely to be dependent on activation of brainstem MC4 receptors and an increase in cardiac vagal activity (Chitravanshi *et al.*, 2009; De Wildt *et al.*, 1994; Li *et al.*, 1996; Pavia *et al.*, 2003; Tai *et al.*, 2007). The present findings, describing unchanged blood pressure in MSH-OE mice under physiological baseline conditions, point to the fact that a universal increase in melanocortin signaling in the brain might, over time, attenuate the acute blood pressure effects of melanocortins. Furthermore, increases in both hypothalamic and brainstem melanocortin signaling could compensate for the opposite effects. However, these are rather speculative views, and further research is warranted to address this issue.

6.4 Heart rate regulation and autonomic balance in chronic models of melanocortin activation

In terms of hemodynamic effects, the most interesting findings from MSH-OE mice were related to autonomic and baroreflex regulation of heart rate. The present findings demonstrate that transgenic MSH overexpression enhances cardiac vagal activity and baroreflex function.

MSH-OE mice displayed consistently decreased heart rate, a change that is most likely dependent on increased cardiac vagal activity. In an attempt to evaluate further the autonomic nervous system status of MSH-OE mice, a pharmacologic approach was adopted in parallel with a spectral analysis of heart rate variability. Findings from HRV analysis revealed an increase in low-frequency oscillations of heart rate in MSH-OE mice which refers to increased cardiac vagal activity based on the concepts discussed in "Methodological considerations". The pharmacological intervention offered verifying data for this concept, since atropine increased heart rate more substantially in MSH-OE mice than in WT mice. Furthermore, the rapid restoration of heart rate in MSH-OE mice after sympathetic stimulation induced by saline injection could be attributed to increased vagal activity. Similarly, in humans, the rate of HR recovery from exercise is used as a measure of parasympathetic reactivation and sympathetic withdrawal (Cole *et al.*, 1999). Altogether, these findings are in good agreement with the studies showing that activation of the brain stem melanocortin circuits elicit bradycardic responses by

increasing vagal activity (Chitravanshi *et al.*, 2009; De Wildt *et al.*, 1994; Pavia *et al.*, 2003; Tai *et al.*, 2007).

Hints towards enhanced baroreceptor-mediated responses came from the pharmacologic and high-sodium diet experiments. First, MSH-OE mice showed an augmented HR response to vasodilatation induced by the α_1-adrenoceptor antagonist. Second, MSH-OE mice responded to dietary sodium excess by displaying a significant drop in their heart rate, evidence of an improved compensatory mechanism protecting the cardiovascular system against volume loading. Under normal conditions, high-sodium diet should induce a volume-related increase in stroke volume and a decrease in HR which allows the heart to operate more economically. Thus, the reduction of HR in MSH-OE mice refers to an efficient reflex control of HR and sustained augmentation of parasympathetic tone, which is likely to reduce the long-term load on the heart. The decrease in HR in response to the high-sodium diet, combined with the augmented HR increase induced by vasodilatation, indicates that the baroreflex system of MSH-OE mice responds more efficiently to baroreceptor stimulation as well as to baroreceptor unloading. These concepts are further supported by the improved index of spontaneous baroreflex effectiveness in MSH-OE mice.

By studying the effects of chronic MT-II administration, it was intended to demonstrate that the characteristics observed in MSH-OE mice are not exclusively inherent to the transgenic model, but can be phenocopied by a pharmacologic approach. Indeed, MT-II treated mice displayed a tendency towards increased cardiac vagal activity and a significant enhancement in HR response after drug-evoked vasodilatation, a phenotype that closely mirrors that seen in the MSH-OE mouse. The enhanced HR responses to prazosin in MSH-OE and MT-II-treated mice are supported by a recent clinical study (Wellhöner *et al.*, 2012). Intranasal administration of the MC receptor agonist MSH/ACTH(4-10) caused lipolysis in humans, but in addition, increased muscle sympathetic nerve activity in response to vasodilatation without affecting the baseline nerve activity, indicating an enhanced baroreceptor-mediated response to the fall in blood pressure.

Although POMC-derived peptides and MC receptors have been reported to be expressed in relevant sites along the baroreflex arc such as the NTS and vagal afferent fibers (Kishi *et al.*, 2003; Liu *et al.*, 2003; Mountjoy *et al.*, 1994; Palkovits *et al.*, 1987; Wan *et al.*, 2008; Williams *et al.*, 2000), it is still an open question how chronic melanocortin activation modulates vagal activity and baroreflex function. Melanocortins have been shown to increase cardiac vagal activity through the activation of MC4 receptors in the brain stem (Chitravanshi *et al.*, 2009; De Wildt *et al.*, 1994; Pavia *et al.*, 2003; Tai *et al.*, 2007), but these findings are based on acute studies and do not necessarily explain the long-term effects of melanocortin activation on vagal activity and baroreflex function which probably ensues from the sensitization of the baroreflex feedback system to the changes in arterial pressure. The baroreflex signal could be modulated at the level of the afferent or efferent arms of the neural baroreflex arc. However, one should not forget that vascular compliance is also an important determinant of baroreflex function and

in the presence of a high compliance, the signal evoked by a change in blood pressure can result in increased baroreceptor firing (Kingwell *et al.*, 1995). Accordingly, another possible explanation is that melanocortin activity could influence baroreflex function by modulating vascular function.

In conclusion, these results demonstrate that a universal and long-term increase in melanocortin activity augments vagal input to the heart and baroreflex-mediated responses. In contrast to the acute pressor and tachycardic actions of melanocortins, chronic melanocortin activation could modulate autonomic and baroreflex control of cardiovascular function in a favourable manner. These findings have significant implications since the risk for cardiovascular disease has been linked to baroreflex dysfunction and decreased cardiac vagal activity. Therefore, the present data seem to point to a protective role for chronic melanocortin activation in heart rate regulation.

6.5 The vascular protective role of α-MSH

Through a series of isometric tension recordings, it became evident that melanocortins have significant effects on the endothelium-dependent control of blood vessel tone. Despite the lack of any direct effects on vascular tone, both *in vitro* and *in vivo* treatments with α-MSH enhanced endothelium-dependent vasodilatation and blunted vasoconstrictor influences. These effects were confined to the large conduit arteries and were strongly dependent on increased NO formation. Since the mechanism of action is NO-dependent, this explains why the melanocortins elicited vessel-specific responses. The endothelial NO system exerts varying influences on the vascular tree: it predominantly modulates vascular tone of large arteries while contributing to EDHF-responses in small resistance arteries. Accordingly, the relative importance of eNOS-derived NO to ACh-induced relaxation is positively correlated with the vessel size, explaining thereby why the effects of α-MSH-treatment on endothelium-dependent relaxation were confined to the larger arteries. It is important to draw attention to the versatility of the vascular effects that were evoked by α-MSH. In addition to enhancing endothelial capacity for NO formation, it improved the sensitivity of vascular smooth muscle cells to exogenous NO and endothelium-independent vasodilatation. Furthermore, melanocortins had a beneficial influence on coronary flow regulation as indicated by improved NO-dependent flow responses in the isolated heart of NDP α-MSH-treated mice and improved coronary flow reserve of MSH-OE mice. Owing to these beneficial vascular effects, treatment with potent and stable melanocortin analogues might hold promise as a therapeutic tool for cardiometabolic diseases.

To shed light on the underlying mechanisms, cultured human endothelial cells were used revealing that α-MSH can regulate NO availability through endothelial MC1 receptors. This could be attributed to several molecular-level mechanisms that augment NO availability: α-MSH increased eNOS mRNA and protein expression, and phosphorylated the Ser1177 residue of eNOS, which is critical for its enzymatic activity (Fleming *et al.*, 2003). Altogether, these molecular-level effects on eNOS expression and

phosphorylation are mechanistically linked to the observations of enhanced endothelium- and NO-dependent vasodilatation in isometric tension recordings. In addition, the local availability of NO in blood vessels is determined by the rate of breakdown of NO by ROS. Contributing to this phenomenon, α-MSH stimulated the expression of Mn-SOD which is responsible for the conversion of superoxide anions into oxygen and hydrogen peroxide. However, the exact role of increased expression of Mn-SOD remains elusive in terms of vascular reactivity. The enzyme might contribute to endothelium-independent relaxation by increasing the vascular sensitivity to exogenous NO but further research will be needed to explore this possibility. Our research group has also unpublished data showing that the MC1 receptor is similarly expressed in vascular smooth muscle cells. This observation merits further research to clarify the effects of α-MSH stimulation e.g. on the expression of soluble guanylyl cyclase in these cells. In conclusion, α-MSH regulates endothelium-dependent control of blood vessel tone and vascular NO availability via MC1-R expressed in the endothelium (Fig. 6.1).

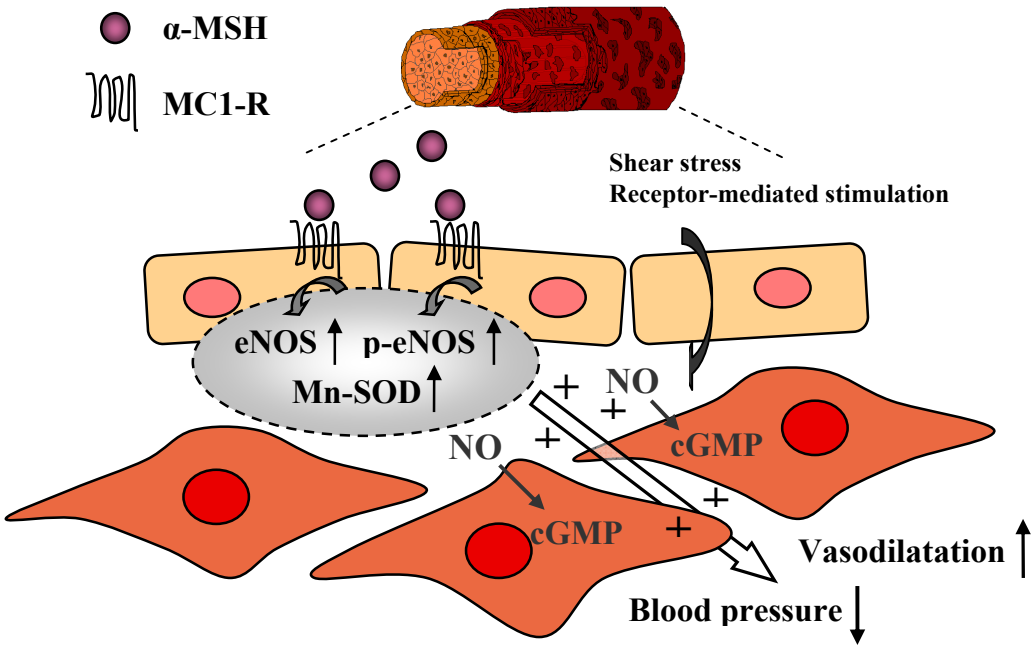

Figure 6.1. Proposed role for endothelial MC1 receptor signaling in the regulation of vascular tone and blood pressure. MC1-R activation by α-MSH increases eNOS expression and its phosphorylation and Mn-SOD expression. This leads to enhanced signaling through the NO-cGMP pathway in response to hemodynamic stimuli (e.g. shear stress).

6.6 Therapeutic perspectives

Melanocortins have been previously shown to participate in the regulation of sodium homeostasis, but the present findings show that pharmacological targeting of

melanocortin receptors with a pan-agonist, NDP-α-MSH, possesses therapeutic efficacy in experimental hypertension. Since sodium accumulation together with impaired vascular function contributes to the development of hypertension and associated cardiovascular pathologies, NDP-α-MSH treatment might have a dual therapeutic effect by interrupting these critical pathophysiological processes. The present data demonstrate that NDP-α-MSH can alleviate sodium retention in the DOCA-salt model of hypertension via MC3-R-mediated diuretic and natriuretic actions. Furthermore, the observation that α-MSH promotes vascular function via MC1 receptors might add therapeutic value for NDP-α-MSH treatment in the management of hypertension (Fig. 6.2). The findings demonstrating that chronic melanocortin activation could increase cardiac vagal activity and enhance baroreflex function might also contribute to the antihypertensive properties of NDP-α-MSH, but the association between these findings is still obscure.

Excessive sodium accumulation contributes to the pathogenesis of the DOCA-salt model, indicating a volume-dependence of the hypertensive state (Schenk *et al.*, 1992). Therefore, it was decided to investigate whether the diuretic and natriuretic actions of NDP-α-MSH could attenuate water and sodium retention and thereby also provide blood pressure control in DOCA-salt hypertensive mice. Although it did not reverse pre-existing hypertension, NDP-α-MSH treatment was able to prevent further elevation of blood pressure and to normalize the circadian blood pressure rhythm in DOCA-salt mice. However, the latter effect might be attributed to the relatively short duration of action of NDP-α-MSH, since the half-life of systemically administered NDP-α-MSH during the elimination phase is only a few hours (Ugwu *et al.*, 1997). In study V, NDP-α-MSH was administered during day-time, which might explain why NDP-α-MSH-treated mice showed better blood pressure control during the day-time, while night-time pressure values did not differ from those of saline-treated mice. Accordingly, longer-acting analogues of α-MSH or modified dosage regimens could provide further blood pressure control in DOCA-salt hypertensive mice. Nevertheless, the used dosage regimen restored sodium balance in DOCA-salt hypertension, since NDP-α-MSH-treated mice seemed to be protected from the hypernatremia. NDP-α-MSH was not able to prevent the hypokalemia of DOCA-salt mice, suggesting that add-on therapy with a potassium-sparing diuretic would be needed to achieve an improved potassium balance. Taken together, NDP-α-MSH showed therapeutic efficacy in the management of hypertension due to its diuretic and natriuretic effects.

In hypertension and many other cardiovascular pathologies, the function of the eNOS enzyme is compromised, leading to impaired endothelium-dependent control of vascular tone and elevated vascular resistance (Förstermann *et al.*, 2011). These features are also captured in the DOCA-salt model of hypertension (Shirasaki *et al.*, 1988; Van de Voorde *et al.*, 1986). Supporting this view, urinary excretion of cGMP excretion was suppressed to a level below the baseline in saline-treated DOCA-salt mice, reflecting disturbed integrity of the NO-cGMP pathway. It is particularly noteworthy that cGMP

excretion was increased in NDP-α-MSH-treated mice, pointing to enhanced signaling through the NO-cGMP pathway. However, urinary cGMP excretion is only a rough and indirect measure of the function of the NO-cGMP-axis, and NO-stimulated cGMP formation does not solely account for the total cGMP output. For instance, atrial natriuretic peptide (ANP) directly stimulates cGMP formation through its G-protein-coupled A- and B-type receptors. This might have relevance to our findings, since plasma ANP concentration has been shown to increase after γ-MSH administration in rats (Chen *et al.*, 1997b). On the other hand, the heightened NO formation in DOCA-salt mice might be largely attributable to an increase in iNOS-derived NO rather than to enhanced NO formation by eNOS (Obst *et al.*, 2004). Thus, although NO excretion was similarly increased in saline- and NDP-α-MSH-treated mice under DOCA-salt challenge, it does not solely represent the local availability of eNOS-derived NO in blood vessels which is critical for the maintenance of vascular homeostasis. Taken together, the disruption of the NO-cGMP pathway contributes to the blood pressure progression in DOCA-salt hypertension and NDP-α-MSH treatment may prevent this process by protecting the normal function of eNOS system and NO-mediated vascular responses. Although the relative contributions of the natriuretic and vascular effects to the antihypertensive efficacy of α-MSH-treatment remain unknown, the underlying mechanism is probably multifactorial, involving influences on both blood vessels and renal function.

In terms of therapeutics, promoting the endothelium to respond appropriately to hemodynamic stimuli might be a better approach rather than relaxing the vessels directly by pharmacological means (e.g. organic nitrates), considering that the development of tolerance is a major therapeutic limitation inherent with organic nitrate therapy. Thus, the lack of an immediate effect of α-MSH on vascular tone might be a benefit from a therapeutic standpoint. Importantly, the therapeutic efficacy of α-MSH is based on its ability to restore endothelial function by promoting the natural function of the endothelium in circulatory regulation. It is worth pointing out that an improvement in endothelial function does not necessarily translate into an antihypertensive effect, and therefore, the evaluation of therapeutic efficacy of drugs that improve endothelial function will need to be based on observations beyond blood pressure control. Certainly, it would be intriguing to combine melanocortin treatment with standard antihypertensive therapy such as ACE inhibition and to investigate whether this combination would act in a synergistic manner in the treatment of more severe disease states.

In addition to its effects in promoting endothelial function, several lines of evidence indicate that α-MSH produces potent anti-inflammatory effects in rodents, as well as in systems with human cells and samples (Catania *et al.*, 2004). From a pathophysiological perspective, α-MSH, either through the activation of MC1-R or MC3-R, has been shown to inhibit inflammation and associated disease burden in various animal models such as experimental colitis and ischemia-reperfusion injury (Leoni *et al.*, 2008; Maaser *et al.*, 2006). This evidence is derived from observations in MC1 and MC3 receptor

KO models that exhibit increased levels of pro-inflammatory cytokines in injured tissues. Conversely, selective MC1 and MC3 receptor agonists exert anti-inflammatory actions and for instance, inhibit cell adhesion and migration by acting on the adherent leucocytes and/or the endothelium in an inflamed vasculature (Lam *et al.*, 2005; Leoni *et al.*, 2010). Given that inflammation and its mediators have been linked to the pathogenesis of hypertension (Harrison *et al.*, 2012), NDP-α-MSH might provide beneficial effects in the treatment of hypertension by suppressing the inflammatory processes involved in the disease progression. Furthermore, it is worth pointing out that NDP-α-MSH treatment could have renoprotective effects that are likely to occur independent of its hemodynamic effects. The MC-R agonist ACTH was widely used in the past for the treatment of nephrotic syndrome owing to its antiproteinuric and renoprotective effects (Gong, 2012). It has been suggested that the protective effects of ACTH in kidney disease are not entirely attributable to its streroidogenic actions occurring via MC2-R activation. One of the potential mechanisms is a direct protection of kidney cells, probably through the activation of MC1-R in podocytes. Although the therapeutic concept has been primarily proven in acute kidney failure and refractory nephrotic syndrome, it might have relevance to the therapeutic qualities of MC-R agonists in hypertension, since kidney disease and failure represent common forms of target-organ damage in hypertensive patients.

It is well-known that oxidative stress plays a crucial role in the pathogenesis of DOCA-salt hypertension (Beswick *et al.*, 2001; Manning *et al.*, 2003). Based on studies showing that α-MSH can suppress oxidative stress in various disease models (Kolgazi *et al.*, 2007; Lindskog *et al.*, 2010), it was expected that NDP-α-MSH treatment would improve the redox balance of DOCA-salt hypertensive mice. However, despite the increased urinary 8-isoprostane levels, DOCA-salt hypertension did not aggravate the oxidative stress at the tissue level as evidenced by the modest increase in vascular ROS production. It is possible that effective antioxidant systems in this mouse strain have maintained adequate redox balance in tissues under observation, which, in turn, may have prevented the appearance of possible antioxidant effects of NDP-α-MSH. Further research will be needed to investigate whether α-MSH analogues could provide protection against oxidative stress and associated cardiovascular pathologies in more severe models of hypertension.

In conclusion, treatment with nonspecific α-MSH analogues that activate multiple MC receptor pathways may hold promise as a potential strategy for treating hypertension (Fig. 6.2.). In addition to the natriuretic and vascular protective effects exerted by the α-MSH analogues, chronic treatment might modulate autonomic balance and baroreceptor-mediated responses in a favourable manner and thereby, reduce the risk for other cardiovascular pathologies that are often associated with persistent hypertension. However, the major challenge of developing MC-R targeted drug treatments is to avoid centrally mediated pressor and tachycardic actions which are likely attributable to the activation of hypothalamic MC4-R (Fig. 6.2).

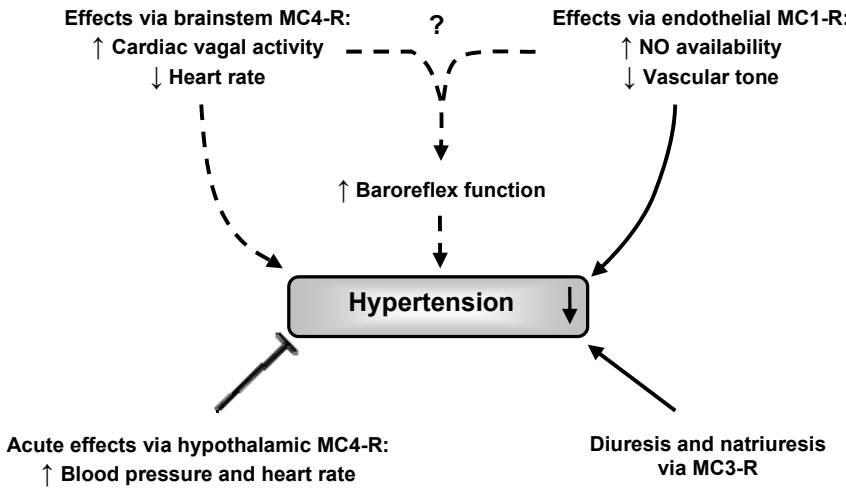

Figure 6.2. Potential mechanisms contributing to the therapeutic efficacy of NDP-α-MSH in experimental hypertension.

7 SUMMARY AND CONCLUSIONS

The studies using genetic and pharmacologic models of increased melanocortin activity showed that melanocortins are likely to promote cardiovascular health in the long term by regulating cardiac autonomic balance, the local control of blood vessel tone and sodium balance. The main findings and conclusions were:

1) Systemic administration of α-MSH analogues elicits a subtle increase in blood pressure and a significant tachycardic effect through the activation of MC3/4 receptors and the sympathetic nervous system.

2) Transgenic MSH overexpression does not affect blood pressure but leads to a subtle decrease in heart rate under physiological baseline conditions.

3) Transgenic and pharmacologic models of chronic melanocortin activation displayed features of increased cardiac vagal activity and enhanced baroreceptor-mediated responses in heart rate dynamics, suggesting that a universal increase in melanocortin activity can favorably modulate autonomic nervous system balance.

4) α-MSH regulates endothelial NO availability via melanocortin 1 receptors. α-MSH was found to promote blood vessel relaxation by enhancing NO formation and by improving sensitivity to NO-induced vasodilatation. Mechanistically, α-MSH by interacting with endothelial MC1 receptors increased the expression and phosphorylation of eNOS, thus enhancing the capacity for NO production. Furthermore, pharmacological treatment with melanocortin analogues ameliorated endothelial dysfunction associated with aging and diet-induced obesity.

5) Chronic treatment with the stable melanocortin analogue NDP- α-MSH attenuated blood pressure development in DOCA-salt hypertensive mice through its ability to improve endothelial function and to prevent sodium retention.

In conclusion, the present results indicate that despite the acute pressor and tachycardic actions of melanocortin analogues, they could be used as therapeutic tools for the treatment of vascular dysfunction and sodium retention, which are associated with many cardiovascular disease states such as hypertension.

8 ACKNOWLEDGEMENTS

This thesis was carried out at the Department of Pharmacology, Drug Development and Therapeutics, Institute of Biomedicine, University of Turku. I am grateful for the Heads of the Department, Professors Mika Scheinin, Markku Koulu and Risto Huupponen, for allowing me to use the facilities of the department. It has been a real pleasure to work at the department under your guidance. I appreciate that you have helped me whenever there has been something to ask about. I also greatly appreciate the Drug Discovery Graduate School and its coordinator Eeva Valve for educational and financial support. This thesis was also financially supported by the Finnish Cultural Foundation, the Finnish Foundation for Cardiovascular Research, the Academy of Finland, and the Finnish Foundation for Diabetes Research.

I wish to express my sincere gratitude to my supervisor Eriika Savontaus for believing in me right from the beginning. I am thankful for your heartfelt encouragement and guidance throughout this project. You have paved my way into the fascinating world of science and taught me everything I should know about making science at this stage. I am still a novice as a scientist, but I feel ready to face new challenges and to continue my academic endeavors. I really appreciate that you have given me independence and freedom to express my own ideas and test them in practice in the lab. The excitement, what you can experience when testing your own hypotheses, is something that has kept me going. In addition, I am very grateful to Professor Markku Koulu for encouraging me to start my PhD studies. Although we have not collaborated so much in this very project, you have thought me to believe in myself and to work hard for my dreams. Your optimism and support gave an essential spark for this project. I really admire your dedication to teaching and research. Even though it feels like reaching for the stars, I wish I could someday be a teacher and mentor like you.

I am very grateful to the official examiners of my thesis, Professor Michael Humphreys and Docent Risto Kerkelä, for their valuable comments that helped me to improve the thesis manuscript and to prepare myself for the public defense of the thesis.

I want to thank all the co-authors of this work: Janne Harjunpää, Mika Scheinin, Sanna Tikka, Satu Mäkelä, Tomi Streng, Wendy Nordlund, Anna-Maija Penttinen, Ilkka Heinonen, Antti Saraste, Suvi Ruohonen, Laura Vähätalo, Katja Kaipio, Victor J. Hruby, Minying Cai, Saku Ruohonen, and Markku Ahotupa. Your contributions mean a lot to me. All for one and one for all! I want to especially thank Ilkka and Saku. Your great sense of humour has always lightened the atmosphere during the long measurement days by the myography. Not forgetting our lively scientific discussions. I think we have learned a lot about vascular biology, cross-country skiing, perivascular fat, hunting, α_2-adrenoceptors, you name it, by spending countless days by the myography. I am also very thankful to Sanna Tikka for her valuable assistance in mouse telemetry and to Janne

Harjunpää for all the guidance and help in statistical analyses. Your contributions to this project were pivotal over the years.

Specials thanks belong to the members of our neuropeptide group: Suvi Ruohonen, Laura Vähätalo, Liisa Ailanen, Satu Mäkelä, Kim Eerola, and Wendy Nordlund. It has been a great pleasure to get to know you and to work with you. I thank you for the friendly atmosphere and memorable moments inside and outside the lab.

I want to thank all my fellow scientists and co-workers at Farmis for creating a warm and professional work environment. I want to express my special thanks to Anja Similä, Hanna Tuominen, Helena Hakala, and Hanna Laine for their valuable secretarial assistance, and Raija Kaartosalmi, Tuula Juhola and Maarit Nikula for the excellent technical assistance. I would also like to thank Francisco López Picón, Jonne Laurila, Henriikka Salomäki, Ville Ranta-Panula, Valtteri Rinne, Milka Hauta-Aho, Sanna Soini, Mikko Uusi-Oukari, Susann Björk, Anna Huhtinen, Anne Vuorenpää, and all the other former and present co-workers at the department. Outside our department, I would especially want to thank Sandra Nyberg, Kalle Mikola, Otso Pulkkinen and Matti Rainio for pushing me forward to complete the project on time.

I want to warmly thank my family, parents Irmeli and Keijo, and sister Minna. I am most grateful for your endless support in my life. Finally, and most importantly, I owe my deepest gratitude to my Salla. Whenever there has been frustration and moments of despair in work, your smile and affection have put all the worries aside and brought the right balance in my life. It means the world to me. Thank you for your support, patience and love. Thank you for being by my side.

Turku, December 2012

Petteri Rinne

REFERENCES

Adachi S, Nakano T, Vliagoftis H, Metcalfe DD (1999). Receptor-mediated modulation of murine mast cell function by alpha-melanocyte stimulating hormone. *J Immunol* **163**(6)**:** 3363-3368.

Akselrod S, Gordon D, Ubel FA, Shannon DC, Berger AC, Cohen RJ (1981). Power spectrum analysis of heart rate fluctuation: a quantitative probe of beat-to-beat cardiovascular control. *Science* **213**(4504)**:** 220-222.

Al-Obeidi F, Castrucci AM, Hadley ME, Hruby VJ (1989). Potent and prolonged acting cyclic lactam analogues of alpha-melanotropin: design based on molecular dynamics. *J Med Chem* **32**(12)**:** 2555-2561.

Arnold WP, Mittal CK, Katsuki S, Murad F (1977). Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. *Proc Natl Acad Sci U S A* **74**(8)**:** 3203-3207.

Balligand JL, Kelly RA, Marsden PA, Smith TW, Michel T (1993). Control of cardiac muscle cell function by an endogenous nitric oxide signaling system. *Proc Natl Acad Sci U S A* **90**(1)**:** 347-351.

Baudrie V, Laude D, Elghozi J (2007). Optimal frequency ranges for extracting information on cardiovascular autonomic control from the blood pressure and pulse interval spectrograms in mice. *Am J Physiol Regul Integr Comp Physiol* **292**(2)**:** R904-912.

Bazzani C, Guarini S, Botticelli A, Zaffe D, Tomasi A, Bini A*, et al.* (2001). Protective effect of melanocortin peptides in rat myocardial ischemia. *J Pharmacol Exp Ther* **297**(3)**:** 1082-1087.

Bertinieri G, Di Rienzo M, Cavallazzi A, Ferrari A, Pedotti A, Mancia G (1988). Evaluation of baroreceptor reflex by blood pressure monitoring in unanesthetized cats. *Am J Physiol* **254**(2 Pt 2)**:** H377-383.

Beswick RA, Dorrance AM, Leite R, Webb RC (2001). NADH/NADPH oxidase and enhanced superoxide production in the mineralocorticoid hypertensive rat. *Hypertension* **38**(5)**:** 1107-1111.

Bhardwaj R, Becher E, Mahnke K, Hartmeyer M, Schwarz T, Scholzen T*, et al.* (1997). Evidence for the differential expression of the functional alpha-melanocyte-stimulating hormone receptor MC-1 on human monocytes. *J Immunol* **158**(7)**:** 3378-3384.

Borst C, Karemaker JM (1983). Time delays in the human baroreceptor reflex. *J Auton Nerv Syst* **9**(2-3)**:** 399-409.

Boston BA, Cone RD (1996). Characterization of melanocortin receptor subtype expression in murine adipose tissues and in the 3T3-L1 cell line. *Endocrinology* **137**(5)**:** 2043-2050.

Bredt DS, Hwang PM, Snyder SH (1990). Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature* **347**(6295)**:** 768-770.

Broberger C (2005). Brain regulation of food intake and appetite: molecules and networks. *J Intern Med* **258**(4)**:** 301-327.

Busse R, Edwards G, Félétou M, Fleming I, Vanhoutte PM, Weston AH (2002). EDHF: bringing the concepts together. *Trends Pharmacol Sci* **23**(8)**:** 374-380.

Butler AA, Kesterson RA, Khong K, Cullen MJ, Pelleymounter MA, Dekoning J*, et al.* (2000). A unique metabolic syndrome causes obesity in the melanocortin-3 receptor-deficient mouse. *Endocrinology* **141**(9)**:** 3518-3521.

Callahan M, Kirby R, Wolff D, Strandhoy J, Lymangrover J, Johnson A*, et al.* (1985). Sympathetic nervous system mediation of acute cardiovascular actions of gamma 2-melanocyte-stimulating hormone. *Hypertension* **7**(3 Pt 2)**:** I145-150.

Catania A, Gatti S, Colombo G, Lipton JM (2004). Targeting melanocortin receptors as a novel strategy to control inflammation. *Pharmacol Rev* **56**(1)**:** 1-29.

Catania A, Rajora N, Capsoni F, Minonzio F, Star RA, Lipton JM (1996). The neuropeptide alpha-MSH has specific receptors on neutrophils and reduces chemotaxis in vitro. *Peptides* **17**(4)**:** 675-679.

Chai B, Li JY, Zhang W, Ammori JB, Mulholland MW (2007). Melanocortin-3 receptor activates MAP kinase via PI3 kinase. *Regul Pept* **139**(1-3)**:** 115-121.

Chen AS, Marsh DJ, Trumbauer ME, Frazier EG, Guan XM, Yu H*, et al.* (2000). Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. *Nat Genet* **26**(1)**:** 97-102.

Chen T, Cai Q, Hong Y (2006). Intrathecal sensory neuron-specific receptor agonists bovine adrenal medulla 8-22 and (Tyr6)-gamma2-MSH-6-12 inhibit formalin-evoked nociception and neuronal Fos-like immunoreactivity in the spinal cord of the rat. *Neuroscience* **141**(2)**:** 965-975.

Chen W, Kelly MA, Opitz-Araya X, Thomas RE, Low MJ, Cone RD (1997a). Exocrine gland dysfunction in MC5-R-deficient mice: evidence for coordinated regulation of exocrine gland function by melanocortin peptides. *Cell* **91**(6)**:** 789-798.

Chen XW, Ying WZ, Valentin JP, Ling KT, Lin SY, Wiedemann E*, et al.* (1997b). Mechanism of the natriuretic action of gamma-melanocyte-stimulating hormone. *Am J Physiol* **272**(6 Pt 2)**:** R1946-1953.

Chen Y, Joaquim L, Farah V, Wichi R, Fazan RJ, Salgado H*, et al.* (2005). Cardiovascular autonomic control in mice lacking angiotensin AT1a receptors. *Am J Physiol Regul Integr Comp Physiol* **288**(4)**:** R1071-1077.

Chhajlani V (1996). Distribution of cDNA for melanocortin receptor subtypes in human tissues. *Biochem Mol Biol Int* **38**(1)**:** 73-80.

Chhajlani V, Muceniece R, Wikberg JE (1993). Molecular cloning of a novel human melanocortin receptor. *Biochem Biophys Res Commun* **195**(2)**:** 866-873.

Chhajlani V, Wikberg JE (1992). Molecular cloning and expression of the human melanocyte stimulating hormone receptor cDNA. *FEBS Lett* **309**(3)**:** 417-420.

Chitravanshi V, Bhatt S, Sapru H (2009). Microinjections of alpha-melanocyte stimulating hormone into the nucleus ambiguus of the rat elicit vagally mediated bradycardia. *Am J Physiol Regul Integr Comp Physiol* **296**(5)**:** R1402-1411.

Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP*, et al.* (1998). Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood* **91**(10)**:** 3527-3561.

Cole CR, Blackstone EH, Pashkow FJ, Snader CE, Lauer MS (1999). Heart-rate recovery immediately after exercise as a predictor of mortality. *N Engl J Med* **341**(18)**:** 1351-1357.

Cone RD (2005). Anatomy and regulation of the central melanocortin system. *Nat Neurosci* **8**(5)**:** 571-578.

Cope G, Flanagan ET, Houghton BL, Walsh SA, Johns EJ, Healy V (2012). The analogue, NDP-γ(2) MSH, possesses the renal excretory but not the cardiovascular actions of the native peptide, gamma2-melanocyte stimulating hormone in anaesthetized rats. *Clin Exp Pharmacol Physiol*.

Cowley MA, Smart JL, Rubinstein M, Cerdán MG, Diano S, Horvath TL*, et al.* (2001). Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* **411**(6836)**:** 480-484.

Crowley VE (2008). Overview of human obesity and central mechanisms regulating energy homeostasis. *Ann Clin Biochem* **45**(Pt 3)**:** 245-255.

da Costa-Goncalves A, Tank J, Plehm R, Diedrich A, Todiras M, Gollasch M*, et al.* (2008). Role of the multidomain protein spinophilin in blood pressure and cardiac function regulation. *Hypertension* **52**(4)**:** 702-707.

Danson EJ, Li D, Wang L, Dawson TA, Paterson DJ (2009). Targeting cardiac sympatho-vagal imbalance using gene transfer of nitric oxide synthase. *J Mol Cell Cardiol* **46**(4)**:** 482-489.

Davrath LR, Goren Y, Pinhas I, Toledo E, Akselrod S (2003). Early autonomic malfunction in normotensive individuals with a genetic predisposition to essential hypertension. *Am J Physiol Heart Circ Physiol* **285**(4)**:** H1697-1704.

de Boer R, Karemaker J, Strackee J (1985). Relationships between short-term blood-pressure fluctuations and heart-rate variability in resting subjects. I: A spectral analysis approach. *Med Biol Eng Comput* **23**(4)**:** 352-358.

De Wildt D, Krugers H, Kasbergen C, De Lang H, Versteeg D (1993). The hemodynamic effects of gamma 2-melanocyte-stimulating hormone and related melanotropins depend on the arousal potential of the rat. *Eur J Pharmacol* **233**(1)**:** 157-164.

De Wildt D, Van der Ven J, Van Bergen P, De Lang H, Versteeg D (1994). A hypotensive and bradycardic action of gamma 2-melanocyte-stimulating hormone (gamma 2-MSH) microinjected into the nucleus tractus solitarii of the rat. *Naunyn Schmiedebergs Arch Pharmacol* **349**(1)**:** 50-56.

Delaey C, Boussery K, Van de Voorde J (2002). Contractility studies on isolated bovine choroidal small arteries: determination of the active and passive wall tension-internal circumference relation. *Exp Eye Res* **75**(3)**:** 243-248.

Dinerman JL, Dawson TM, Schell MJ, Snowman A, Snyder SH (1994). Endothelial nitric oxide synthase localized to hippocampal pyramidal cells: implications for synaptic plasticity. *Proc Natl Acad Sci U S A* **91**(10)**:** 4214-4218.

Dorr RT, Lines R, Levine N, Brooks C, Xiang L, Hruby VJ*, et al.* (1996). Evaluation of melanotan-II, a superpotent cyclic melanotropic peptide in a pilot phase-I clinical study. *Life Sci* **58**(20)**:** 1777-1784.

Dunbar J, Lu H (2000). Proopiomelanocortin (POMC) products in the central regulation of sympathetic and cardiovascular dynamics: studies on melanocortin and opioid interactions. *Peptides* **21**(2)**:** 211-217.

Ellacott KL, Halatchev IG, Cone RD (2006). Characterization of leptin-responsive neurons in the caudal brainstem. *Endocrinology* **147**(7)**:** 3190-3195.

Eller MS, Ostrom K, Gilchrest BA (1996). DNA damage enhances melanogenesis. *Proc Natl Acad Sci U S A* **93**(3)**:** 1087-1092.

Eves P, Haycock J, Layton C, Wagner M, Kemp H, Szabo M*, et al.* (2003). Anti-inflammatory and anti-invasive effects of alpha-melanocyte-stimulating hormone in human melanoma cells. *Br J Cancer* **89**(10)**:** 2004-2015.

Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD (1997). Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature* **385**(6612)**:** 165-168.

Fan W, Ellacott K, Halatchev I, Takahashi K, Yu P, Cone R (2004). Cholecystokinin-mediated suppression of feeding involves the brainstem melanocortin system. *Nat Neurosci* **7**(4)**:** 335-336.

Fleming I, Busse R (2003). Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *Am J Physiol Regul Integr Comp Physiol* **284**(1)**:** R1-12.

Fox K, Borer JS, Camm AJ, Danchin N, Ferrari R, Lopez Sendon JL*, et al.* (2007). Resting heart rate in cardiovascular disease. *J Am Coll Cardiol* **50**(9)**:** 823-830.

Furchgott RF, Zawadzki JV (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288**(5789)**:** 373-376.

Félétou M, Vanhoutte PM (2006). Endothelial dysfunction: a multifaceted disorder (The Wiggers Award Lecture). *Am J Physiol Heart Circ Physiol* **291**(3)**:** H985-1002.

Förstermann U, Li H (2011). Therapeutic effect of enhancing endothelial nitric oxide synthase (eNOS) expression and preventing eNOS uncoupling. *Br J Pharmacol* **164**(2)**:** 213-223.

Förstermann U, Pollock JS, Schmidt HH, Heller M, Murad F (1991). Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells. *Proc Natl Acad Sci U S A* **88**(5)**:** 1788-1792.

Gantz I, Konda Y, Tashiro T, Shimoto Y, Miwa H, Munzert G*, et al.* (1993a). Molecular cloning of a novel melanocortin receptor. *J Biol Chem* **268**(11)**:** 8246-8250.

Gantz I, Miwa H, Konda Y, Shimoto Y, Tashiro T, Watson SJ*, et al.* (1993b). Molecular cloning, expression, and gene localization of a fourth melanocortin receptor. *J Biol Chem* **268**(20)**:** 15174-15179.

Geschwind II, Huseby RA, Nishioka R (1972). The effect of melanocyte-stimulating hormone on coat color in the mouse. *Recent Prog Horm Res* **28:** 91-130.

Getting SJ, Di Filippo C, Christian HC, Lam CW, Rossi F, D'Amico M*, et al.* (2004). MC-3 receptor and the inflammatory mechanisms activated in acute myocardial infarct. *J Leukoc Biol* **76**(4)**:** 845-853.

Gibbs J, Falasco JD, McHugh PR (1976). Cholecystokinin-decreased food intake in rhesus monkeys. *Am J Physiol* **230**(1)**:** 15-18.

Giuliani D, Leone S, Mioni C, Bazzani C, Zaffe D, Botticelli AR*, et al.* (2006a). Broad therapeutic treatment window of [Nle(4), D-Phe(7)]alpha-melanocyte-stimulating hormone for long-lasting protection against ischemic stroke, in Mongolian gerbils. *Eur J Pharmacol* **538**(1-3)**:** 48-56.

Giuliani D, Mioni C, Altavilla D, Leone S, Bazzani C, Minutoli L*, et al.* (2006b). Both early and delayed treatment with melanocortin 4 receptor-stimulating melanocortins produces neuroprotection in cerebral ischemia. *Endocrinology* **147**(3)**:** 1126-1135.

Giuliani D, Mioni C, Bazzani C, Zaffe D, Botticelli AR, Capolongo S*, et al.* (2007). Selective melanocortin MC4 receptor agonists reverse haemorrhagic shock and prevent multiple organ damage. *Br J Pharmacol* **150**(5)**:** 595-603.

Gong R (2012). The renaissance of corticotropin therapy in proteinuric nephropathies. *Nat Rev Nephrol* **8**(2)**:** 122-128.

Graham M, Shutter JR, Sarmiento U, Sarosi I, Stark KL (1997). Overexpression of Agrt leads to obesity in transgenic mice. *Nat Genet* **17**(3)**:** 273-274.

Greenfield JR, Miller JW, Keogh JM, Henning E, Satterwhite JH, Cameron GS*, et al.* (2009). Modulation of blood pressure by central melanocortinergic pathways. *N Engl J Med* **360**(1)**:** 44-52.

Gross V, Luft FC (2003). Exercising restraint in measuring blood pressure in conscious mice. *Hypertension* **41**(4)**:** 879-881.

Gross V, Plehm R, Tank J, Jordan J, Diedrich A, Obst M*, et al.* (2002). Heart rate variability and baroreflex function in AT2 receptor-disrupted mice. *Hypertension* **40**(2)**:** 207-213.

Gross V, Tank J, Obst M, Plehm R, Blumer K, Diedrich A*, et al.* (2005). Autonomic nervous system and blood pressure regulation in RGS2-deficient mice. *Am J Physiol Regul Integr Comp Physiol* **288**(5)**:** R1134-1142.

Gruber K, Callahan M (1989). ACTH-(4-10) through gamma-MSH: evidence for a new class of central autonomic nervous system-regulating peptides. *Am J Physiol* **257**(4 Pt 2)**:** R681-694.

Guo L, Münzberg H, Stuart RC, Nillni EA, Bjørbaek C (2004). N-acetylation of hypothalamic alpha-melanocyte-stimulating hormone and regulation by leptin. *Proc Natl Acad Sci U S A* **101**(32)**:** 11797-11802.

Guyenet PG (2006). The sympathetic control of blood pressure. *Nat Rev Neurosci* **7**(5)**:** 335-346.

Guyton A, Hall J (2006). *Textbook of Medical Physiology, 11th edition*. Philadelphia: Elsevier Saunders.

Guyton AC (1980). *Circulatory Physiology III: Arterial Pressure and Hypertension*. W.B. Saunders Company.

Gödecke A, Ziegler M, Ding Z, Schrader J (2002). Endothelial dysfunction of coronary resistance vessels in apoE-/- mice involves NO but not prostacyclin-dependent mechanisms. *Cardiovasc Res* **53**(1)**:** 253-262.

Hadley ME, Dorr RT (2006). Melanocortin peptide therapeutics: historical milestones, clinical studies and commercialization. *Peptides* **27**(4)**:** 921-930.

Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D*, et al.* (1995). Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* **269**(5223)**:** 543-546.

Harrison DG, Marvar PJ, Titze JM (2012). Vascular inflammatory cells in hypertension. *Front Physiol* **3:** 128.

Hartley C, Reddy A, Madala S, Michael L, Entman M, Taffet G (2008). Doppler estimation of reduced coronary flow reserve in mice with pressure overload cardiac hypertrophy. *Ultrasound Med Biol* **34**(6)**:** 892-901.

Hartley C, Reddy A, Madala S, Michael L, Entman M, Taffet G (2007). Effects of isoflurane on coronary blood flow velocity in young, old and ApoE(-/-) mice measured by Doppler ultrasound. *Ultrasound Med Biol* **33**(4)**:** 512-521.

Hartmeyer M, Scholzen T, Becher E, Bhardwaj RS, Schwarz T, Luger TA (1997). Human dermal microvascular endothelial cells express the melanocortin receptor type 1 and produce increased levels of IL-8 upon stimulation with alpha-melanocyte-stimulating hormone. *J Immunol* **159**(4)**:** 1930-1937.

Haynes W, Morgan D, Djalali A, Sivitz W, Mark A (1999). Interactions between the melanocortin system and leptin in control of sympathetic nerve traffic. *Hypertension* **33**(1 Pt 2)**:** 542-547.

Hill C, Dunbar J (2002). The effects of acute and chronic alpha melanocyte stimulating hormone (alphaMSH) on cardiovascular dynamics in conscious rats. *Peptides* **23**(9)**:** 1625-1630.

Hradec J, Horký K (1979). Natriuretic and kaliuretic effect of melanocyte-stimulating hormones in hamsters. *Endocrinol Exp* **13**(3)**:** 145-152.

Hummel KP, Dickie MM, Coleman DL (1966). Diabetes, a new mutation in the mouse. *Science* **153**(3740)**:** 1127-1128.

Humphreys M (2004). Gamma-MSH, sodium metabolism, and salt-sensitive hypertension. *Am J Physiol Regul Integr Comp Physiol* **286**(3)**:** R417-430.

Humphreys MH, Ni XP, Pearce D (2011). Cardiovascular effects of melanocortins. *Eur J Pharmacol* **660**(1)**:** 43-52.

Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR*, et al.* (1997). Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* **88**(1)**:** 131-141.

Ignarro LJ, Harbison RG, Wood KS, Kadowitz PJ (1986). Activation of purified soluble guanylate cyclase by endothelium-derived relaxing factor from intrapulmonary artery and vein: stimulation by acetylcholine, bradykinin and arachidonic acid. *J Pharmacol Exp Ther* **237**(3)**:** 893-900.

Ingalss AM, Dickie MM, Snell GD (1950). Obese, a new mutation in the house mouse. *J Hered* **41**(12)**:** 317-318.

Jacobowitz D, O'Donohue T (1978). alpha-Melanocyte stimulating hormone: immunohistochemical identification and mapping in neurons of rat brain. *Proc Natl Acad Sci U S A* **75**(12)**:** 6300-6304.

Janssen B, Leenders P, Smits J (2000). Short-term and long-term blood pressure and heart rate variability in the mouse. *Am J Physiol Regul Integr Comp Physiol* **278**(1)**:** R215-225.

Janssen B, Smits J (2002). Autonomic control of blood pressure in mice: basic physiology and effects of genetic modification. *Am J Physiol Regul Integr Comp Physiol* **282**(6)**:** R1545-1564.

Japundzic N, Grichois M, Zitoun P, Laude D, Elghozi J (1990). Spectral analysis of blood pressure and heart rate in conscious rats: effects of autonomic blockers. *J Auton Nerv Syst* **30**(2)**:** 91-100.

Juni A, Cai M, Stankova M, Waxman AR, Arout C, Klein G*, et al.* (2010). Sex-specific mediation of opioid-induced hyperalgesia by the melanocortin-1 receptor. *Anesthesiology* **112**(1)**:** 181-188.

Just A, Faulhaber J, Ehmke H (2000). Autonomic cardiovascular control in conscious mice. *Am J Physiol Regul Integr Comp Physiol* **279**(6)**:** R2214-2221.

Kathpalia PP, Charlton C, Rajagopal M, Pao AC (2011). The natriuretic mechanism of Gamma-

Melanocyte-Stimulating Hormone. *Peptides* **32**(5)**:** 1068-1072.

Kingwell B, Cameron J, Gillies K, Jennings G, Dart A (1995). Arterial compliance may influence baroreflex function in athletes and hypertensives. *Am J Physiol* **268**(1 Pt 2)**:** H411-418.

Kishi T, Aschkenasi CJ, Lee CE, Mountjoy KG, Saper CB, Elmquist JK (2003). Expression of melanocortin 4 receptor mRNA in the central nervous system of the rat. *J Comp Neurol* **457**(3)**:** 213-235.

Kleinbongard P, Schulz R, Rassaf T, Lauer T, Dejam A, Jax T*, et al.* (2006). Red blood cells express a functional endothelial nitric oxide synthase. *Blood* **107**(7)**:** 2943-2951.

Koizumi K, Terui N, Kollai M (1985). Effect of cardiac vagal and sympathetic nerve activity on heart rate in rhythmic fluctuations. *J Auton Nerv Syst* **12**(2-3)**:** 251-259.

Kokot A, Metze D, Mouchet N, Galibert MD, Schiller M, Luger TA*, et al.* (2009). Alpha-melanocyte-stimulating hormone counteracts the suppressive effect of UVB on Nrf2 and Nrf-dependent gene expression in human skin. *Endocrinology* **150**(7)**:** 3197-3206.

Kolgazi M, Arbak S, Alican I (2007). The effect of alpha-melanocyte stimulating hormone on gentamicin-induced acute nephrotoxicity in rats. *J Appl Toxicol* **27**(2)**:** 183-188.

Konda Y, Gantz I, DelValle J, Shimoto Y, Miwa H, Yamada T (1994). Interaction of dual intracellular signaling pathways activated by the melanocortin-3 receptor. *J Biol Chem* **269**(18)**:** 13162-13166.

Krude H, Biebermann H, Luck W, Horn R, Brabant G, Grüters A (1998). Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. *Nat Genet* **19**(2)**:** 155-157.

Kuo J, da Silva A, Tallam L, Hall J (2004). Role of adrenergic activity in pressor responses to chronic melanocortin receptor activation. *Hypertension* **43**(2)**:** 370-375.

Kuo J, Silva A, Hall J (2003). Hypothalamic melanocortin receptors and chronic regulation of arterial pressure and renal function. *Hypertension* **41**(3 Pt 2)**:** 768-774.

Kurtz TW, Griffin KA, Bidani AK, Davisson RL, Hall JE, Association SoPaPEotAH (2005). Recommendations for blood pressure measurement in humans and experimental animals. Part 2: Blood pressure measurement in experimental animals: a statement for professionals from the subcommittee of professional and public education of the American Heart Association council on high blood pressure research. *Hypertension* **45**(2)**:** 299-310.

Lam CW, Getting SJ (2004). Melanocortin receptor type 3 as a potential target for anti-inflammatory therapy. *Curr Drug Targets Inflamm Allergy* **3**(3)**:** 311-315.

Lam CW, Getting SJ, Perretti M (2005). In vitro and in vivo induction of heme oxygenase 1 in mouse macrophages following melanocortin receptor activation. *J Immunol* **174**(4)**:** 2297-2304.

Laude D, Baudrie V, Elghozi J (2008). Applicability of recent methods used to estimate spontaneous baroreflex sensitivity to resting mice. *Am J Physiol Regul Integr Comp Physiol* **294**(1)**:** R142-150.

Laude D, Baudrie V, Elghozi J (2009). Tuning of the sequence technique. *IEEE Eng Med Biol Mag* **28**(6)**:** 30-34.

Leoni G, Patel HB, Sampaio AL, Gavins FN, Murray JF, Grieco P*, et al.* (2008). Inflamed phenotype of the mesenteric microcirculation of melanocortin type 3 receptor-null mice after ischemia-reperfusion. *FASEB J* **22**(12)**:** 4228-4238.

Leoni G, Voisin MB, Carlson K, Getting S, Nourshargh S, Perretti M (2010). The melanocortin MC(1) receptor agonist BMS-470539 inhibits leucocyte trafficking in the inflamed vasculature. *Br J Pharmacol* **160**(1)**:** 171-180.

Lerner AB, McGuire JS (1961). Effect of alpha- and betamelanocyte stimulating hormones on the skin colour of man. *Nature* **189:** 176-179.

Levin ER (1995). Endothelins. *N Engl J Med* **333**(6)**:** 356-363.

Levy MN, Martin PJ, Iano T, Zieske H (1970). Effects of single vagal stimuli on heart rate and atrioventricular conduction. *Am J Physiol* **218**(5)**:** 1256-1262.

Levy MN, Zieske H (1969). Autonomic control of cardiac pacemaker activity and atrioventricular transmission. *J Appl Physiol* **27**(4)**:** 465-470.

Li S, Varga K, Archer P, Hruby V, Sharma S, Kesterson R*, et al.* (1996). Melanocortin antagonists define two distinct pathways of cardiovascular control by alpha- and gamma-melanocyte-stimulating hormones. *J Neurosci* **16**(16)**:** 5182-5188.

Lin SY, Chaves C, Wiedemann E, Humphreys MH (1987). A gamma-melanocyte stimulating hormone-like peptide causes reflex natriuresis after acute unilateral nephrectomy. *Hypertension* **10**(6)**:** 619-627.

Lin SY, Wiedemann E, Humphreys MH (1985). Role of the pituitary in reflex natriuresis following acute

unilateral nephrectomy. *Am J Physiol* **249**(2 Pt 2)**:** F282-290.

Lindblom J, Opmane B, Mutulis F, Mutule I, Petrovska R, Klusa V*, et al.* (2001). The MC4 receptor mediates alpha-MSH induced release of nucleus accumbens dopamine. *Neuroreport* **12**(10)**:** 2155-2158.

Lindskog A, Ebefors K, Johansson ME, Stefánsson B, Granqvist A, Arnadottir M*, et al.* (2010). Melanocortin 1 receptor agonists reduce proteinuria. *J Am Soc Nephrol* **21**(8)**:** 1290-1298.

Lingueglia E, Champigny G, Lazdunski M, Barbry P (1995). Cloning of the amiloride-sensitive FMRFamide peptide-gated sodium channel. *Nature* **378**(6558)**:** 730-733.

Lingueglia E, Deval E, Lazdunski M (2006). FMRFamide-gated sodium channel and ASIC channels: a new class of ionotropic receptors for FMRFamide and related peptides. *Peptides* **27**(5)**:** 1138-1152.

Liu H, Kishi T, Roseberry AG, Cai X, Lee CE, Montez JM*, et al.* (2003). Transgenic mice expressing green fluorescent protein under the control of the melanocortin-4 receptor promoter. *J Neurosci* **23**(18)**:** 7143-7154.

Loir B, Pérez Sánchez C, Ghanem G, Lozano JA, García-Borrón JC, Jiménez-Cervantes C (1999). Expression of the MC1 receptor gene in normal and malignant human melanocytes. A semiquantitative RT-PCR study. *Cell Mol Biol (Noisy-le-grand)* **45**(7)**:** 1083-1092.

Lorenz JN (2002). A practical guide to evaluating cardiovascular, renal, and pulmonary function in mice. *Am J Physiol Regul Integr Comp Physiol* **282**(6)**:** R1565-1582.

Lu D, Willard D, Patel IR, Kadwell S, Overton L, Kost T*, et al.* (1994). Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. *Nature* **371**(6500)**:** 799-802.

Luger TA, Scholzen T, Grabbe S (1997). The role of alpha-melanocyte-stimulating hormone in cutaneous biology. *J Investig Dermatol Symp Proc* **2**(1)**:** 87-93.

Lymangrover JR, Buckalew VM, Harris J, Klein MC, Gruber KA (1985). Gamma-2MSH is natriuretic in the rat. *Endocrinology* **116**(3)**:** 1227-1229.

Maaser C, Kannengiesser K, Specht C, Lügering A, Brzoska T, Luger TA*, et al.* (2006). Crucial role of the melanocortin receptor MC1R in experimental colitis. *Gut* **55**(10)**:** 1415-1422.

Malliani A, Pagani M, Lombardi F, Cerutti S (1991). Cardiovascular neural regulation explored in the frequency domain. *Circulation* **84**(2)**:** 482-492.

Manning RD, Meng S, Tian N (2003). Renal and vascular oxidative stress and salt-sensitivity of arterial pressure. *Acta Physiol Scand* **179**(3)**:** 243-250.

Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K*, et al.* (2000). Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest* **106**(12)**:** 1521-1530.

Matsumura K, Tsuchihashi T, Abe I, Iida M (2002). Central alpha-melanocyte-stimulating hormone acts at melanocortin-4 receptor to activate sympathetic nervous system in conscious rabbits. *Brain Res* **948**(1-2)**:** 145-148.

Mayan H, Ling KT, Lee EY, Wiedemann E, Kalinyak JE, Humphreys MH (1996). Dietary sodium intake modulates pituitary proopiomelanocortin mRNA abundance. *Hypertension* **28**(2)**:** 244-249.

Minder EI (2010). Afamelanotide, an agonistic analog of α-melanocyte-stimulating hormone, in dermal phototoxicity of erythropoietic protoporphyria. *Expert Opin Investig Drugs* **19**(12)**:** 1591-1602.

Mogil JS, Wilson SG, Chesler EJ, Rankin AL, Nemmani KV, Lariviere WR*, et al.* (2003). The melanocortin-1 receptor gene mediates female-specific mechanisms of analgesia in mice and humans. *Proc Natl Acad Sci U S A* **100**(8)**:** 4867-4872.

Morgan C, Cone RD (2006). Melanocortin-5 receptor deficiency in mice blocks a novel pathway influencing pheromone-induced aggression. *Behav Genet* **36**(2)**:** 291-300.

Morgan C, Thomas RE, Cone RD (2004). Melanocortin-5 receptor deficiency promotes defensive behavior in male mice. *Horm Behav* **45**(1)**:** 58-63.

Mountjoy K, Robbins L, Mortrud M, Cone R (1992). The cloning of a family of genes that encode the melanocortin receptors. *Science* **257**(5074)**:** 1248-1251.

Mountjoy KG, Mortrud MT, Low MJ, Simerly RB, Cone RD (1994). Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. *Mol Endocrinol* **8**(10)**:** 1298-1308.

Muceniece R, Zvejniece L, Liepinsh E, Kirjanova O, Baumane L, Petrovska R*, et al.* (2006). The MC3 receptor binding affinity of melanocortins correlates with the nitric oxide production inhibition in mice brain inflammation model. *Peptides* **27**(6)**:** 1443-1450.

Nakanishi S, Inoue A, Kita T, Nakamura M, Chang AC, Cohen SN*, et al.* (1979). Nucleotide sequence of cloned cDNA for bovine corticotropin-beta-lipotropin precursor. *Nature* **278**(5703)**:** 423-427.

Ni X, Butler A, Cone R, Humphreys M (2006a). Central receptors mediating the cardiovascular actions of melanocyte stimulating hormones. *J Hypertens* **24**(11)**:** 2239-2246.

Ni X, Pearce D, Butler A, Cone R, Humphreys M (2003). Genetic disruption of gamma-melanocyte-stimulating hormone signaling leads to salt-sensitive hypertension in the mouse. *J Clin Invest* **111**(8)**:** 1251-1258.

Ni XP, Bhargava A, Pearce D, Humphreys MH (2006b). Modulation by dietary sodium intake of melanocortin 3 receptor mRNA and protein abundance in the rat kidney. *Am J Physiol Regul Integr Comp Physiol* **290**(3)**:** R560-567.

Ni XP, Kesterson RA, Sharma SD, Hruby VJ, Cone RD, Wiedemann E*, et al.* (1998). Prevention of reflex natriuresis after acute unilateral nephrectomy by melanocortin receptor antagonists. *Am J Physiol* **274**(4 Pt 2)**:** R931-938.

Nijsen MJ, de Ruiter GJ, Kasbergen CM, Hoogerhout P, de Wildt DJ (2000). Relevance of the C-terminal Arg-Phe sequence in gamma(2)-melanocyte-stimulating hormone (gamma(2)-MSH) for inducing cardiovascular effects in conscious rats. *Br J Pharmacol* **131**(7)**:** 1468-1474.

Niswender KD, Baskin DG, Schwartz MW (2004). Insulin and its evolving partnership with leptin in the hypothalamic control of energy homeostasis. *Trends Endocrinol Metab* **15**(8)**:** 362-369.

Nogueiras R, Wiedmer P, Perez-Tilve D, Veyrat-Durebex C, Keogh JM, Sutton GM*, et al.* (2007). The central melanocortin system directly controls peripheral lipid metabolism. *J Clin Invest* **117**(11)**:** 3475-3488.

Obst M, Gross V, Bonartsev A, Janke J, Müller DN, Park JK*, et al.* (2004). Nitric oxide synthase expression in AT2 receptor-deficient mice after DOCA-salt. *Kidney Int* **65**(6)**:** 2268-2278.

Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I*, et al.* (1997). Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science* **278**(5335)**:** 135-138.

Orias R, McCann SM (1972). Natriuresis induced by alpha and beta melanocyte stimulating hormone (MSH) in rats. *Endocrinology* **90**(3)**:** 700-706.

Ottani A, Giuliani D, Galantucci M, Spaccapelo L, Novellino E, Grieco P*, et al.* (2010). Melanocortins counteract inflammatory and apoptotic responses to prolonged myocardial ischemia/reperfusion through a vagus nerve-mediated mechanism. *Eur J Pharmacol* **637**(1-3)**:** 124-130.

Palkovits M, Mezey E, Eskay R (1987). Pro-opiomelanocortin-derived peptides (ACTH/beta-endorphin/alpha-MSH) in brainstem baroreceptor areas of the rat. *Brain Res* **436**(2)**:** 323-338.

Palmer RM, Ferrige AG, Moncada S (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327**(6122)**:** 524-526.

Pavia J, Schiöth H, Morris M (2003). Role of MC4 receptors in the depressor and bradycardic effects of alpha-MSH in the nucleus tractus solitarii of the rat. *Neuroreport* **14**(5)**:** 703-707.

Peiper U, Schmidt E, Laven R, Griebel L (1973). Length-tension relationships in resting and activated vascular smooth muscle fibres. *Pflugers Arch* **340**(2)**:** 113-122.

Penttilä J, Vesalainen R, Helminen A, Kuusela T, Hinkka S, Rahi K*, et al.* (2001). Spontaneous baroreflex sensitivity as a dynamic measure of cardiac anticholinergic drug effect. *J Auton Pharmacol* **21**(2)**:** 71-78.

Pomeranz B, Macaulay RJ, Caudill MA, Kutz I, Adam D, Gordon D*, et al.* (1985). Assessment of autonomic function in humans by heart rate spectral analysis. *Am J Physiol* **248**(1 Pt 2)**:** H151-153.

Price DA, Greenberg MJ (1977). Structure of a molluscan cardioexcitatory neuropeptide. *Science* **197**(4304)**:** 670-671.

Ramaekers D, Beckers F, Demeulemeester H, Bert C, Denef C, Aubert A (2002). Effects of melanocortins on cardiovascular regulation in rats. *Clin Exp Pharmacol Physiol* **29**(7)**:** 549-558.

Roselli-Rehfuss L, Mountjoy KG, Robbins LS, Mortrud MT, Low MJ, Tatro JB*, et al.* (1993). Identification of a receptor for gamma melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. *Proc Natl Acad Sci U S A* **90**(19)**:** 8856-8860.

Saraste A, Kytö V, Laitinen I, Saraste M, Leppänen P, Ylä-Herttuala S*, et al.* (2008). Severe coronary artery stenoses and reduced coronary flow velocity reserve in atherosclerotic mouse model: Doppler echocardiography validation study. *Atherosclerosis* **200**(1)**:** 89-94.

Saraste A, Kytö V, Saraste M, Vuorinen T, Hartiala J, Saukko P (2006). Coronary flow reserve and heart failure in experimental coxsackievirus myocarditis. A transthoracic Doppler echocardiography study. *Am J Physiol Heart Circ Physiol* **291**(2)**:** H871-875.

Sase K, Michel T (1995). Expression of constitutive endothelial nitric oxide synthase in human blood platelets. *Life Sci* **57**(22)**:** 2049-2055.

Savontaus E, Breen T, Kim A, Yang L, Chua SJ, Wardlaw S (2004). Metabolic effects of transgenic

melanocyte-stimulating hormone overexpression in lean and obese mice. *Endocrinology* **145**(8)**:** 3881-3891.

Sawyer TK, Sanfilippo PJ, Hruby VJ, Engel MH, Heward CB, Burnett JB*, et al.* (1980). 4-Norleucine, 7-D-phenylalanine-alpha-melanocyte-stimulating hormone: a highly potent alpha-melanotropin with ultralong biological activity. *Proc Natl Acad Sci U S A* **77**(10)**:** 5754-5758.

Sayers BM (1973). Analysis of heart rate variability. *Ergonomics* **16**(1)**:** 17-32.

Schenk J, McNeill JH (1992). The pathogenesis of DOCA-salt hypertension. *J Pharmacol Toxicol Methods* **27**(3)**:** 161-170.

Schiöth HB, Chhajlani V, Muceniece R, Klusa V, Wikberg JE (1996). Major pharmacological distinction of the ACTH receptor from other melanocortin receptors. *Life Sci* **59**(10)**:** 797-801.

Scholzen TE, Brzoska T, Kalden DH, Hartmeyer M, Fastrich M, Luger TA*, et al.* (1999). Expression of functional melanocortin receptors and proopiomelanocortin peptides by human dermal microvascular endothelial cells. *Ann N Y Acad Sci* **885:** 239-253.

Schwartz M, Woods S, Porte DJ, Seeley R, Baskin D (2000). Central nervous system control of food intake. *Nature* **404**(6778)**:** 661-671.

Shaul PW, North AJ, Wu LC, Wells LB, Brannon TS, Lau KS*, et al.* (1994). Endothelial nitric oxide synthase is expressed in cultured human bronchiolar epithelium. *J Clin Invest* **94**(6)**:** 2231-2236.

Shirasaki Y, Kolm P, Nickols GA, Lee TJ (1988). Endothelial regulation of cyclic GMP and vascular responses in hypertension. *J Pharmacol Exp Ther* **245**(1)**:** 53-58.

Smith AI, Funder JW (1988). Proopiomelanocortin processing in the pituitary, central nervous system, and peripheral tissues. *Endocr Rev* **9**(1)**:** 159-179.

Somers MJ, Mavromatis K, Galis ZS, Harrison DG (2000). Vascular superoxide production and vasomotor function in hypertension induced by deoxycorticosterone acetate-salt. *Circulation* **101**(14)**:** 1722-1728.

Spyer KM (1994). Annual review prize lecture. Central nervous mechanisms contributing to cardiovascular control. *J Physiol* **474**(1)**:** 1-19.

Stauss HM (2007). Power spectral analysis in mice: What are the appropriate frequency bands? *Am J Physiol Regul Integr Comp Physiol* **292**(2)**:** R902-903.

Stuehr DJ, Cho HJ, Kwon NS, Weise MF, Nathan CF (1991). Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc Natl Acad Sci U S A* **88**(17)**:** 7773-7777.

Tai M, Weng W, Lo W, Chan J, Lin C, Lam H*, et al.* (2007). Role of nitric oxide in alpha-melanocyte-stimulating hormone-induced hypotension in the nucleus tractus solitarii of the spontaneously hypertensive rats. *J Pharmacol Exp Ther* **321**(2)**:** 455-461.

Tallam LS, Stec DE, Willis MA, da Silva AA, Hall JE (2005). Melanocortin-4 receptor-deficient mice are not hypertensive or salt-sensitive despite obesity, hyperinsulinemia, and hyperleptinemia. *Hypertension* **46**(2)**:** 326-332.

Tanabe K, Gamo K, Aoki S, Wada K, Kiyama H (2007). Melanocortin receptor 4 is induced in nerve-injured motor and sensory neurons of mouse. *J Neurochem* **101**(4)**:** 1145-1152.

Taylor JA, Carr DL, Myers CW, Eckberg DL (1998). Mechanisms underlying very-low-frequency RR-interval oscillations in humans. *Circulation* **98**(6)**:** 547-555.

Thireau J, Zhang BL, Poisson D, Babuty D (2008). Heart rate variability in mice: a theoretical and practical guide. *Exp Physiol* **93**(1)**:** 83-94.

Touyz RM, Briones AM (2011). Reactive oxygen species and vascular biology: implications in human hypertension. *Hypertens Res* **34**(1)**:** 5-14.

Ugwu SO, Blanchard J, Dorr RT, Levine N, Brooks C, Hadley ME*, et al.* (1997). Skin pigmentation and pharmacokinetics of melanotan-I in humans. *Biopharm Drug Dispos* **18**(3)**:** 259-269.

Vaisse C, Clement K, Guy-Grand B, Froguel P (1998). A frameshift mutation in human MC4R is associated with a dominant form of obesity. *Nat Genet* **20**(2)**:** 113-114.

Van Bergen P, Janssen P, Hoogerhout P, De Wildt D, Versteeg D (1995). Cardiovascular effects of gamma-MSH/ACTH-like peptides: structure-activity relationship. *Eur J Pharmacol* **294**(2-3)**:** 795-803.

Van Bergen P, Kleijne J, De Wildt D, Versteeg D (1997). Different cardiovascular profiles of three melanocortins in conscious rats; evidence for antagonism between gamma 2-MSH and ACTH-(1-24). *Br J Pharmacol* **120**(8)**:** 1561-1567.

Van Bergen P, Vleeming W, Van Heijst B, Versteeg D, De Wildt D (1998). A study on possible modulating and direct effects of gamma2-MSH and ACTH-(1-24) on the cardiovascular system of the rat. *Naunyn Schmiedebergs Arch Pharmacol* **358**(2)**:** 220-229.

Van de Voorde J, Leusen I (1986). Endothelium-dependent and independent relaxation of aortic rings

from hypertensive rats. *Am J Physiol* **250**(5 Pt 2)**:** H711-717.

Versteeg D, Van Bergen P, Adan R, De Wildt D (1998). Melanocortins and cardiovascular regulation. *Eur J Pharmacol* **360**(1)**:** 1-14.

Voss-Andreae A, Murphy JG, Ellacott KL, Stuart RC, Nillni EA, Cone RD*, et al.* (2007). Role of the central melanocortin circuitry in adaptive thermogenesis of brown adipose tissue. *Endocrinology* **148**(4)**:** 1550-1560.

Wallingford N, Perroud B, Gao Q, Coppola A, Gyengesi E, Liu ZW*, et al.* (2009). Prolylcarboxypeptidase regulates food intake by inactivating alpha-MSH in rodents. *J Clin Invest* **119**(8)**:** 2291-2303.

Wan S, Browning KN, Coleman FH, Sutton G, Zheng H, Butler A*, et al.* (2008). Presynaptic melanocortin-4 receptors on vagal afferent fibers modulate the excitability of rat nucleus tractus solitarius neurons. *J Neurosci* **28**(19)**:** 4957-4966.

Wardlaw SL (2011). Hypothalamic proopiomelanocortin processing and the regulation of energy balance. *Eur J Pharmacol*.

Watson S, Akil H, Richard Cr, Barchas J (1978). Evidence for two separate opiate peptide neuronal systems. *Nature* **275**(5677)**:** 226-228.

Wellhöner P, Hörster R, Jacobs F, Sayk F, Lehnert H, Dodt C (2012). Intranasal application of the melanocortin 4 receptor agonist MSH/ACTH(4-10) in humans causes lipolysis in white adipose tissue. *Int J Obes (Lond)* **36**(5)**:** 703-708.

Wikberg J, Muceniece R, Mandrika I, Prusis P, Lindblom J, Post C*, et al.* (2000). New aspects on the melanocortins and their receptors. *Pharmacol Res* **42**(5)**:** 393-420.

Wikberg JE, Mutulis F (2008). Targeting melanocortin receptors: an approach to treat weight disorders and sexual dysfunction. *Nat Rev Drug Discov* **7**(4)**:** 307-323.

Williams D, Schwartz M (2005). The melanocortin system as a central integrator of direct and indirect controls of food intake. *Am J Physiol Regul Integr Comp Physiol* **289**(1)**:** R2-3.

Williams DL, Bowers RR, Bartness TJ, Kaplan JM, Grill HJ (2003). Brainstem melanocortin 3/4 receptor stimulation increases uncoupling protein gene expression in brown fat. *Endocrinology* **144**(11)**:** 4692-4697.

Williams DL, Kaplan JM, Grill HJ (2000). The role of the dorsal vagal complex and the vagus nerve in feeding effects of melanocortin-3/4 receptor stimulation. *Endocrinology* **141**(4)**:** 1332-1337.

Wilson BD, Ollmann MM, Kang L, Stoffel M, Bell GI, Barsh GS (1995). Structure and function of ASP, the human homolog of the mouse agouti gene. *Hum Mol Genet* **4**(2)**:** 223-230.

Wolff GL, Roberts DW, Mountjoy KG (1999). Physiological consequences of ectopic agouti gene expression: the yellow obese mouse syndrome. *Physiol Genomics* **1**(3)**:** 151-163.

Xia Y, Dawson VL, Dawson TM, Snyder SH, Zweier JL (1996a). Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. *Proc Natl Acad Sci U S A* **93**(13)**:** 6770-6774.

Xia Y, Wikberg JE (1996b). Localization of ACTH receptor mRNA by in situ hybridization in mouse adrenal gland. *Cell Tissue Res* **286**(1)**:** 63-68.

Xia Y, Wikberg JE, Chhajlani V (1995). Expression of melanocortin 1 receptor in periaqueductal gray matter. *Neuroreport* **6**(16)**:** 2193-2196.

Yaswen L, Diehl N, Brennan MB, Hochgeschwender U (1999). Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. *Nat Med* **5**(9)**:** 1066-1070.

Yemane H, Busauskas M, Burris SK, Knuepfer MM (2010). Neurohumoral mechanisms in deoxycorticosterone acetate (DOCA)-salt hypertension in rats. *Exp Physiol* **95**(1)**:** 51-55.

Yeo GS, Farooqi IS, Aminian S, Halsall DJ, Stanhope RG, O'Rahilly S (1998). A frameshift mutation in MC4R associated with dominantly inherited human obesity. *Nat Genet* **20**(2)**:** 111-112.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature* **372**(6505)**:** 425-432.

Zheng H, Patterson LM, Phifer CB, Berthoud HR (2005). Brain stem melanocortinergic modulation of meal size and identification of hypothalamic POMC projections. *Am J Physiol Regul Integr Comp Physiol* **289**(1)**:** R247-258.

Zvejniece L, Muceniece R, Krigere L, Dambrova M, Klusa V (2006). The differential influences of melanocortins on nociception in the formalin and tail flick tests. *Pharmacol Biochem Behav* **85**(2)**:** 287-291.