Calcitonin gene-related peptide (CGRP) in age-associated vascular inflammation and leukocyte trafficking

King, Ross

Awarding institution:
King's College London

Calcitonin gene-related peptide (CGRP) in age-associated vascular inflammation and leukocyte trafficking

Thesis submitted for the degree of

Doctor of Philosophy

King's College London

Ross King

Cardiovascular Division

BHF Centre of Research Excellence

King's College London

Franklin-Wilkins Building

Waterloo Campus

London SE1 9NH

| Abstract

Calcitonin gene-related peptide (CGRP) is a 37 amino acid neuropeptide that is predominantly synthesised and secreted by small, thinly-myelinated and unmyelinated sensory neurons that richly innervate the vasculature. It is perhaps best known for its ability to act as a potent dilator of the microvasculature and its role as a vasculoprotectant is gradually being discovered within a range of cardiovascular disease models. However, the role of CGRP in the ageing cardiovascular system and current information regarding CGRP and the ageing process in general is unknown. This study is particularly relevant as ageing is the single biggest independent risk factor for the development of future cardiovascular complications.

We hypothesised that CGRP may have a beneficial role in protecting the cardiovascular system against age-associated cardiovascular disease, such as hypertension, and related vascular inflammatory processes mediated by aberrant inflammatory cell activity. We initially characterised the vascular phenotype of ageing (15 months) αCGRP wild type (WT) and knockout (KO) mice, in comparison to respective juveniles (3 months). We found that aged animals had comparable blood pressures to juvenile animals and KO mice did not differ significantly from WTs at either timepoint. Furthermore, markers of leukocytic vascular inflammation were significantly raised within the aortae of ageing mice but the genetic deletion of αCGRP had no bearing on this process.

To study direct influences of CGRP on inflammatory processes driven by leukocytes, we employed a reductive approach and studied leukocyte-endothelial cell interactions *in vitro*, under physiological flow conditions. Here, we showed that treatment of endothelial cells with CGRP for six hours is sufficient to attenuate the adhesion of monocytic cells to tumour necrosis factor alpha (TNFα)-activated HUVEC (HUVEC) by around 50%. The mechanisms regulating this phenomenon did not appear to be related to alterations in the whole-cell expression of classical cell adhesion molecules, including vascular cell adhesion molecule-1(VCAM-1). Furthermore, microarray analysis of HUVEC transcriptome revealed that CGRP did not influence gene expression induced by TNFα in these cells.

We finally sought to establish a novel geriatric pathology model in mice that may be more relevant to the human disease condition. To do this, we infused aged αCGRP WT and KO mice with 1.1 mg/kg/day angiotensin II (ATII) for 14 days to induce hypertension and vascular inflammation. However, despite some level of pharmacological activity by ATII, we did not witness the induction of hypertension and we observed no clear vascular inflammatory phenotype.

This thesis provides evidence that ageing in mice is sufficient to induce vascular inflammation in the absence of an overt maladaptive cardiovascular phenotype, where the deletion of αCGRP appears to have minimal effect. However, we have uncovered novel evidence to suggest that CGRP might be protective against leukocytic inflammation by virtue of its ability to attenuate leukocyte-endothelial cell interactions under physiological flow conditions.

| Acknowledgements

I would firstly like to express my greatest thanks to Sue for giving me the opportunity to work in her lab and allowing me to develop myself as a scientist. It would be fair to say that we've had our ups and downs but despite this, I am truly grateful for your unwavering support and constant looking out for my best interests – I hope you don't have another student like me in the future! Secondly, I have to thank Alex for hosting me in his lab and agreeing to be part of this project. You've always had belief in me, even at times when I didn't have any in myself. I couldn't have asked for a better mentor and I hope we stay good pals! To both of you, I wish the best of luck for the future – you deserve it. I am also very grateful for the generous funding of this PhD by The British Heart Foundation.

Of course, this entire PhD has been propelled and cultivated by a substantial rogues gallery. Tremendous thanks have to be given to Patrick Fox and the hardworking members of staff of the BSU – none of us could have done any of this work without you and your work here sadly appears to be greatly underappreciated. Many thanks also to Dr. Matthew Arno for his assistance with microarray experiments and finding time for "just one more thing". I owe much to Sarah-Jane Smillie, in particular for showing me the way in the Brain lab, helping me find my feet and not yelling at me for spilling QIAzol all over the bench, never mind being an excellent confidante and sounding board in between times. I'd also like to acknowledge all current and former members of the Brain and Keeble labs for putting up with me over the years – in particular Maria, Aisah, Fiona, Khadjia, Elena, Pratish, Jenny, Lihuan and Beth. Finally, thanks to other members of Vascular Biology at Franklin-Wilkins Building, both past and present, for making it fun to come to work.

Despite the great celebration of completing the PhD, there is a large part of it that I will dearly miss. I am so lucky to have travelled down this road with Claire Sand, the best partner in crime I could ever have hoped for. We've enjoyed (far too many) long leisurely lunches, cry-laughed at (far too many) inappropriate situations and drank (far too many) a booze. I knew from the beginning we'd be great friends and I hope we continue to smell many more new smells.

Finally, I absolutely have to thank my family for being there throughout and dealing with my chronic imposter's syndrome, not just throughout the PhD but also throughout life. I am so thankful for every opportunity you've given me and facilitated at the sacrifice of your own and I hope I can repay you one day. I hope I've been able to make you proud.

| List of Figures

| List of Tables

| List of Publications and Published Abstracts

1.1 Publications

- Russell FA, **King R**, Smillie S-J, Kodji X & Brain SD (2014). The role of CGRP in biology and pathophysiology. *Physiological Reviews.* **94**(4): 1099-1142.
- Smillie S-J, **King R**, Kodji X, Outzen E, Pozsgai G, de Winter P, Fernandes E, Heads R, Marshall N, Dessapt-Baradez C, Shah A, Siow R & Brain SD (2014) An ongoing role of αCGRP as part of a protective network against hypertension, vascular hypertrophy and oxidative stress. *Hypertension.* **63(5)**: 1056-62.
- **King R**. & Brain SD (2013). Calcitonin gene-related peptide. Book chapter in: *Handbook of Biologically Active Peptides, 2nd Edition.* Chapter 189.

1.2 Abstracts

- **King R**, Smillie S-J, Ivetic A, Brain SD (2014). Calcitonin gene-related peptide deletion does not promote an adverse cardiovascular phenotype in aged mice but inhibits monocyte recruitment *in vitro*. *CGRP Family Peptides Meeting, Ascona, Switzerland*.
- **King R**, Smillie S-J, Kodji X, Ivetic A, Brain SD (2014). Investigating a role for calcitonin gene-related peptide in age-associated vascular inflammation. *British Microvascular Society, Bristol, UK.*
- **King R**, Smillie S-J, Brain SD (2013). Investigating a role for CGRP in the ageing cardiovascular system. *International Union of Physiological Sciences Meeting. Birmingham, UK.*
- Smillie S-J, **King R**, Pozsgai G, Liang L, Fernandes E, de Winter P, Siow R, Brain SD (2013). Alpha calcitonin gene-related peptide plays a long-lasting protective role in both the onset and sustained models of angiotensin-II induced hypertension and vascular inflammation/remodelling. *International Union of Physiological Sciences Meeting. Birmingham, UK.*
- Smillie S-J, **King R**, Pozsgai G, de Winter P, Siow R, Brain SD (2013). Alpha calcitonin gene-related peptide plays a protective role in both an acute and sustained model of hypertension, a mechanism which may be linked to the loss of endothelial nitric oxide synthase (eNOS) as the hypertension progresses. *International Hypertension Society Meeting, Sydney, Australia.*
- Aubdool AA, Bodkin JV, Kodji X, **King R**, Gentry C, Liang L, Fernandes ES, Bevan S, Brain SD (2012). Neurovascular effects of cinnamaldehyde through activation of TRPA1: mechanisms underlying TRPA1-induced vasodilatation in the peripheral vasculature. *Proceedings of the British Pharmacological Society http://www.pa2online.org/abstracts/vol10issue4abst156p.pdf*
- Aubdool AA, Bodkin JV, Kodji X, **King R**, Gentry C, Fernandes ES, Bevan S, Brain SD (2012). Role of TRPA1 in the peripheral vasculature in vivo: increasing evidence for a CGRP and nitric oxide-sensitive mechanism. *International Workshop on Transient Receptor Potential (TRP) Channels.*
- **King R**, Smillie S-J, Bodkin JV, Brain SD (2012). Impaired vascular responses to calcitonin gene-related peptide in the ageing αCGRP knockout mouse. *Proceedings of the British Pharmacological Society. http://bps.conference-services.net/resources/344/3146/pdf/BSPNEURO2012_0024.pdf*

| List of Abbreviations

2K1C	2-kidney-1-clip
4-HNE	4-hydroxynonenal
ACE	Angiotensin converting-enzyme
AGE	Advanced glycation endproducts
AM	Adrenomedullin
AP-1	Adaptor protein-1
ApoE	Apolipoprotein E
AT1R	Angiotensin type 1 receptor
ATII	Angiotensin II
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate (cAMP)
cDNA	Complimentary DNA
CGRP	Calcitonin gene-related peptide
CLR	Calcitonin receptor-like receptor
CRP	C-reactive protein
CTR	Calcitonin receptor
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
DOCA	Deoxycorticosterone acetate
DRG	Dorsal root ganglion
EC	Endothelial cell
ECE-1	Endothelin-converting enzyme-1

EDD	Endothelial-dependent dilatation
eNOS	Endothelial nitric oxide synthase
EPAC	Exchange nucleotide protein directly activated by cAMP
EPC	Endothelial progenitor cell
ERK	Extracellular signal-related kinase
ET	Endothelin
FDR	False discovery rate
GPCR	G protein-coupled receptor
GPx	Glutathione peroxidase
HO-1	Haem oxygenase-1
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule-1
ICER	Inducible cAMP early repressor
IFN	Interferon
IκB	Inhibitor of nuclear factor kappa light chain polypeptide enhancer in B cells
IL	Interleukin
JAM	Junctional adhesion molecule
KC	Keratinocyte-derived chemokine
KO	Knockout
L-NAME	NG-nitro-L-arginine methyl ester
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1

MPO	Myeloperoxidase
mRNA	Messenger RNA
NA	Noradrenaline
NFκB	Nuclear factor kappa light chain polypeptide enhancer in B cells
NGF	Nerve growth factor
NHS	National Health Service
NO	Nitric oxide
NOX	NADPH oxidase
NQO1	NAPDH dehydrogenase quinone-1
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
PBMC	Peripheral blood mononuclear cell
PE	Phenylephrine
PECAM-1	Platelet-endothelial cell adhesion molecule-1
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
rAGE	Receptor for advanced glycation endproducts
RAMP	Receptor activity-modifying protein
RCP	Receptor component protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTqPCR	Quantitative reverse-transcription polymerase chain reaction
SASP	Senescence-associated secretory phenotype

SEM	Standard error of the mean
SNARE	Soluble NSF Attachment Protein
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
SP	Substance P
TNFα	Tumour necrosis factor alpha
TRPA1	Transient receptor potential ankyrin-1
TRPV1	Transient receptor potential vanilloid-1
UK	United Kingdom
VCAM-1	Vascular cell adhesion molecule-1
VSMC	Vascular smooth muscle cell
WT	Wild type

| Table of contents

1. | Chapter One: Introduction

Advances in modern medicine, thanks to breakthrough discoveries in biomedical research, have undoubtedly shaped the lives of those privileged enough to have access to it for the better. However, in ensuring that we are far less susceptible to falling ill than we once were, another problem has been created. We are now living much longer than ever before, and this presents a different set of problems related to how we care for the elderly. In order to understand how we might treat the geriatric population, efforts must be made to understand the basic biology of ageing. The aetiology of ageing is heterogeneous in nature and some interventions (both pharmacological or lifestyle-oriented) have shown promise with respect to extension of lifespan and, more critically, extension of healthspan (i.e. the proportion of time spent at the end of life in good health) (Kolovou et al., 2014).

Calcitonin gene-related peptide (CGRP) is the most potent microvascular vasodilator known to date (Brain et al., 1985) and possesses diverse activity throughout the cardiovascular and other body systems (Russell et al., 2014). It is widely expressed throughout the central and peripheral nervous systems, with a particularly rich expression within small-diameter thinly myelinated and amyelinated sensory Aδ/C neuronal fibres. These fibres densely innervate the cardiovascular system, in particular the arteriolar side of the vascular tree. CGRP is a 37 amino acid peptide, discovered in 1982 (Amara et al., 1982) and is a member of the calcitonin family of peptides, which also comprises calcitonin, amylin, adrenomedullin (AM) and intermedin/adrenomedullin 2. CGRP is thought to possess a protective role in a wide range of pathologies, including those relevant to the cardiovascular system. Indeed, many have shown cardioprotective (Covino and C Spadaccio, 2011) roles of CGRP and also vasculoprotective roles, especially within the context of systemic hypertension (Smillie and Brain, 2011). However, little is known of how CGRP influences the pathophysiological processes of ageing.

The introduction to this thesis will begin by introducing both the phenomenon of ageing with respect to its socioeconomic burden, how it came to be through an evolutionary biological perspective, and also discussing several of the classical mechanisms that drive such a heterogeneous phenotype, with particular emphasis on those impinging upon the cardiovascular system. The introduction will then present a review of the literature concerning CGRP, including its basic biology, effects on organ systems and how it mediates these effects though specific receptor systems. The central hypotheses and aims of this

thesis will then be discussed, before progressing to subsequent methods and results chapters.

1.1 Ageing

1.1.1 The Ageing Epidemic

The United Kingdom (UK) is facing a looming epidemic with respect to its ageing population. In a 2008 population census, roughly 10 million people aged 65 years or older were living in the UK. This is projected to reach around 15 million by 2033 and by 2050 this population demographic is set to double to around 19 million people. While one-in-six of the population are currently aged 65 and over, by 2050, one-in-four will be. Figure 1 illustrates the spread of the UK population in 2008 *versus* predicted population growth by 2033. It is quite clear from these projections that the population that is set to grow the most is that of the over 65s, with substantial increases in the number of nonagenarians and even those living as long as to receive their centenarial telegrams(Cracknell, 2008).

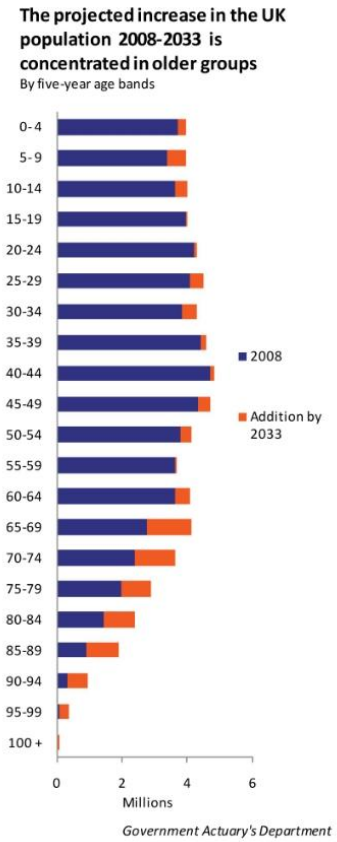

Figure 1: Graph depicting 2008 population demographics and 2033 population projections, divided into five-year age bands (obtained from Richard Cracknell, UK Government's Actuary's Department)

The prospect of an ageing population brings with it real socioeconomic concern. Of great importance is the cost this will impose on the National Health Service (NHS). Currently, the average NHS spending for retired households is nearly double that for non-retired households: in 2007/08, the average cost of NHS services for retired households was £5,200, compared with £2,800 for non-retired. This cost further increases the older one gets, where NHS services are thought to cost around three times as much for someone aged 85 years and older, *versus* those aged between 65 to 74 years. Thus, there is a real need to understand what sort of health problems the older generation is likely to face and how we can begin to tackle these problems.

According to the UK Office for National Statistics, a 2012 consensus revealed that cardiovascular disease was the leading cause of death in England and Wales for men and women combined. This finding is also reflected throughout the European Union and the United States. The main forms of CVD are coronary heart disease, heart attack and stroke. A common feature of these diseases is the development of atherosclerotic plaques within the walls of blood vessels, which can lead to vessel obstruction either by arterial stenosis, or the formation of a thrombus, which can dislodge to obstruct distal vessels. As one ages, the risk of developing some form of CVD substantially increases and, in fact, the single biggest independent risk factor for the development of CVD is getting older. To put this into context, the Framingham score for the risk of coronary heart disease allocates seven points to men between the ages of 70-74, compared with only three for men with high cholesterol and high blood pressure and only two points for those who are smokers or are diabetic (Wilson et al., 1998). Not only does the advancement of age significantly increase the risk of developing CVD, many other age-associated degenerative diseases such as dementia have been shown to have a substantial vascular component in their aetiology. With these factors in mind, there is a clear need to understand the basic biological mechanisms of ageing with reference to the cardiovascular system, in order to be able to develop new therapeutics that can either extend lifespan or, more importantly, compress end of life morbidity.

Ageing is almost always not an ‘isolated’ condition and high blood pressure, or hypertension, is very often comorbid with advancing age. Hypertension is diagnosed in the UK as a persistently high blood pressure of 140/90 mmHg and is broadly classified as two distinct types, depending on its aetiology. These are referred to as primary (or ‘essential’) and secondary hypertension. Secondary hypertension is much less common than essential hypertension, affecting around 5% of hypertensive patients and its genesis is dependent on

another known medical condition that drives the increase in blood pressure. This may be as a result of endocrine dysfunction, cardiac and renal diseases or certain tumours, such as phaeochromocytomas. Therefore, the treatment of secondary hypertension must be first targeted to the underlying condition in order to bring about a reduction in blood pressure(Rimoldi et al., 2014). In contrast to this, essential hypertension is largely idiopathic, having no obvious underlying cause. Despite this, there are various risk factors that can contribute to increased likelihood of developing primary hypertension. These include genetic factors, hyperactivity of the sympathetic nervous system and hyperactivity of the renin-angiotensin-aldosterone axis. Lifestyle factors such as high alcohol intake, smoking, high salt intake, little or no exercise and stress can also significantly increase one's risk of developing the disease. However, the single biggest independent risk factor for the development of essential hypertension (and other CVDs as a result) is increased age (Carretero and Oparil, 2000).

As previously discussed, in the UK, stroke and coronary heart disease are the major causes of death in people aged 65 years and older. The most common treatable risk factor for these is hypertension. Advancing age as a risk factor for hypertension has been known for some time. Data obtained from the Framingham Heart Study demonstrated that systolic blood pressure shows a continuous increase from the age of 34 onwards, until death(Fleg and Strait, 2011). Diastolic blood pressure, on the other hand, is much more variable, increasing until the fifth decade and slowly decreasing from around the age of 60 to at least 84 years of age. This divergence in systolic *versus* diastolic pressure leads to a substantially increased pulse pressure with age, which is thought to be a powerful predictor of future adverse events.

Whilst hypertension is clearly definedby pathophysiological elevations in blood pressure, it is also associated with widespread vascular inflammation and remodelling. The inflammation associated with hypertension overlaps greatly with that associated with the ageing process, albeit to an exaggerated extent and this will be discussed in subsequent sections.

1.1.2 Evolutionary Theories of Ageing

Ageing is arguably the most familiar yet least well-understood aspect of human biology. It is broadly defined as the time-dependent functional decline in the capacity of the body to maintain proper homeostasis. To ask how or even why ageing occurs requires an understanding of the evolutionary biology of ageing and the question remains as to why such an apparently disadvantageous trait is allowed to be perpetuated in the face of the widely-accepted Darwinian theory of natural selection.

The most common hypotheses for the evolution of ageing processes work at the level of group selection, stating that age-related decline leading to death prevents population overcrowding and facilitates the progression of further evolution by ensuring proper population turnover. These hypotheses echo Weissmann, who is noted to have said "Worn out individuals are not only valueless to the species, but they are even harmful, for they take the place of those which are sound."(Weissmann, 1891). Thus, ageing in this manner is said to be "programmed", largely for utilitarian purposes. There are many reasons as to why this idea is flawed. Firstly, data obtained from organisms living in the wild show that many individuals rarely survive to ages where age-associated deterioration actually becomes apparent; most organisms succumb to extrinsic mortality (such as predation or environmental challenges such as cold or starvation) before senescence becomes a factor. This means that there is no pressure for ageing to "weed out" older individuals. Secondly, there'd be little opportunity for evolution to select genes specifically 'for' ageing, given natural selection would not normally see them in action. In addition, these hypotheses stress that ageing benefits the group rather than the individual and this is flawed. Traits that benefit the good of the group are open to the problem of individuals being dishonest. For example, individuals possessing mutations that inactivate or lengthen the ageing programme would be favoured over those who succumb to death earlier and this phenotype would be expected to spread throughout the group, thus diminishing the original effect of ageing itself(Kirkwood, 2002). Even though the argument of programmed ageing seems compelling, further research performed in *C. elegans* has shown that no single gene mutation or combination of mutations has produced a phenotype that completely abolishes ageing altogether, despite being able to extend longevity. This suggests that programmed ageing either does not exist or is *incredibly* robust. Thus the question remains, if ageing is not programmed, how has it evolved? There are three main evolutionary theories of ageing and these shall now be discussed in turn.

1.1.2.1 Mutation Accumulation

Peter Medawar put forward one of the first modern explanations as to why ageing occurs in a 1952 lecture at University College London, entitled "An Unsolved Problem of Biology". In this, he suggested that the force of natural selection tended to wane the longer an individual lived. For instance, if an individual possessed a trait as a result of random germline mutation that made it likely for it to die before it reached reproductive age, this trait was likely to be subjected to a very large negative selection pressure as the 'fatal gene' would be unlikely to be passed on to subsequent generations. If, however, an individual possessed a lethal trait that only manifested itself *after* the individual was likely to have reproduced, then it could conceivably be selected for, provided the individual looked after its progeny well. An example of this model would be human sufferers of Huntingdon's disease – such an apparently disadvantageous disease (with respect to survival, at least) can be propagated throughout the gene pool because its lethal phenotype strikes individuals aged between 35-44 years of age, when most individuals will have already reproduced and have passed the faulty gene on. Medawar postulated that such late-acting alleles might accumulate over time as natural selection is weak to act against them and such mutations may facilitate negative phenotypes. Thus, any individual fortunate enough to escape extrinsic mortality would feel the full force of these mutations as "ageing" (Medawar, 1952).

1.1.2.2 Antagonistic Pleiotropy

In keeping with Medawar's theory of mutation accumulation is that of antagonistic pleiotropy, proposed by George Williams in a 1957 paper entitled "*Pleiotropy, Natural Selection, and the Evolution of Senescence*" (Williams, 1957). Genetic pleiotropy refers to the ability of a gene to exert different effects at different stages of an individual's lifespan. Williams proposed that ageing was caused by genetic pleiotropy, where genes 'for' ageing were selected due to their ability to produce a beneficial effect from youth until reproductive age was reached, in spite of their ability to produce deleterious effects in old age, when selection pressures are negligible. To illustrate, one can imagine a gene that enhances calcification processes to be beneficial in youth as the skeletal system develops, thus enhancing fitness. However, once the skeleton is fully developed, such a gene may become deleterious in as much as it may promote aberrant calcification of other tissues, such as those of the cardiovascular system, which is unwanted and would subsequently lower fitness. However, such a gene would evade negative selection due to its beneficial nature in early life.

1.1.2.3 Disposable Soma Theory

Whilst the ideas of Medawar and Williams detail most of the current thinking about the genetic evolutionary theories of ageing, there is a severe lack of empirical evidence to back these up. A more testable theory of ageing was proposed by Thomas Kirkwood in 1977 (Kirkwood, 1977), in which he put forward the idea of the 'disposable soma'. In this, he envisioned an energetic "trade-off" between germline and somatic cells, where the individual should optimally allocate its resources between maintenance of the body (the soma) to ensure fitness of the individual, increasing chances of reaching reproductive age, and the germline to ensure protection of the gametes and thus increasing their fitness. As metabolic resources are finite, clearly spending resources on one reduces the amount one can spend on the other and thus a trade-off is established. The idea of the disposable soma is certainly very relevant when we examine some of the molecular mechanisms that are associated with the ageing process.

1.1.3 The Ageing Vasculature

The physiology of the ageing process is incredibly complex: a multitude of factors can contribute and interact to produce the phenotype and not all of these factors need be involved to produce the end result. Thus, the phenotype of the ageing vasculature can be quite heterogenous depending on a variety of factors, particularly in humans where the total combinations of environmental interactions one may encounter over time to influence vascular health can be numerous. However, whilst complex, there is a broad group of hallmarks that appear to be well conserved throughout this process and these will be discussed in turn below. These hallmarks can largely be grouped under the single idea of a type of low-grade, sterile injury known as “inflammaging”, first coined by Franceschi in a 2003 paper investigating the biological characteristics of centenarians (Franceschi and Bonafè, 2003). In humans, the effect of ageing on the arterial tree is very heterogeneous, depending on which vascular bed is studied. Macroscopically, however, the effects of age on the larger elastic arteries appear to include increased collagen retention, covalent cross-linking of collagen fibres, elastin fibre fragmentation and calcification and reduction in elastin content. There are also significant changes to endothelial function, wall thickness media to lumen ratio, and arterial stiffness. The smaller resistance arteries experience similar changes, though less emphasis is placed on changes to the proteins of the extracellular matrix, given their low expression and relevance within these vascular networks. Endothelial dysfunction, however, is a key problem within the microvasculature as the organism ages. As previously mentioned, the ageing vascular phenotype can be quite multifaceted. With this in mind, the following section will discuss common molecular mechanisms that appear to drive the ageing phenotype, with special reference to those that are related to the theory of “inflammaging”.

1.1.4 Molecular Mechanisms of Vascular Ageing

1.1.4.1 Circulating Cells and Soluble Factors

Moving from the luminal compartment of the vessel through the vascular wall, it is possible to identify several hallmark changes that occur with advancing age in both humans and animal models. Accumulating evidence suggests that a type of circulating stem cell, the endothelial progenitor cell (EPC), can contribute to vascular repair and re-endothelialisation. In settings of vascular damage, such as ischaemia, EPCs are mobilised from the bone marrow into the circulation, whereby they can migrate to regions of damage to initiate the repair process. Reductions in circulating EPC counts have been identified as an independent predictor of future cardiovascular events (Werner et al., 2005). In normal, 'healthy' ageing, a reduction in circulating EPC populations has not been identified. Rather, EPCs isolated from 'old' patients (mean age 61 years) were less *functional* than those isolated from 'young' patients (mean age 25 years). For example, the old EPCs were less capable of proliferation, migration and survival (Heiss et al., 2005). Interestingly, it has been shown that transplantation of EPCs from young, non-atherosclerotic apolipoprotein E (ApoE) KOmice into elderly ApoEKOrecipients maintained on a high cholesterol diet was sufficient to reduce atherosclerotic plaque size in these animals (Rauscher, 2003). Such a reduction in functionality with age may be partially ascribed to telomere attrition (described in more detail in a subsequent section), leading to initiation of cell cycle arrest – EPCs harvested from older males have been shown to have telomeres around 20% shorter than those taken from younger adults (Kushner et al., 2009). Further to this, EPCs from older patients have been shown to have a significant reduction in telomerase activity resulting in a proapoptotic EPC phenotype (Kushner et al., 2011). Whilst young EPCs naturally appear to have quite high expression of antioxidant defense enzymes, He *et al* have shown that aged EPCs are less capable of tolerating oxidative damage through a reduction in expression and activity of the enzyme glutathione peroxidase-1 (GPx-1) (He et al., 2009). This, coupled with an increase in proinflammatory circulating factors with age, such as angiotensin II (ATII), which are known to stimulate reactive oxygen species (ROS) production in EPCs means that EPCs in the ageing organism may be more susceptible to oxidative damage. Indeed, blockade of AT1Rs in coronary artery disease patients has been shown to significantly increase the number of circulating EPCs available (Endtmann et al., 2011). Therefore, EPCs derived from older patients may be less capable of mediating endothelial repair and maintaining vascular health, as they are more likely to succumb to cellular senescence.

Ageing is also associated with an increased bioavailability of advanced glycation endproducts (AGEs), which are detectable in plasma. AGEs are formed by the irreversible post-translational modification of proteins, lipids and nucleic acids by glucose *via* a chain of spontaneous, non-enzymatic chemical reactions, known as the Maillard reaction. These species are perhaps best known for their role in driving the pathogenesis of diabetes, secondary to the hyperglycaemia that is characteristic of diabetic patients(Simm, 2013). However, owing to the spontaneous nature of AGE formation, time is a critical element in their formation and over a person's lifespan, AGE bioavailability has been seen to increase, either by increased rate of formation or by increased stability of glycated macromolecules (Farmer and Kennedy, 2009). Common examples of AGE-modified proteins are those that comprise the extracellular matrix and also plasma albumins, having the longest half-life of all circulating proteins andtherefore are consequently ripe targets for glycation(Garlick and Mazer, 1983). At first glance, it may appear that the formation of AGEs is largely random, leading to the formation of swathes of modified moieties, each with very different structural characteristics. However, it has since been discovered that there is areceptor that recognises AGEs, known as the receptor for advanced glycation endproducts, or rAGE. rAGE is a multiligand receptor of the immunoglobulin superfamily of cell-surface molecules and is expressed widely throughout the vascular system, including on endothelial cells (EC), vascular smooth muscle cells (VSMC) and leukocytes(Farmer and Kennedy, 2009). Activation of rAGE by its ligands is classically considered to be proinflammatory through several distinct mechanisms. Commonly, rAGE activation has been shown to reduce nitric oxide (NO) bioavailability within ECs either through direct quenching (Bucala et al., 1991) or through deactivation/lowering of expression of NO synthases (Rojas et al., 2000). Furthermore, AGEs have been demonstrated to induce oxidative stress *via* increased NADPH oxidase (NOX) activity (Coughlan et al., 2009), which in turn may activate pro-inflammatory transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) through ROS production(Bierhaus et al., 2001).

Circulating autacoids may also markedly influence the ageing process. Two of the most influential circulating factors in driving the vascular ageing phenotype are ATII and endothelin (ET). Local, bioactive concentrations of ATII have consistently been shown to be elevated in both ageing humans and laboratory animals (Wang et al., 2013). This is as a result of increased transcription, translation and activity of the angiotensin converting enzyme (ACE), which is responsible for the cleavage of angiotensin I to the bioactive product ATII. (Michel et al., 1994; Wang et al., 2003; 2007a). In tandem with increased bioavailability

of the ligand ATII in aged subjects, there is also increased expression of the so-called "inflammatory" angiotensin receptor subtype, AT1R (Wang et al., 2013). Furthermore, messenger RNA (mRNA) expression of the vasoconstrictor peptide precursor, pro-ET and its activating protein, endothelin-converting enzyme-1 (ECE-1) has been shown to be elevated in the aged aorta (Goel et al., 2010). This was associated with an increased level of exocytosis of ET from the EC. Furthermore, increased ET receptor mRNA expression has been documented in ageing animals, in conjunction with an increase in the total binding capacity of the ligand to its receptor, as determined by radioligand binding (Ishihata and Katano, 2006). In summary, it is evident that the bioavailability and activity of both ATII and ET are elevated with age. Whilst acutely this may promote vasoconstriction, the effects of long-term elevation of these peptides may have more serious repercussions, owing to the signalling pathways both molecules converge upon. These pathways can promote the induction of endothelial dysfunction by aberrant ROS generation, for example, or can promote vascular remodelling through their effects onVSMC activity, causing them to proliferate and migrate in a pathophysiological manner(Savoia et al., 2011).

Cytokines are another group of circulating factors that are differentially regulated as part of the ageing process. Of these, those that have consistently been shown to change and contribute to age-associated pathologies and increased risk of mortality in humans are members of the acute phase protein family, TNFα. A study examining a large cohort of mixed-gender centenarians showed that soluble TNFα plasma concentrations more than doubled from 1.4 pg/ml in 18-30 year-old healthy controls to 3.7 pg/ml in centenarians(Bruunsgaard et al., 1999). Whilst statistically significant, this is a reasonably modest increase that is far from that associated with inflammation seen in acute infection, reflecting the widely held tenet that ageing is associated with chronic *low-grade* inflammation. Plasma levels of TNFα also correlated positively with increased levels of interleukin-6 (IL), the soluble TNFα receptor sTNFR-II and C-reactive protein (CRP). sTNFR is an endogenous inhibitor of TNFα activity, yet rises in circulating levels of this inhibitory protein appeared to have no effect on the association between high TNFα plasma concentrations and increased risk of dementia and/or atherosclerosis in the centenarian group (Bruunsgaard et al., 1999). *In vitro*, peripheral blood mononuclear cells (PBMC) isolated from elderly donors had increased TNFα secretion under basal conditions *versus* younger donors (Myśliwska et al., 1999) and following viral stimulation (Saurwein-Teissl et al., 2000). In a mouse model of neuroinflammation following intracerebroventricular injection of lipopolysaccharide (LPS), there was an age-dependent increase in mortality that

was associated with increased brain and plasma concentrations of TNFα over time and increased cerebral microglial/macrophage activation (Kalehua et al., 2000).

Increased bioavailability of TNFα within the circulation may increase the likelihood of it binding to one of two cognate receptors: TNFR1 (CD120a) and TNFR2 (CD120b), both expressed by ECs – one of the first cell types to 'sample' circulating cytokines. Signalling from these receptors is initiated by ligand binding and subsequent recruitment of a wide array of intracellular adaptor proteins to the activated receptor. These adaptor proteins have been linked to two common signalling convergence points, namely activation of the transcription factors activator protein-1 (AP-1) and NF-κB(Sedger and McDermott, 2014). NF-κB is often thought of as being the 'master regulator' of inflammation. It has been identified as the transcription factor most associated with mammalian aging, based on patterns of gene expression(Adler et al., 2007). In quiescent cells, NF-κB transcriptional activity is usually low, owing to the inhibitory sequestering effect on the complex by cytosolic inhibitors of κB (IκB). Following activation of the EC by TNFα, IκB proteins become phosphorylated at critical serine residues that allows for polyubiquitination of the protein and subsequent targeting for proteosomal degradation, thus releasing the suppressor effect it has on NF-κB(Csiszar et al., 2008). This process can happen in HUVEC as rapidly as 15 minutes following TNFα stimulation and NFκB activity in HUVEC following stimulation by this ligand is maximal at 15-60 minutes and is sustained for >20h. Once IκB proteins are degraded, NFκB is free to translocate to the nuclear compartment where it can initiate gene transcription. Binding of the transcription factor to DNA response elements drives pro-inflammatory gene expression and typically promotes long-term cell surface expression of cell adhesion molecules, such as E-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1)(Johnson et al., 1996; Spiecker et al., 2000). Other cytokines that are thought to be found at increased levels in the plasma and contribute to increased risk of frailty are IL-6 and CRP(Harris et al., 1999; Alley et al., 2007).

1.1.4.2 Vascular Cells, Telomere Attrition and Cellular Senescence

The 2009 Nobel Prize in Physiology or Medicine was awarded to three eminent scientists: Elizabeth Blackburn, Carol Greider and Jack Szostak for their seminal work examining how DNA can be faithfully copied during cell division without loss of information – addressing the so-called 'end replication problem'. The end replication problem refers to how the 5' ends of DNA strands cannot be replicated during mitosis, meaning that with each cell division, the length of each daughter chromosome is shortened, which should result in progressive loss of genomic information. However, in most cases this is shown not to occur. The reason this does not occur is due to extended repeat regions of nucleotide sequences (GGGTTA in vertebrates) at the end of each chromosome, known as telomeres, which are highly conserved throughout the tree of life(Zhao et al., 2014). As an analogy, the telomeres are often thought of as the protective capping at the end of a shoelace to prevent damage to the lace itself. With each round of mitosis, the telomeres shorten and cells can tolerate this attrition to an extent. However, when telomeres shorten to a certain threshold, this signals the arrest of cellular division and cells become senescent. This event helps explain a well-known event known as the Hayflick phenomenon, first described by Hayflick and Moorhead in 1961 (Hayflick and Moorhead, 1961). When cells are isolated and serially subcultured *in vitro*, they can only undertake a certain number of cell divisions before they stop dividing, a point known as the 'Hayflick limit' or 'Hayflick number'. This happens to most cells in culture and many cells *in vivo* and can be attributed to excessive telomere shortening. As this shortening can be described as a time-dependent effect, where 'older' cells are more likely to have shortened telomeres, there is a case to be made for telomere attrition being an important contributing factor to the ageing process.

What is interesting, however, is that some cells *in vivo* are virtually exempt from telomere shortening and associated cellular senescence. Of particular note are germline cells, which actively express a family of genes that encode proteins required to form a reverse transcriptase enzyme complex known as telomerase, which works to actively extend the length of the telomere and replace eroded nucleotide repeats(Zhao et al., 2014). The fact that germline cells are given this senescence-avoiding advantage and yet somatic cells are exempt from it is a strong supporter of the disposable soma theory of ageing – preferentially maintaining a healthy germline in order to pass on one's genes to the next generation. Whilst somatic cells might seem to be hindered by lacking expression of this enzyme and thus risking falling into senescence, this might be somewhat advantageous. Uncontrolled

and unlimited cellular division could result ultimately in the excision of all telomere sequences from the daughter DNA strand and eventual excision of important genomic information from the ends of each chromosome. This could result in an abnormal cellular phenotype that senescence actively protects against, namely oncogenesis. However, it has become evident that not all cell types fall into senescence, for example cardiomyocytes, as these cells are largely mitotically-inactive. Whilst telomere attrition is a significant contributory factor the initiation of cellular senescence, it is worth noting that other stimuli can induce this phenotypic switch in the absence of shortened telomeres. Factors that contribute to genomic instability (for example direct damage to nucleotide bases or formation of double-strand breaks or interstrand crosslinking, for instance) can also induce telomere-independent senescence. Most factors that contribute to the initiation of cellular senescence mediate this process *via* engagement of p53/p21 or p16/retinoblastoma protein tumour suppressor pathways as the final effectors of this senescence programme(Zhao et al., 2014).Despite being beneficial in protecting against tumorigenesis, cellular senescence does not come without its costs, highlighting an antagonistically pleiotropic role for this mechanism. An increased ratio of senescent to non-senescent cells with ageing can be detrimental, owing to the so-called 'senescence-associated secretory phenotype' or 'SASP' (Kuilman et al., 2010). This will be discussed in subsequent sections.

Both of the major cell types resident to the vascular wall have been shown to be capable of falling into senescence both *in vitro* and *in vivo*. Human studies have shown that shorter telomeres have been shown to correlate strongly with the lifespan of vascular cells and, as an extension, those with short telomeres are more likely to develop coronary artery disease and also have stiffer arteries, as measured by higher pulse pressures and pulse wave velocities (Benetos et al., 2001). Even in healthy middle-aged individuals with no apparent overt risk of CVD, shorter telomeres correlated with increased circulating inflammatory markers, such as CRP and IL-6 (Bekaert et al., 2007). In hypertensive patients, short telomeres are associated with increased prevalence of carotid artery atheromas (Benetos et al., 2004). Additonally, normotensive *and* hypertensive patients who were found to have an increased renin to aldosterone ratio (indicative of increased renin-angiotensin-aldosterone activity) were shown to have shorter telomeres (Vasan et al., 2008), hinting that ATII can be a potent inducer of telomere attrition even in the absence of elevated blood pressures.

In patients with atherosclerosis (a disease highly comorbid with increased age), populations of senescent cells have been shown to be elevated in the affected vasculature compared to

other vessels obtained from the same individual. This staining is largely restricted to cells of the tunica intima, indicating the presence of senescent ECs (Minamino et al., 2002). In this study, senescent aortic ECs were shown to have a reduction in endothelial nitric oxide synthase (eNOS) expression and activity (potential markers of endothelial dysfunction, discussed below), which led to an increase in cellular ICAM-1 expression, a key molecule involved in leukocyte (specifically, monocyte) firm adhesion. This phenotype was rescued by introducing telomerase enzyme into the cells, which presumably had a positive effect on telomere length (Minamino et al., 2002). A number of studies have shown that many of the changes in senescent vascular cell behaviour are consistent with known changes seen in age-related vascular diseases, suggesting a critical role of cellular senescence in driving age-associated vascular pathophysiology. For instance, NO bioavailability is markedly reduced in senescent ECs, which may lead to endothelial dysfunction (discussed in a later section). This occurs both in relation to basal NO production (Sato et al., 1993) and shear-induced NO production (Matsushita et al., 2001) and the mechanism behind this is thought to be related to an overall reduction in eNOS protein expression in conjunction with a decrease in Akt-mediated phosphorylation of critical serine residues within eNOS that positively regulate its activity (Hoffmann et al., 2001). Senescent ECs also decrease prostacyclin production (Nakajima et al., 1997) and increase plasminogen activator inhibitor-1 expression (Comi et al., 1995) as they become senescent, promoting a vasoconstrictor and prothrombogenic environment. Further to this, interactions between monocytes and ECs are significantly enhanced when ECs become senescent (Maier et al., 1993).

It is clear that senescent ECs are present within arteries affected by atherosclerosis. Senescent VSMCs have also been identified within the intimal but not medial layers of the vessel (migrating VSMCs are a common feature of atheroma) (Minamino et al., 2003). Senescent VSMCs synthesise and secrete an array of pro-inflammatory factors, which together comprise the so-called ‘SASP’, mentioned previously. Such releasable factors may include cytokines and cell adhesion molecules, such as IL-1, IL-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1). The SASP phenotype in these cells has been shown to be dependent on increased extracellular signal-related kinase (ERK) activity (Minamino et al., 2003).

1.1.4.3 Endothelial Dysfunction

It was once thought that the endothelial monolayer served only as a structural barrier, separating the cells of the blood compartment from those located within the vascular wall. With time, we have come to appreciate that the endothelium is in fact a key regulator of vascular tone, structure and function as well as being a barrier. This polymodal function is controlled by a host of endothelial-derived autacoids, including prostacyclin and endothelial-derived hyperpolarising factor but is perhaps best ascribed to the activity of NO. Seminal experiments in the 1980s by Furchgott and Zawadzki highlighted the obligatory role of the endothelium in the generation of VSMC relaxation in response to acetylcholine: rabbit aortae with intact endothelia produced a dose-dependent relaxation to acetylcholine, whilst those vessels with the endothelium mechanically or chemically removed lost this vasodilator response (Furchgott and Zawadzki, 1980). However, it wasn't until 1987 that the identity of this "endothelium-derived relaxing factor" was identified as being NO (Ignarro et al., 1987; Palmer et al., 1987). Within the EC, NO is produced mainly by eNOS via the metabolism of L-arginine to L-citrulline, producing NO as a by-product. eNOS activity can be influenced by the action of various agonists that mobilise intracellular calcium (such as acetylcholine), leading to the binding of calcium to calmodulin, increasing the affinity of this molecule for the enzyme. However, eNOS can also be activated by stimuli independent of long-term elevation in intracellular calcium and one such stimulus important for vascular homeostasis is laminar shear stress(Förstermann and Sessa, 2012). Continuous flow of blood parallel to the vessel wall is an important stimulus of eNOS phosphorylation at a critical residue for its activation: Ser^{1177}. Phosphorylation of this amino acid residue increases the activity of eNOS severalfold (Fleming and Busse, 2003). The constant release of NO from the endothelium promotes a vascular environment that is vasodilatory, anti-coagulative, anti-proliferative and anti-inflammatory (Förstermann and Sessa, 2012). Any negative alteration in NO bioavailability can severely impair vascular function by shifting its phenotype to that of a vasoconstrictor, pro-coagulative, proliferative and pro-inflammatory system. Such a decrease in NO bioavailability is often described as "endothelial dysfunction", which is diagnosed in humans by a reduced ability of the peripheral arteries to produce an endothelial-dependent dilatation (EDD) in response to chemical or mechanical stimuli(Seals et al., 2011).

Endothelial dysfunction is a phenomenon that increases in prevalence and severity with age, as characterised by EDD (Seals et al., 2011). Reduction in EDD does not appear to be mediated by a reduction in sensitivity of vascular smooth muscle to NO, as vasodilator

responses to NO donors are unchanged (Taddei et al., 1995). Data suggest that age-associated endothelial dysfunction is mediated by a decrease in NO bioavailability. This may be due to a decrease in NO synthesis or an increase in its removal, or both. However, the exact contributions of altered NO production compared with its removal during endothelial dysfunction are yet unknown and, indeed, they may vary considerably between individuals. Despite consistent observation that NO bioavailability is reduced with advancing age, data in animal models are inconsistent when examining arterial eNOS protein expression and "active" eNOS expression using phospho-specific antibodies to Serine^{1177} – reports have shown increased, decreased and unchanged levels of expression and activity (Cernadas et al., 1998b; Yang et al., 2009; Rippe et al., 2010). Such discrepancies might be attributable to species and strain differences, making results difficult to interpret. Furthermore, differences in age of animals used are likely to have a major impact on results. In healthy humans however, studies in the brachial artery have shown increased eNOS expression and activity in ECs of older versus younger patients, accompanied by the hallmark reduction in EDD (Donato et al., 2009). Therefore, this increase in eNOS activity might be a compensatory response to diminished levels of total NO.

1.1.4.4 Oxidative Stress

Another important factor that may contribute to a reduction in NO bioavailability in the ageing vasculature is that of an increased production of ROS. The evolution of aerobic respiration in eukaryotic cells was clearly critical for the development of complex, multi-cellular organisms. However, oxygen metabolism comes at a price, owing to its capacity to be transformed into toxic metabolites such as superoxide (O_2^-), hydroxyl anion (OH^-) and hydrogen peroxide (H_2O_2). Cells constantly produce ROS as a result of oxidative phosphorylative processes to generate ATP within the mitochondria and, as a result, they have evolved multiple mechanisms to defend themselves against the deleterious effects of aberrant ROS production(Puca et al., 2013). This balance between ROS production and defense is often described as the redox state of the cell. Vascular cells are capable of producing ROS from sources other than the mitochondria, such as from cytochrome P450, xanthine oxidase, the NOXes and also from uncoupled NO synthase (Bachschmid et al., 2012). NO synthase enzymes can become uncoupled under reduced availability of the substrate, L-arginine, or its essential co-factor tetrahydrobiopterin (Landmesser et al., 2003). Uncoupling of the enzyme leads to a switch from NO production to superoxide production. This direct suppression of NO formation is accompanied by an ability of superoxide to react rapidly with remaining NO to form the highly reactive nitrogen species, peroxynitrite ($ONOO^-$), thus further driving down NO bioavailability by a quenching mechanism. Generation of ROS can further feedback onto the NOS enzyme by oxidising tetrahydrobiopterin, thereby causing it to be uncoupled from the enzyme and initiating a positive feedback circuit of dysregulated ROS formation.

In tandem with a propensity to develop endothelial dysfunction with increasing age is an increased incidence of oxidative stress. Almost a century ago it was noted that animals with high metabolic rates had shorter lifespan, though the reason for this was unknown. In the mid-1950s, however, Denham Harman proposed a “free-radical theory” of ageing that stands today as one of the most convincing arguments to explain which mechanisms govern the ageing process. Harman hypothesised that cells had the capacity to generate endogenous oxygen radicals whose action resulted in a pattern of cumulative damage to cellular components (Harman, 1956). As discussed previously, ROS are generated as a by-product of mitochondrial oxidative phosphorylative processes that govern ATP synthesis. Movement of electrons down the mitochondrial electron transport chain can result in the donation of electrons to molecular oxygen to form superoxide radical. As the production of

superoxide is non-enzymatic, the rate of its formation from the mitochondria is directly related to metabolic rate (Hou et al., 2011; Dai et al., 2012). As superoxide is relatively incapable of crossing plasma membranes, mitochondrial-derived superoxide is often retained within this compartment and is therefore allowed to react with mitochondrial proteins and DNA, leading to mitochondrial dysfunction. Mitochondrial dysfunction can promote malfunction of the respiratory chain and an even greater production of superoxide (Xin et al., 2003). As already mentioned, other sources of ROS exist throughout the cell but perhaps the most interesting source of free radicals emanates from the NOX family of enzymes. The NADPH oxidase was first described in phagocytic cells, where it plays a pivotal role in regulating the innate immune response to pathogen via the generation of a large burst of superoxide (the "oxidative burst") during the process of phagocytosis(Cave et al., 2006). It has since been discovered that other NOX isoforms exist within cells of the cardiovascular system and indeed it is thought that they represent the largest source of free radicals in the vasculature. This is an intriguing discovery, given that the apparent sole function of the enzyme family is to generate ROS – indicating that generation of free radicals from this source is likely to have some form of evolutionary benefit and therefore may play an important physiological role (Cave et al., 2006). Several members of the NOX family of enzymes have been detected in vascular cells. NOX1 is expressed in smooth muscle cells, and to a lesser extent in ECs. NOX2 is found in ECs and adventitial fibroblasts, whilst NOX4 is far more ubiquitously expressed throughout all levels of the vascular wall (Takac et al., 2012). It has been shown that old rats have an increased capacity to generate NOX-derived superoxide anion when compared with young rats and this increase in activity is accompanied by an elevation in protein expression of NOX subunits (Oudot et al., 2006). Furthermore, certain NOX isoforms (particularly NOX2) can be stimulated by pro-inflammatory cytokines, such as TNFα, which are known to be upregulated with advancing age (Bruunsgaard et al., 2000). However, others hypothesise that the xanthine oxidase enzyme is a more important source of ROS in the ageing cardiovascular system (Vida et al., 2011).

Together with an increased likelihood of ROS formation within vascular cells to induce a pro-oxidant environment with age, there is also an increased likelihood for these cells to have an impaired oxidant handling system. The major removal mechanism for superoxide anion is by the superoxide dismutase (SOD) family of enzymes, of which there are three distinct isoforms: the cytosolic copper-zinc SOD (Cu/ZnSOD, SOD-1), mitochondrial manganese (MnSOD, SOD-2) and extracellular SOD (ecSOD, SOD-3). These enzymes function to convert

superoxide to hydrogen peroxide and molecular oxygen (Faraci and Didion, 2004). Despite considerable effort, it is not at this stage clear how the expression of these three SOD isoforms are regulated as part of the ageing process. Other antioxidants include the enzymatic antioxidants catalase, haem oxygenase-1 (HO-1) and glutathione peroxidase (GPx). Non-enzymatic ROS scavengers also exist and include glutathione, urate and some vitamins (van der Loo et al., 2009).

Transcriptional regulators of the oxidative stress response can also be dysregulated as a function of age. The nuclear factor erythroid-2-related factor-2 (Nrf2) is an evolutionarily highly conserved redox-sensitive transcription factor that can be activated by ROS production within the vasculature. Activation of Nrf2 induces the transcription of multiple genes that are involved in ROS detoxification processes or those that have direct antioxidant properties(Baird and Dinkova-Kostova, 2011). Despite increases in vascular superoxide production, ageing rats have been shown to have a dampened Nrf2 response, as assayed by measurement of Nrf2-dependent gene transcription and protein production (including NADPH quinone oxidoreductase-1 (NQO1), ϒ-glutamylcystein synthase and HO-1. The reduction in Nrf2-dependent gene transcription was observed in parallel with an upregulation of NFκB-dependent gene targets (Mármol et al., 2007). Similar results were observed in vascular tissues obtained from the nonhuman primate, *Macaca mulatta* (Ungvari et al., 2011). Further studies investigating the biology of the naked mole rat bolster this hypothesis. The naked mole rat is extremely long-lived in comparison to other rodents, despite having a similar degree of ROS production and a similar intracellular oxidative environment (Lewis et al., 2010). However, the key to this organism's longevity and *healthy* longevity appears to be a consistent high level of Nrf2 activity, even into old age. This animal is more resistant to oxidant-induced apoptosis of vascular cells and also DNA fragmentation (Labinskyy et al., 2006). Therefore, maintenance of Nrf2 activity could represent a viable target in preventing the onset of age-associated vascular inflammation secondary to dysregulated redox balance. All things considered, it is widely accepted that as an organism ages, its ability to handle oxidative stress declines and this translates to an inflammatory phenotype as a result. Figure 2 illustrates common signalling pathways that occur in the EC as a function of ageing, with specific reference to processes influencing endothelial redox balance.

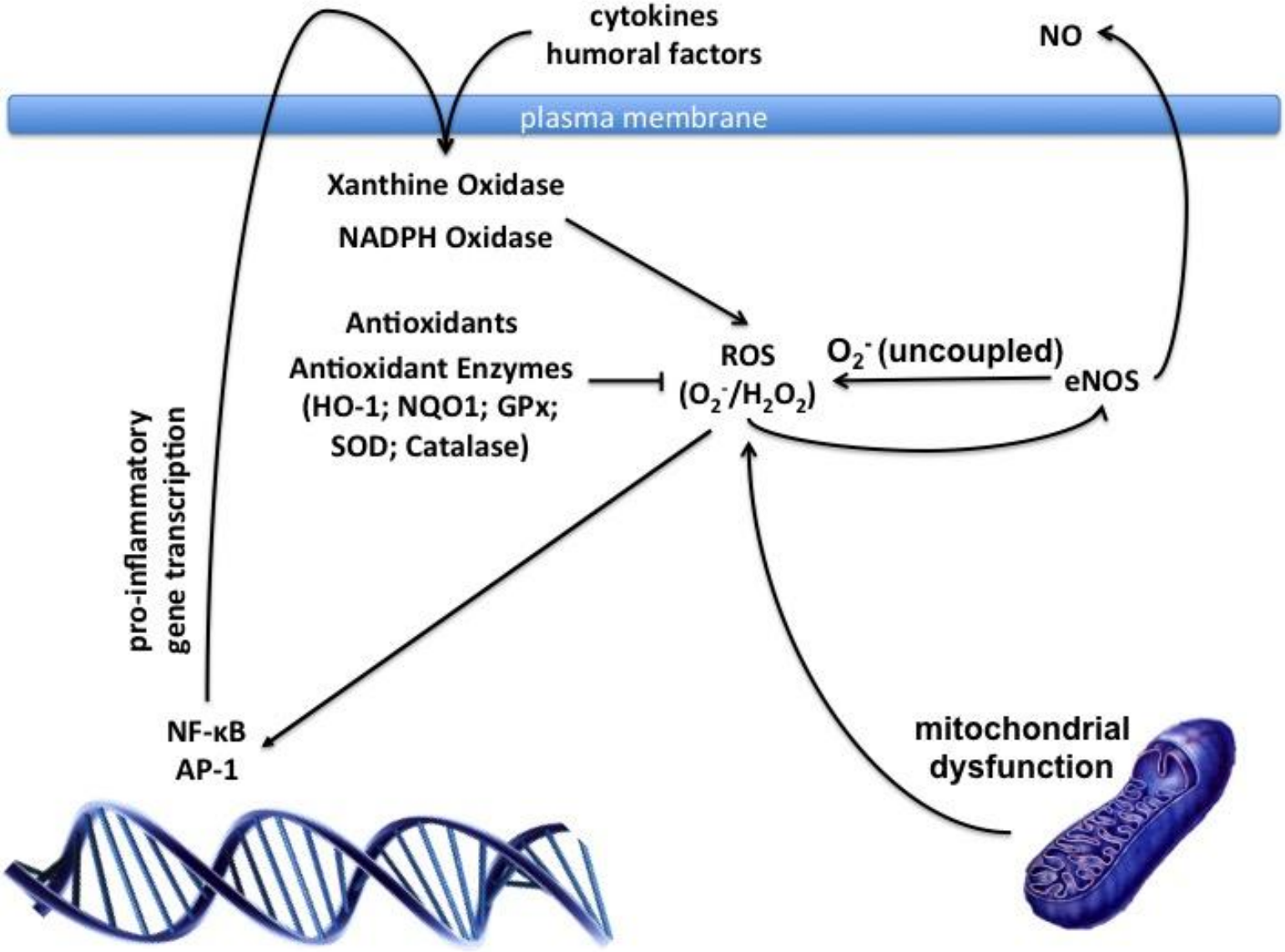

Figure 2: Signalling schematic denoting typical pro- and anti-oxidant pathways important in mediating age-associated endothelial dysfunction. ROS: reactive oxygen species; O_2^-: superoxide; H_2O_2: hydrogen peroxide; NO: nitric oxide; HO-1: haem oxygenase-1; NQO1: NAD(P)H dehydrogenase quninone-1; GPx: glutathione peroxidase; SOD: superoxide dismutase; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; AP-1: activator protein-1.

1.1.4.5 Vascular Smooth Muscle Cells

The predominant cell type to populate the medial layer of the vascular wall is the VSMC. The impaired generation and maintenance of the EC combined with an increased capacity of the vascular smooth muscle to proliferate and migrate significantly influence vessel remodeling with age. Traditionally, VSMCs have either been classed as one of two extremes: 'contractile' or 'synthetic'. However, the current paradigm recognises that VSMCs exist on a spectrum, and can adopt properties of both cellular phenotypes. Contractile smooth muscle cells are largely quiescent and are responsible for maintaining an appropriate level of vascular tone and respond to a range of vasoactive stimuli in order to do so. The phenotype of the VSMC can change, however, in response to environmental cues, whereby the cell moves from a contractile to a synthetic state. As their name suggests, synthetic VSMCs are capable of synthesising and secreting an array of factors including adhesion molecules, matrix metalloproteases, pro-fibrotic molecules and chemokines. Their synthesis and release can substantially contribute towards the formation of vascular inflammation mediated by the VSMC(Davis-Dusenbery et al., 2011). Within the aged arterial wall, these factors can impinge upon signalling pathways that may lead to arterial inflammation, stiffening and fibrosis. This may produce arteries that are overall less distensible. Thus, VSMCs at the level of the resistance arteries are less capable of regulating peripheral resistance and therefore blood pressure. Additionally, less distensible VSMCs that comprise the medial wall of the large resistance arteries may contribute to lowered compliance, meaning pressure waves emanating from the heart are more likely to be reflected backwards rather than propagated forwards, leading to increased systolic pressures(Zhu et al., 2012). A significant factor in mediating VSMC dysfunction with age is the increased propensity for cells to cease proliferating and to enter a senescent state, which can significantly alter the phenotype of the cell.

With these factors in mind, aspects of the ageing vasculature and the role of inflammation in driving age-associated cardiovascular pathology will be investigated. This will primarily be investigated within the context of CGRP biology, which will be introduced in the next section of this thesis.

1.2 Calcitonin gene-related peptide

1.2.1 Brief History

Calcitonin gene-related peptide is a 37 amino acid peptide that is primarily expressed by peripheral sensory neurons. Specifically, it is expressed by the small-diameter, unmyelinated and small-diameter, thinly-myelinated C and Aδ nociceptive fibres. These nerves have their cell bodies in the dorsal root ganglia (DRG) and project afferent fibres to the dorsal horn of the spinal cord to transmit nociceptive signals to the central nervous system. They also possess efferent fibres, which innervate throughout the body, with a rich degree of innervation to the cardiovascular system, particularly at the perivascular level. CGRP is also to a lesser extent expressed non-neuronally, although much less is known about these sources of the peptide(Russell et al., 2014).

Evolutionarily, CGRP is fairly well conserved and appears to have an ancient origin among species of the metazoan era, existing before the emergence of deuterostomes, including modern vertebrates. The emergence of the related calcitonin gene did not happen until later, indicating that CGRP might actually be the precursor gene to calcitonin and not vice-versa (Lafont et al., 2007). Despite being relatively ancient, the existence of CGRP was first discovered by humans in 1982 following the observation that rat medullary thyroid cells decreased the production of mRNA encoding the calcium-regulating hormone, calcitonin, in favour of another distinct RNA transcript, which was then christened as CGRP. This initial finding also hinted at the differential expression pattern of calcitonin and CGRP mRNAs. Amara *et al* showed that calcitonin was produced mainly by the parafollicular cells of the thyroid gland, whilst CGRP production was predominant within the hypothalamus (Amara et al., 1982). Until 1984, the production of CGRP had only been observed in rats and it was unclear whether this was a peptide that was synthesised also by humans. Using an antibody raised against a fragment of rat CGRP, Morris *et al* detected a cross-reacting peptide in human medullary thyroid carcinoma biopsies. The isolated peptide was sequenced by mass spectrometry and was found to differ to the rat peptide by four amino acid residues, resulting in an 89% homology between both species (Morris et al., 1984). Shortly after the discovery of human CGRP, an additional CGRP-encoding gene was identified, that was shown to contain sequences highly homologous to exons 3, 5 and 6 of the CALC I gene (CGRP-encoding) but not to exon 4 of the same gene (calcitonin-encoding). The gene was found to be located nearby on the same chromosome of CALC I (chromosome 11 in humans; 7 in mice) but only facilitated the transcription of mRNA for a CGRP-like product, as opposed

to both calcitonin and CGRP (Höppener et al., 1985; Steenbergh et al., 1985). The product of this gene is now recognised as an alternative isoform of CGRP: βCGRP. Thus, CGRP is now known to exist as two distinct isoforms: alpha and beta. Given its similarity and proximity to the CALC I gene, it is likely that the CALC II gene arose through duplication. The structure of the CALC I gene is depicted in Figure 3. CGRP belongs to a much wider family of related peptide hormones that includes calcitonin, CGRP, AM, AM2 (or intermedin) and amylin(Russell et al., 2014). Whilst expressed by very different cell types, these peptides share similarities in their structure and also in the receptor molecules they bind to in order to produce a biological effect.

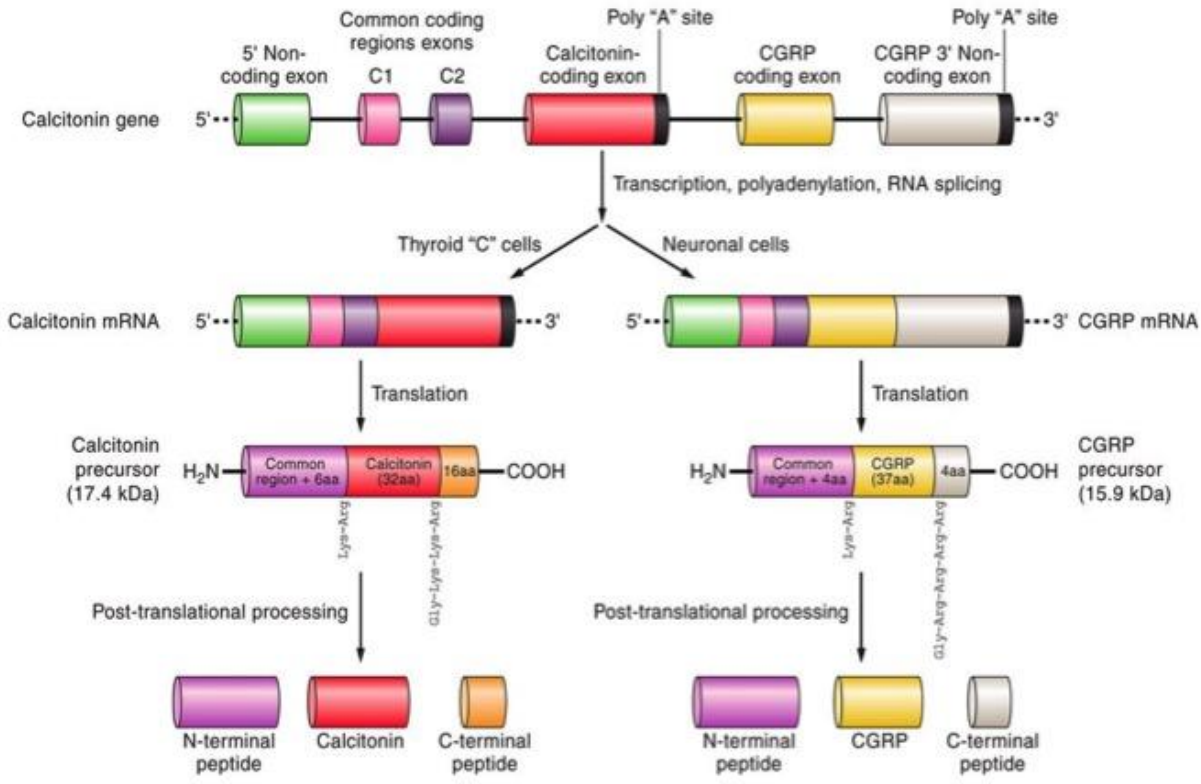

Figure 3: Illustration of the CALC I gene and exons required to encode mRNA for calcitonin and αCGRP, including peptide processing mechanisms. Adapted from (Russell et al., 2014).

1.2.2 Synthesis, structure and release

The regulation of CGRP synthesis is still poorly understood. CGRP synthesis is known to be upregulated in models of nerve damage, such as peripheral axotomy, and it is thought that synthesis of the peptide is enhanced in tissues that are undergoing an inflammatory response (Donnerer and Stein, 1992). This may be linked to local release of nerve growth factor (NGF) from cells such as macrophages and keratinocytes. NGF is vitally important for the growth of sensory nerves and for the maintenance of function of mature nerves (Terenghi, 1999). After depletion of sensory neuropeptides from nerve terminals following treatment with the pungent chilli pepper extract and transient receptor potential vanilloid 1 (TRPV1) agonist capsaicin, NGF is required for the synthesis of new peptide (Donnerer et al., 1992). Interestingly, NGF has been linked to the upregulation of CGRP production within the DRG and promotion of CGRP expression in the spontaneously hypertensive rat (SHR)(Supowit et al., 2001).

As previously discussed, CGRP exists in two distinct isoforms, encoded for by two distinct genes located on the same chromosome (although the chromosome where these genes are located differs between mice and humans). Each isoform shares >90% sequence homology with the other, differing by three amino acid residues in the human (Morris et al., 1984; Amara et al., 1985; Steenbergh et al., 1986). Human αCGRP is comprised of four principal domains (Conner et al., 2002) that have been deduced mainly through nuclear magnetic resonance spectroscopic and other physical techniques. The N-terminus of the peptide is constrained within a ring-like structure owing to a disulphide bond existing between amino acid residues 2 and 7. Removal of the N-terminus of the molecule ablates its activity as an agonist at the CGRP receptor and instead produces a competitive, reversible antagonist at the same receptor. The second domain appears to form an alpha helix in hydrophobic solvents between residues 8 and 18 (Hubbard et al., 1991; Mimeault et al., 1992). The third domain appears to be a little less well-structured, comprising a hinge region that may adopt the form of a β or Y twist (Conner et al., 2002). Residues 28-37 constitute the final domain, which appears to be important in generating an overall high-affinity conformation of the peptide for its cognate receptor, with two critical bends in the peptide structure occurring at residues 29 and 34. Human βCGRP peptide appears to adopt a largely similar molecular topology (Morris et al., 1984). The primary sequences of human, mouse and rat αCGRP and βCGRP are denoted in Figure 4.

After synthesis, CGRP is stored in large, dense-core vesicles within the sensory nerve terminal (Matteoli et al., 1988). Following neuronal depolarization, CGRP is released from the terminal via calcium-dependent exocytosis mediated by classical exocytotic pathways that involve members of the SNARE (Soluble NSF Attachment Protein Receptor) family of proteins (Meng et al., 2007). The release of CGRP from sensory neurons was first demonstrated using capsaicin, which selectively activates sensory neurons (Diez Guerra et al., 1988). However, as capsaicin is an exogenous compound, it is unlikely that the release of CGRP is regulated via this mechanism alone. The main pharmacological target of capsaicin is TRPV1, an ion channel that is also activated by noxious heat (>43°C) and is sensitised by low pH (Caterina et al., 1997). It therefore seems more likely that these sorts of stimuli are more relevant in terms of TRPV1 activation and subsequent CGRP release *in vivo*, especially in areas of inflammation that are likely to have raised local temperature and increased proton concentrations. More recently, it has been shown that mediators of the endocannabinoid system, such as anandamide, are endogenously produced by the body and are capable of binding and activating TRPV1(Fernandes et al., 2012). Aside from TRPV1, other members of the TRP family have been shown to release CGRP following stimulation, such as thetransient receptor potential ankyrin 1 (TRPA1) channel. TRPA1 is often coexpressed alongside TRPV1 in certain populations of sensory neurons. Indeed, it is estimated that 50% of TRPV1-expressing neurons also express TRPA1 (Bodkin and Brain, 2011). The release of CGRP from novel, non-neuronal cells has also been linked to the activation of TRPV1 (Luo et al., 2008a). Undoubtedly, other mechanisms and receptors will be linked to the release of CGRP from various cell types and this warrants future research into this area.

Figure 4: Primary sequences of human, rat and mouse αCGRP and βCGRP. Residues highlighted in blue indicate where residues differ from human αCGRP. Adapted from (Russell et al., 2014).

1.2.3 Metabolism and Removal

Several aspects concerning the regulation of CGRP activity are well understood but it is still unclear as to how the peptide is metabolised following release from the cell. Initial studies investigating the capacity of the neuropeptide substance P (SP) to elicit neurogenic inflammation showed that low concentrations of CGRP were able to potentiate plasma extravasation induced by SP in rat abdominal skin (Gamse and Saria, 1985). Undoubtedly, this result may have been as a result of many different pharmacological effects but it was later suggested that CGRP was capable of inhibiting SP degradation and therefore augmenting its bioactivity by competing for degradation by the same endopeptidase, though the identity of this molecule was not determined (Le Grevès et al., 1985; 1989). The lack of an identified candidate for this effect may be attributed to a false interpretation of data from these groups. Indeed, it is perhaps more likely that the potentiation of oedema formation in this model was due to the potent vasodilator activity of CGRP working in synergy with the potent endothelial junction destabilisation activity of SP (Brain and Williams, 1985). In order to provide some more information as to the type of protease involved in breaking down both CGRP and SP, experiments were performed using purified mast cell tryptase from human lung samples. This study showed that whilst the tachykinins tested were not metabolised by mast cell tryptase, CGRP was hydrolysed by this system with favourable kinetic parameters (Brain and Williams, 1989). Subsequent to this study, the identity of a shared removal enzyme common to both SP and CGRP had been identified as neutral endopeptidase (Katayama et al., 1991). More recently, a proteomic approach has been used to identify novel CGRP fragments in mouse tissue and to reveal the endogenous cleavage sites within the primary sequence of the peptide. Mass spectrometry revealed the presence of 10 endogeously-produced peptide fragments in murine spinal cord homogenates that highlighted two primary cleavage sites *in vivo*: Serine^{17}-Arginine^{18} and Asparagine^{26}-Phenylalanine^{27}. Subsequent biochemical assays identified a role for a metalloprotease in the regulation of CGRP proteolysis, of which insulin-degrading enzyme (IDE) was found to be particular important in processing of the peptide at the aforementioned cleavage sites (Kim et al., 2012). Another enzyme, ECE-1 has also been implicated in the degradation of CGRP and this has been suggested to be of functional importance in murine pulmonary fibrosis (Hartopo et al., 2013).

An alternative mechanism has been proposed to regulate CGRP removal. In the mouse vas deferens, application of the TRPV1 agonist capsaicin was shown to attenuate electrically-induced twitch responses, an effect that was attributable to the release of CGRP from the

sensory nerve terminal. However, following a second exposure to capsaicin, its ability to blunt muscle twitch was markedly reduced, suggesting sensory afferents had depleted their stores of CGRP following the first stimulation. This effect was rescued by pre-incubating the preparation for a short time with exogenous CGRP. The authors suggest that this restoration of function is attributable to reuptake of CGRP into the neuron by an active transport system (Sams-Nielsen et al., 2001; Sheykhzade et al., 2011) Indeed, this aspect of reuptake is similar to the reuptake process in sympathetic nerves (Mandela and Ordway, 2006). Similar results for CGRP have been shown in the guinea pig basilar artery and in rat dura mater encephali, where reuptake may prove to be an important mechanism by which CGRPergic signalling is regulated in conditions such as migraine (Gupta et al., 2010). In summary, a wide range of removal mechanisms have been proposed to be important in the removal or breakdown of CGRP following its release from nerves or vascular cells. Specific targeting of each of these mechanisms may yield a novel means by which to augment or suppress CGRP bioactivity for therapeutic gain.

1.2.4 Distribution of CGRP

1.2.4.1 Sensory neurons

CGRP is widely distributed throughout the central and peripheral nervous systems. In the periphery, it is most commonly found within sensory neurons: it has long been known that the small, unmyelinated C-fibres and the larger, myelinated Aδ fibres contain CGRP. These fibres do not express CGRP alone and have been shown to differentially express other co-transmitters, depending on anatomical location and tissue innervation. Common co-transmitters include SP and the neurokinins (Bell and McDermott, 1996). These sensory neurons have nerve terminals located within a wide range of tissues and organ systems, including those of the cardiovascular system. Intriguingly, peripheral neuronal somata have recently been identified within the adventitial layers of rat mesenteric arteries and these neurons are thought to express the beta isoform of CGRP (Somasundaram et al., 2006; 2012). This direct neuronal source of CGRP stemming from the DRG within the vascular wall makes a significant contribution to CGRP within the vasculature and remains the biggest source of bioactive CGRP within the vessel wall.

Sensory neurons richly innervate multiple tissues throughout the body and the cardiovascular system is no exception. It has been known for some time that the cardiovascular system received peptidergic innervation, with particular reference to SP-positive neurons. Following the discovery of CGRP in 1982, much work was done to characterise the distribution of this peptide throughout the peripheral nervous system and its target tissues. An initial, chiefly immunohistochemical study performed by Mulderry *et al* in 1985 in the rat showed that CGRP-positive nerve fibres had a widespread and close association around the vasculature, in a manner that was largely similar to the localisation of SP-positive nerves. Innervation of cardiac tissue was minimal but within the limit of detection (Mulderry et al., 1985). The following year, a similar yet more comprehensive study was performed in guinea pig that showed again that the large vessels, such as the carotid and thoracic aorta, received a rich innervation of CGRP-immunoreactive fibres and innervation appeared to decrease as one descended into the microvascular network(Lundberg et al., 1985; Dhall et al., 1986). Generally, the arteriolar side of the circulation appeared to receive a higher degree of innervation than that of the venous side. These nerve fibres extended to the tunica adventitia of the vessel and sometimes as far as the adventitial-medial border. The heart, once again, was demonstrated to receive sensory

innervation but more at the level of the epi- and endo-cardium, with little innervation in the myocardium itself (Uddman et al., 1986).

Aside from neuronal sources of the peptide, however, a recent body of evidence has emerged to suggest that the production of CGRP is not strictly limited to sensory neurons and might in fact be directly synthesised by some cells of the blood and vasculature, as discussed in the subsequent section of this thesis.

1.2.4.2 Endothelial Cells

There is accumulating evidence for expression of CGRP by the ECs that form the innermost layer of cells within blood vessels. This was first reported in 1992 by Cai *et al* who used immunogold labelling techniques to demonstrate that CGRP was expressed by around 10% of HUVEC*in situ* (Cai et al., 1993). This finding was later confirmed in the rat carotid body using a similar detection technique, except that further evidence now pointed to the localisation of CGRP within the Weibel-Palade bodies of the EC, which might indicate a potential storage site for the peptide within the endothelium (Ozaka et al., 1997). Building upon this, CGRP was again shown to be localised to the Weibel-Palade bodies (and rough endoplasmic reticulum) of ECs obtained from conduit *and* resistance arteries of rats (Doi et al., 2001).

However, expression of CGRP by the EC does not mean that it acts in a functionally relevant manner in this cell type. Since the identification of CGRP in the endothelium, it has been shown that acute heat stress (42°C) is capable of releasing CGRP from HUVEC, secondary to endothelial TRPV1 activation, an effect that was recapitulated *in vivo*(Ye et al., 2007). This source of CGRP was found to be beneficial in protecting the endothelium against damage induced by lysophosphatidylcholine. A subsequent paper also examining the effects of heat stress on the EC showed both alpha and beta CGRP mRNA expression by HUVEC that decreased with subsequent passaging of the cells. This was also released by TRPV1 stimulation and protected the cells from damage by improving their overall viability (Luo et al., 2008a). Further to this, the α_2-adrenoceptor agonist, clonidine, has been shown to induce gene transcription of both isoforms of CGRP and the release of CGRP from cultured ECs in a NO-dependent manner (Zhang et al., 2009). In summary, evidence for expression of CGRP by ECs is not as robust as that of sensory nerves, but certainly warrants further research in order to confirm existing findings and expand upon the role of local sources of CGRP within the vessel wall.

1.2.4.3 Endothelial Progenitor Cells

EPCs have been of great interest of late to cardiovascular researchers, given their ability to repair damaged regions of endothelium and their contribution to neovascularisation processes. Early publications investigating links between CGRP and EPCs actually took advantage of CGRP's potential vascular protection and stably transfected EPCs with CGRP DNA in an attempt to ameliorate pulmonary hypertension and associated pathology (Zhao et al., 2007). However, this study did not show endogenous expression of CGRP in this cell type.

This issue has since been addressed. In 2010, Zhou *et al* showed that EPCs derived from human patients did indeed express αCGRP mRNA and this level was decreased significantly in EPCs derived from hypertensive patients (Zhou et al., 2010b). A similar phenotype was seen in EPCs derived from SHRs, where further mechanistic insight could be gleaned. Rats administered with a reported phytoalkaloid TRPV1 agonist, rutaecarpine(derived from the plant *Evodia rutaecarpa*), had dose-dependent increases in EPC αCGRP transcript that correlated with a reduction in EPC senescence (Zhou et al., 2010b). In a related study from the same group, human EPCs were maintained in culture for a period of up to 21 days, where it was shown that αCGRP mRNA expression increased up to day 10, where levels then declined. These cells also expressed mRNA for CGRP receptor genes. Once again, rutaecarpine was shown to inhibit EPC senescence induced by ATII*via* upregulation of the "anti-aging" hormone klotho and subsequent reduction in ROS-producing enzymes (Zhou et al., 2010a). Others have also confirmed expression and release of CGRP by EPCs (Fang et al., 2011). Indeed, whilst it seems as though EPCs both synthesise *and* release CGRP following stimulation, the relevance of this source of the peptide is still unclear and, as such, EPCs represent an exciting new research avenue for CGRP.

1.2.4.4 Cells and Tissues of the Immune System

Little is known about the endogenous expression of the peptide by the cells of the immune system. However, what is perhaps better established is the existence of a sensory nerve-immune system synapse. Early histochemical studies performed in this field identified CGRP-positive sensory nerve fibres infiltrating non-inflamed primary and secondary lymphoid tissues, including the thymus, bone marrow and many peripheral lymph nodes (Weihe et al., 1991). Many of these fibres were found to be in close association with the vasculature feeding the tissue but perhaps more interestingly, some were found to innervate the tissue directly, unassociated with blood vessels. Within the lymph nodes themselves, non-vascular associated CGRP-positive fibres tend to be found within the paracortical T-cell regions of the lymph node. Other studies have highlighted a more intense interaction between the sensory nervous system and *individual* immune cells. Interactions have been documented with cell types including macrophages and epidermal Langerhans cells (Hosoi et al., 1993). More convincingly, there is a larger body of evidence to support direct interactions between sensory neurons containing CGRP and tissue mast cells (Stead et al., 1987; Weihe et al., 1989; Naukkarinen and Jaervikallio, 1996). Given mast cell degranulation can lead to the release of a variety of substances that can sensitise and activate sensory nerves to lead to the perception of pain, this interaction is perhaps not so surprising (Steinhoff et al., 2000).

However, despite interactions between CGRP-positive neurons and cells of the immune system, less is known about the endogenous expression of the peptide by these types of cells. Subsets of cells from both the adaptive and innate immune systems appear to express CGRP following stimulation. For instance, both B cells and T cells produce CGRP following mitogen/cytokine stimulation (Holzmann, 2011) and in some cases following stimulation by the endocannabinoid, anandamide (Li et al., 2009; Shi et al., 2012). Further to this, human monocytes have been shown to upregulate the expression of CGRP following stimulation by NGF or LPS.Additionally, upregulation of CGRP following LPS challenge could be attenuated with the coincubation of an anti-NGF antibody (Bracci-Laudiero et al., 2005). It was unclear, however, which isoform of CGRP these cells were expressing. In summary, our knowledge of the expression of CGRP by immune cells is quite weak, but there exists a level of interaction between the sensory nervous system and regions that are highly-populated with immune cells, indicating a possible regulatory role of sensory-derived neuropeptides in immune cell function.

1.2.5 Biological Activity

1.2.5.1 Vascular

CGRP is perhaps best known for its ability to act as a potent microvascular vasodilator. This effect was first characterised by Brain *et al* in 1985. This seminal study showed that CGRP was capable of dose-dependently relaxing strips of rabbit aorta within the picomolar to nanomolar range *via* a mechanism that appeared to be largely dependent on the presence of endothelial-dependent NO activity. Furthermore, femtomolar and picomolar injections of CGRP into the human volar skin of the forearm elicited a marked reddening of the injection site that lasted for several hours. This effect was attributable to increased microvascular vasodilatation and increased blood flow as a result (Brain et al., 1985). Early studies examining the effect of CGRP in human dermal blood flow showed similar results, whereby picomolar concentrations of peptide injected intradermally into the human forearm produced an erythema that was slow in onset but persisted up to 10-12 hours post-injection (Brain et al., 1986). CGRP's ability to act as a potent microvascular vasodilator has been reproducibly demonstrated in a wide range of vascular beds (ranging from skin microvasculature(Brain et al., 1985) and gastric mucosa (Whittle et al., 1992)) from a wide range of model organisms and also humans(Russell et al., 2014). Shortly after the discovery of the vasodilator properties of CGRP, it was discovered that the peptide was able to increase the production of the second messenger cAMP in rat VSMCs *via* adenylate cyclase, whilst having no effect on the generation of cGMP in the same preparation (Kubota et al., 1985). A more mechanistic insight into CGRP-induced arteriolar dilatation showed that the K^+_{ATP} channel blocker, glibenclamide, was capable of attenuating the CGRP relaxation response in mesenteric arteries obtained from the rabbit. In single cell preparations, CGRP was shown to hyperpolarise VSMC obtained from the same vessel type and that glibenclamide could also reverse this hyperpolarization (Nelson et al., 1990). The current thinking remains that conduit vessel vasodilatation mediated by CGRP is largely dependent on endothelial-derived NO production whilst the smaller resistance vessels are more dependent on the activity of K^+_{ATP} channels present within the VSMC(Russell et al., 2014).

In keeping with the ability of CGRP to relax blood vessels *in vitro* and also at a local level *in vivo*, CGRP produces a hypotensive response when given intravenously. Integrated *in vivo* responses to CGRP were shown initially in guinea pigs, where intravenous injection of the peptide in anaesthetised animals produced hypotension and reflex tachycardia that mimicked the response to capsaicin infusion, providing an early hint at the link between

TRPV1 activation and CGRP release (Lundberg et al., 1985). Subsequent experiments in rats further characterised the effects of *in vivo* CGRP infusion by measuring responses in conscious animals, where a similar response of vasodilatation coupled with increased heart rate was shown (Sirén and Feuerstein, 1988). Early experiments in man showed that i.v. infusion of 100µg CGRP produced a drop in blood pressure of around 20 mmHg that recovered after roughly two hours post-injection. This hypotensive response was accompanied by a reflex tachycardia and elevation of plasma catecholamines (Gennari and Fischer, 1985). These results are in contrast to when CGRP is delivered intracerebroventricularly, which leads to enhanced sympathetic outflow and, as a result, marked hypertension and tachycardia in rats (Amara et al., 1983).

Given that CGRP acts as a very potent vasodilator, it is certainly plausible that it is capable of being released to act as a homeostatic controller of physiological blood pressure. However, the current paradigm suggests that CGRP has little role to play in baseline blood pressure regulation and appears to become more important as the cardiovascular system is exposed to stress, such as during a pre-hypertensive state. Early studies investigating a role for CGRP in blood pressure maintenance were mixed, owing to differences in the generation of "CGRP knockout" mice. Groups using mice with the sole deletion of αCGRP found no change in baseline cardiovascular parameters (Lu et al., 1999), whilst other groups using a mixed calcitonin/αCGRP model found a dramatic and significant elevation in resting blood pressure of around 40 mm Hg (Gangula et al., 2000). As a result of the mixed outcome, however, further studies have been performed, taking advantage of other investigative tools. For example, administration of CGRP receptor antagonists to anaesthetised rats resulted in no change in systemic blood pressure (Arulmani et al., 2004; Lynch et al., 2010) and, further, administration of CGRP function-blocking antibodies to conscious rats was shown to have little effect on either blood pressure or heart rate (Zeller et al., 2008). Providing more convincing evidence still, Petersen *et al* showed that in a clinical trial for the use of BIBN4096BS as an anti-migraine therapy, healthy patients that received the antagonist treatment had no detectable change in haemodynamic parameters (Petersen et al., 2005). However, it remains to be seen what effect CGRP receptor antagonists will have on migraineurs with existing cardiovascular complications.

1.2.5.2 Cardiac

Although the best-characterised effects of CGRP have been studied within the context of the vasculature, there is undoubtedly a role for CGRP in maintaining cardiac homeostasis, particularly under times of stress. Prior to the functional characterisation of CGRP in cardiovascular tissues, it was known that CGRP-containing nerve fibres were present in the heart, with a particularly dense innervation of the coronary arteries, papillary muscle and also excitatory regions such as the sinoatrial and atrioventricular node (Mulderry et al., 1985). Other experiments investigating the effect of capsaicin on the spontaneously beating guinea pig auricle showed that application of the TRPV1 agonist capsaicin resulted in a positive chronotropic and inotropic response from the atrial tissue (Lundberg et al., 1984). This was an effect that could not be blocked by antagonists of any classical transmitter, including SP and ATP, hinting at the presence of a yet unknown transmitter being released from sensory nerves into cardiac tissue. It wasn't long before the transmitter was identified as CGRP (Lundberg et al., 1985). In fact, since the identification of CGRP as a cardiac signalling molecule, it has been shown that it is capable of acting as a very potent endogenous mediator of preconditioning – the phenomenon whereby pre-exposure of the heart to a preconditioning agent can attenuate subsequent damage incurred by an ischaemic episode (Li et al., 2000). In the Langendorff-perfused rat heart, exogenous CGRP mimics preconditioning induced by transient ischaemia via ligation of the left anterior descending coronary artery. Furthermore, application of the small molecule CGRP antagonist BIBN4096BS inhibited the preconditioning response from either exogenous CGRP or transient ischaemia, indicating that CGRP plays an important role in the physiological response to myocardial ischaemia (Chai et al., 2006). The overall clinical weight of CGRP's role in mediating the preconditioning response has still to be evaluated. However, it may be especially relevant in myocardial ischaemia suffered by diabetic patients. In experimental models of diabetes, the ischaemic preconditioning response is markedly attenuated (Lu et al., 2001b), an effect that can be reversed via adenoviral transfection of CGRP gene to myocardial tissue (Zheng et al., 2011). In addition, deletion of the TRPV1 gene produces a similar loss of preconditioning in the heart, suggesting that the beneficial effects of CGRP are secondary to TRPV1 stimulation and neuropeptide release (Zhong and Wang, 2007). The ability of CGRP to produce a preconditioning effect also appears to be attenuated with the advancement of age (Lu et al., 2001a). Therefore, as both diabetes and ageing are associated with sensory nerve degeneration, it is conceivable that a loss of myocardial sensory innervation is a key process in increasing susceptibility to myocardial damage following

ischaemia. Furthermore, in cellular models of cardiac hypertrophy, CGRP has been shown to inhibit norepinephrine-induced apoptosis of cardiomyocytes, suggesting a further level of protection aside from increased perfusion (Zhao et al., 2010).

1.2.5.3 Immune System

During inflammatory responses, both local and systemic levels of CGRP rapidly increase *in vivo*. The overall result of this increase appears to be to inhibit the activity of cells of the immune system. This anti-inflammatory effect has been shown in a series of *in vitro* experiments. Peritoneal macrophages isolated from mouse were stimulated with LPS to encourage cytokine production, among which were IL-10 and IL-1β. Co-stimulation of these macrophages with LPS and CGRP facilitated the exacerbated production of IL-10, largely considered to be 'anti-inflammatory', and decreased the production of IL-1β (considered 'pro-inflammatory') (Torii et al., 1997). This enhanced production of IL-10 by CGRP has also been confirmed in isolated human PBMCs (Fox et al., 1997). The elevation in IL-10 concentration has been suggested by both groups to inhibit antigen presentation by professional antigen-presenting cells. However, others argue that this inhibition of immunocyte function occurs through an IL-10-independent pathway and instead occurs through CGRP receptor-mediated activation of protein kinase A (PKA) and subsequent downstream activation of the transcription factor inducible cAMP early repressor (ICER) (Harzenetter et al., 2007). However, it should be noted that these studies utilized different cell types to analyse CGRP-dependent immune cell suppression and this may partially explain discrepancies in the reported results.

The aforementioned studies were performed in isolated cultures of cells of the innate immune system and therefore are not necessarily representative of the integrated immune system *in vivo*. Thus, several studies have been performed in models of infection, in particular sepsis, to assess the role of CGRP in modulating the body's response to systemic inflammation. A 2005 study by Gomes *et al* showed that treating septic mice with CGRP improved their survival post-endotoxaemia by limiting peritoneal neutrophil invasion and upregulation of pro-inflammatory plasma cytokines such as keratinocyte-derived chemokine (KC) and TNFα, whilst encouraging the circulation of anti-inflammatory cytokines IL-6 and IL-10 (Gomes et al., 2005). In a similar model of endotoxin-induced hepatic dysfunction, CGRP was shown to be protective *via* induction of ICER activity and subsequent reduction in pro-inflammatory cytokine expression (Kroeger et al., 2009). Whilst difficult to interpret the direct role of CGRP, studies performed in receptor activity modifying protein 1 (RAMP1)-deficient mice (a key molecule related to the CGRP receptor, discussed in a later section) have supported the notion that mice lacking this part of the CGRP receptor are more susceptible to tissue immune cell infiltration and increases in plasma inflammatory cytokines

following endotoxaemia and systemic polymicrobial sepsis (Tsujikawa et al., 2007; Jusek et al., 2012).

Whilst there exists a body of evidence supporting an immunosuppressant role for CGRP *in vitro* and *in vivo*, it is relatively unclear at this stage how and if this neuropeptide influences leukocyte trafficking through the endothelial monolayer and into the surrounding tissue. An early study investigating this process by Sung *et al* showed that stimulation of quiescent HUVEC monolayers with CGRP facilitated adhesion of U937 monocytic cells and human PBMCs under static conditions (Sung et al., 1992). This pro-adhesive phenotype was subsequently confirmed for neutrophils by a separate group in a similar model (Zimmerman et al., 1992). In contrast to this, Huang *et al* showed that HUVEC activated by LPS and co-stimulated with CGRP resulted in an inhibition of transmigration of neutrophils and mononuclear cells through the endothelial monolayer in a chemotaxis assay under similar static conditions (Huang et al., 2011). This inhibition of migration was attributable to attenuation in the production of pro-inflammatory chemokines CXCL8, CXCL1 and CCL2 *via* stabilization of the transcription factor NFκB in the cytosol, thus preventing its transmigration into the nuclear compartment of the cell to promote gene transcription (Huang et al., 2011). Additionally, acute stimulation of LPS- and formyl-met-leu-phe-treated neutrophils and monocytes with exogenous CGRP lowered CD11b protein expression by both cell types (2003). Furthermore, treatment of LPS-activated microglia with CGRP resulted in the reduction of their capacity to produce pro-inflammatory cytokines such as IL-6 and TNFα and chemokines such as MCP-1 (Consonni et al., 2011). These data may point to a role of CGRP in regulating leukocyte transmigration on the side of the white blood cell. In an*in vivo* model of peripheral ischaemic preconditioning, CGRP was shown to inhibit leukocyte rolling and adhesion in reperfused vessels, to a similar extent to that of preconditioned vessels, indicating it may be a key mediator of leukocyte trafficking in this setting (Hartmann et al., 2011) and in ethanol-induced preconditioning (Kamada et al., 2006). Put together, however, there is a general absence of information regarding the role of CGRP in the very initial stages of leukocyte adhesion and transmigration and this certainly warrants future research in this area.

1.2.6 CGRP and Ageing

The ageing process involves a complex series of interactions between multiple organ systems that results in a deterioration of normal physiological function and an inability to properly maintain whole-body homeostasis. Sensory neurons are not exempt from the gradual deterioration in function and, as such, it is important to consider some of the changes that may happen to these cells if we wish to understand how CGRP bioactivity may change with advancing age. This is especially pertinent, given that sensory nerves are considered to be the biggest source of CGRP within the body. Furthermore, if it is true that CGRP is an important mediator of cardiovascular health, any change in bioactivity as part of the ageing process could result in a change in cardiovascular phenotype, for better or for worse, and as such, CGRP may represent a valid therapeutic target.

Peripheral neurons in general are negatively affected by the ageing process in terms of their ability to conduct action potentials and to sprout collaterals in regenerating fibres (Verdú et al., 2000). One of the first studies investigating the developmental changes that occur to peptidergic sensory neurons was performed in the ageing guinea pig, where it was discovered that density of peptidergic neurons surrounding the mesenteric and carotid arteries increased with foetal development, with a peak at birth. CGRP-ergic nerve plexuses then declined with age, waning to about half-maximal density once the animal had reached around 2 years of age (Dhall et al., 1986). Interestingly, a study investigating peptidergic innervation of the ageing rat aorta showed that CGRP-positive neuronal fibres were present in animals younger than 6 months, but gradually disappeared to complete absence when animals reached one year of age (Connat et al., 2001). Conversely, a separate study has indicated no change in DRG αCGRP mRNA expression in rats aged 6 months *versus* 24 months (Lu et al., 2002). Therefore, it is still unclear what happens to peptidergic perivascular innervation as time progresses. However, some aspects of neuronal physiology are compromised as part of ageing. As CGRP is a peptide that is synthesised directly by the nucleus (unlike small molecules that require complex metabolic pathways for synthesis, often present within the cytosol) but has the majority of its effects when released from the nerve terminal, it requires axoplasmic transport from the cell body to the axon terminals by means of the neuronal microtubular network. The rate of active transport from nucleus to terminal is significantly diminished with age and this may represent a reduction in CGRP release as age advances (Fernandez and Hodges-Savola, 1994).

In addition to stored CGRP, serum levels of released CGRP have been noted to both increase and decrease with advancing age. One possible reason why serum levels of CGRP are seen to be increased as age increases may be related to the change in CALC I gene splicing in favour of CGRP rather than calcitonin (recall that CGRP was first discovered in the thyroid tissue of ageing rats and isolated in elderly humans from thyroid carcinoma cells). Increased production of CGRP by a highly-vascularised endocrine gland such as the thyroid would presumably explain increases in plasma concentrations (Kendall et al., 1986; Wimalawansa, 1991). However, as circulating levels of CGRP are so low, it is unlikely that this source of peptide has any significant biological effect. It seems more likely that controlled localised release of CGRP is more important than this suggested 'spillover' and as the local sources are more often seen to be reduced than increased as one ages, it is possible that a very valuable store of CGRP is being lost as part of the ageing process.

Functional experiments investigating changes in CGRP bioactivity with age have been published, though are often somewhat confounded by other aspects, such as the induction of experimental hypertension in laboratory animals. As such, results obtained cannot be directly attributed to ageing alone. Nonetheless, some interesting results have been uncovered. In aged female rats, circulating levels of CGRP have been shown to decline slightly in comparison to younger animals and the content of bioavailable CGRP found within the mesenteric vascular bed shows an even more profound drop. This reduction in CGRP could be reversed with supplementation of female sex steroid hormones (Gangula et al., 2009). Several longitudinal studies have also been performed utilising the SHR. In these animals, αCGRP mRNA expression within DRG neurons was found to decrease as the animal aged whilst no change in expression was found in normotensive rats (Yamaga et al., 2001). This decline in CGRP mRNA expression in hypertensive animals appears to be accompanied by a measurable decrease in CGRP-positive sensory nerves that innervate the mesenteric resistance arteries (Kawasaki et al., 1990a; 1991). This reduction in neuronal density was found to result in an overall decrease of releasable CGRP following peripheral nerve electrical stimulation (Kawasaki and Takasaki, 1992) and also following capsaicin stimulation (resulting in the activation of TRPV1 channels and subsequent exocytosis of CGRP-loaded vesicles) (Sun et al., 1998). There has been proposed a strong interaction between the angiotensin signalling system and CGRP and its receptors within the SHR model. One longitudinal study showed that long-term inhibition of AT1Rs using selective antagonists, such as temocapril and losartan, prevented the reduction in perivascular sensory nerve innervation in a blood pressure-dependent manner (Hobara et al., 2005). Thus, it is possible

that as hypertension becomes more prolonged as one ages, this has a negative effect on CGRP-containing nerves and the amount ofbioavailiable CGRP.

CGRP-containing sensory nerves also appear to be important in governing the cardiac preconditioning response to intermittent periods of ischaemia, in order to prepare for an impending ischaemic episode that may precede myocardial infarction. It is true that older humans are more susceptible to myocardial infarction and isolated heart preparations from older laboratory animals appear to lose this preconditioning response (Lu et al., 2001b; Hu et al., 2002). This has been attributed to a reduction in release of endogenous stores of CGRP found within the heart and, curiously, a reduction in the ability of exogenous CGRP to also have a protective effect (Lu et al., 2001a). A similar impairment in CGRP bioactivity with increasing age has been demonstrated in vascular preparations from ageing rats. The ability of CGRP to relax aortic and caudal artery rings was significantly impaired as the animal grew older and intravenous injections of CGRP to older animals resulted in an attenuated hypotensive blood pressure response (Chan and Fiscus, 2002). A similar reduction in sensitivity to CGRP has been shown in the isolated perfused mesenteric bed in both normotensive and hypertensive ageing rats, though this effect was not exclusive to CGRP as vasodilatation to other relaxing agents was also impaired (Amerini et al., 1994).

It is still relatively unclear how the expression, release and bioactivity of CGRP changes with advancing age. This lack of clarity mainly arises from the use of tissue derived from different species of laboratory animal, differing lengths of longitudinal study employed and the majority of information stemming from the use of the SHR. That said, enough information has been uncovered to warrant future research into this area, particularly as the current consensus appears to be that local stores of CGRP are depleted as age advances and this results in a decrease in its bioactivity. If CGRP is indeed an important mediator of cardiovascular (and otherwise) health, then it is conceivable that we are losing a very important protective mediator and this may present with a viable future therapeutic strategy.

1.2.7 CGRP and Hypertension

The majority of our knowledge concerning the role of CGRP as a potential protective mediator working within the cardiovascular system comes from preclinical models of experimental hypertension. Indeed, our understanding has deepened considerably with the development of CGRP knockout mice, which will be discussed further in this section.

1.2.7.1 Human Studies

Circulating levels of CGRP are often attributed to overspill from sensory nerves and plasma levels are not considered to be a major source of biologically relevant peptide. However, this reservoir represents a suitable means to investigate changes in CGRP dynamics under different (patho)physiological and experimental settings. With relevance to hypertension, it has been shown that a subpopulation of Chinese citizens who possess a single nucleotide polymorphism in the CALC I gene (T-692C) were more likely to have essential hypertension than those who possessed the normal allele (Luo et al., 2008b). Measurement of plasma levels of CGRP in hypertensive patients has provided mixed results, though the majority of studies seem to favour a reduction in circulating CGRP from patients with essential hypertension *versus* normotensive controls (Tang et al., 1989; Portaluppi et al., 1992; Wang et al., 2007b; Li et al., 2009). One study has shown that CGRP levels tend to correlate positively with blood pressure and levels were highest in the case of hypertension secondary to primary aldosteronism or phaeochromocytoma. Of the patients suffering from secondary hypertension, elevated levels of CGRP could be reversed following adrenalectomy (Masuda et al., 1992). In the setting of severe hypertension and pre-eclampsia, levels have been shown to be lower than normotensive controls (Edvinsson et al., 1989; Dong et al., 2006). Other studies have shown no change in circulating plasma levels of the peptide, though it is of note that these studies were carried out in borderline and mildly-hypertensive patients, where the role of CGRP in regulating the hypertensive response may not have been fully realised (Schifter et al., 1991; Lemne et al., 1994). At the vascular level, reports have shown that hypertensive arterioles can either have a significantly blunted response to exogenous CGRP application (measured *in vivo* by laser Doppler flowmetry) (Lindstedt et al., 2006) or an augmented response to the peptide (measured *ex vivovia* myography) (Lind and Edvinsson, 2002).

1.2.7.2 Rodent Studies

Whilst human studies can be enlightening, they often lack mechanistic insight and this is where experiments performed in animal models can be of great utility. Certainly, this is the case for investigating the role of CGRP within the cardiovascular system, with respect to the initiation and progression of pathophysiology associated with hypertension. Investigations into circulating levels of CGRP in humans have yielded mixed results, though the general consensus seems to point to a decline in plasma levels in hypertensive patients. Using animal models, CGRP dynamics can be investigated as part of this disease process, and changes in tissue expression measured, which might be a more relevant analysis. Extensive work has been carried out in this area and a similar mixed result has emerged, though such discrepancies in findings may be attributed to the use of different models, analysis of various tissues and examination of different experimental timepoints. As previously stated, the majority of studies tend to find a general reduction in CGRP bioavailability or bioactivity, to one extent or another. In the SHR, laminae I/II spinal cord expression of CGRP is diminished (Westlund et al., 1991), mRNA expression is decreased (Supowit et al., 1993; Kawasaki et al., 2000; Li et al., 2008) and the density of CGRP-positive sensory neurons innervating the vasculature appears to decrease, along with their ability to release peptides into the vascular environment (Kawasaki et al., 1990b; 1990a; 1991). Levels of CGRP have been shown to be decreased in other models of hypertension, such as hypoxia-induced hypertension (Tjen-A-Looi et al., 1998), Dahl-salt (Katki et al., 2001) and phenol-induced neurogenic hypertension (Deng et al., 2004a). In general, two-kidney-one-clip (2K1C) rats appear to have elevated CGRP expression, both at the mRNA level within the DRG and when measured *via* immunoreactivity in the plasma (Deng et al., 2003; Qin et al., 2007). Interestingly, a study has been published examining both the 2K1C model of renovascular hypertension and the SHR model. This study showed that CGRP plasma concentrations were lowered in the SHR versus control WKY but in the 2K1C model, plasma levels were shown to be elevated (Li et al., 2009). This study highlights the importance of considering which hypertensive model to use in order to investigate CGRP-dependent effects, as changes in CGRP activity may be strongly dependent on the nature of the hypertensive stimulus, rather than just general hypertension itself. Others have shown no change in CGRP bioavailability throughout hypertension in deoxycorticosterone acetate (DOCA)-salt (Supowit et al., 1995), subtotal nephrectomy (Supowit et al., 1998) and L-NAME (Gangula et al., 1997) models of hypertension.

With this in mind, some groups have studied the relative importance of endogenous ATII in driving changes in CGRP expression and activity during hypertension. Indeed, it has been shown that ATII can directly inhibit the release of CGRP from perivascular sensory neurons within the mesenteric circulation, *via* its action on AT1Rs. This effect was only present within SHRs and not in the normotensive controls (Kawasaki et al., 1998). Further to this, long-term treatment of SHRs with ACE inhibitors or AT1 receptor blockers was shown to be effective in restoring electrically-induced CGRP release and vasodilatation to a normal level (Kawasaki et al., 1999), improving CGRP DRG mRNA and circulating plasma expression (Kawasaki et al., 2003; Shi et al., 2012) and reducing perivascular sensory denervation (Hobara et al., 2005).

As well as being a potent vasodilator *in vivo* under normotensive conditions, CGRP can also act as a vasodilator during hypertension, first demonstrated in anaesthetised SHRs in 1989 (Xu et al., 1989). Its potency as a vasodilator initially appeared to be unaltered by systemic blood pressure (Ando et al., 1990). However, isolated pial vessels from SHR appear to show increased sensitivity to the peptide (Hong et al., 1997) and the *in vivo* hypotensive response to the peptide was also increased in SHRs (Yamada et al., 1998). More recently it has been shown that in a subtotal nephrectomy model of hypertension, *both* the hypotensive and vasodilator response in isolated vessels to exogenous CGRP was increased, an effect that was attributable to increased vascular receptor component protein (RCP, a CGRP receptor-associating protein) expression, thus boosting receptor-dependent signalling(Supowit et al., 2011). One other study has found a similar result in ATII-induced hypertension in rats, where the vasodilator response to CGRP was improved in hypertension, owing to an increased expression of calcitonin receptor-like receptor (CLR) and RAMP1 (molecules required in forming CGRP receptors, see later section for more detail)(Li and Wang, 2005). Administration of CGRP receptor antagonists have no effect on the basal blood pressures of Sprague-Dawley rats but when these animals are rendered hypertensive *via* an array of techniques including L-NAME (Gangula et al., 1997), deoxycorticosterone-salt delivery (Supowit et al., 1997) and subtotal nephrectomy (Supowit et al., 1998), antagonism of the CGRP receptor further exacerbates experimental hypertension, strengthening the idea that CGRP may become more important as the cardiovascular system becomes stressed. In a similar fashion, targeted blockade of CGRP receptor gene expression, CGRP peptide immunoneutralisation and CGRP receptor antagonism have been shown to worsen pulmonary hypertension (Tjen-A-Looi et al., 1992; Qing and Keith, 2003). Furthermore, sensory nerve degeneration induced by neonatal capsaicin treatment has been shown to exacerbate experimental hypertension (Tjen-A-Looi et al., 1998; Deng et al., 2003). A handful

of investigations have been carried out employing the use of thephytoalkaloid rutaecarpine. Rutaecarpine is a reported TRPV1 agonist that can facilitate the release of CGRP to produce vasodilatation that is inhibited by TRPV1 antagonism, CGRP receptor antagonism and depletion of neuropeptide stores by capsaicin pretreatment (Hu et al., 2003). Chronic rutaecarpine treatment to renal phenol injury hypertensive rats reduces blood pressure in line with increased CGRP expression and release (Deng et al., 2004b). In addition to its blood pressure lowering effect, rutaecarpine has also been shown to prevent adverse arterial remodeling following the 2K1C procedure (Qin et al., 2007) and to prevent aberrant platelet activation in SHRs (Li et al., 2008), both phenotypes thought to be mediated by CGRP activity.

1.2.7.3 Transgenic Studies

The development of genetically engineered mouse models has significantly advanced our understanding of the role of CGRP in the cardiovascular system, and otherwise. Owing to the complexity of the receptor system and the duplicity of the CGRP genes, there exists many potential ways by which one can investigate this system. Most commonly used is the CGRP mouse. The first study to publish an investigation into the endogenous cardiovascular regulatory role of CGRP was by Lu *et al* in 1999. This group showed that both blood pressure and heart rate were unaffected by CGRP deletion under resting conditions. Following a forced swimming exercise, blood pressure was indeed found to be raised but with no difference between WT and KO animals. Interestingly, however, there was a small but significant decrease in the heart rate of mice lacking CGRP versus WT following exercise, though this is a phenomenon yet to be expanded on. In addition, both groups of mice appeared to have normal baroreceptor activity (Lu et al., 1999). This study found no level of developmental compensation from the related peptide, βCGRP, following αCGRP removal. Conversely, a strikingly different result was obtained by Gangula *et al*, published in the Journal of Hypertension mere months after Lu *et al*. This group showed increased blood pressures in both male and female αCGRP-null mice under baseline conditions (systolic blood pressure of 120 mm Hg in WT males *versus* 160 mm Hg in KO males) (Gangula et al., 2000). The hypertensive phenotype of these mice was shown to be as a result of hyperactivation of the renin-angiotensin system, specifically an increase in renin expression (Li et al., 2004). Such a stark difference in findings may be attributed to the generation of different types of KO mice by each group. For instance, generation of the KO used by Lu *et al* involved the targeted disruption of exon 5 of the CALCI gene with a neomycin resistance cassette, in order to remove CGRP but not calcitonin mRNA synthesis. On the other hand, the KO mice studied by Gangula *et al* are deficient in *both* calcitonin *and* αCGRP. Therefore, it is difficult to interpret studies published by this group using these mice, given any phenotype observed could be as a result of calcitonin deletion, αCGRP deletion or, indeed, an interaction resulting from deletion of both genes. Others have studied CGRP KO mice bred onto genetic backgrounds other than C57BL/6. For example, Oh-Hashi *et al* (2001) and Kurihara *et al* (2003) have generated αCGRP KO mice bred onto a 129/Sv x C57BL/6 hybrid background and these have also been shown to be hypertensive, owing to an increase in sympathetic nervous system activity (Oh-hashi et al., 2001; Kurihara et al., 2003). With these disparate results in mind, it would be prudent to choose a model similar to that of Lu *et al*,

using a mouse that solely carries αCGRP and not calcitonin deletion, that shows no baseline changes in haemodynamic parameters.

Despite an unlikely role for CGRP in the regulation of cardiovascular parameters under resting conditions, there is a rapidly growing body of evidence to suggest that the peptide may become more important as the cardiovascular system becomes stressed, particularly within the setting of hypertension. As previously discussed, several studies have shown an eminent role for CGRP in the pathophysiological response to high blood pressure. However, these studies did not take advantage of the CGRP-null mouse. In 2005, Supowit *et al* utilized the CALC I KO to investigate its role in DOCA-salt-induced hypertension. Whilst they reproduced initial findings of elevated baseline blood pressure in these mice, they also found that αCGRP-deficient animals had higher blood pressures following intervention. This was accompanied by histopathological changes to both cardiac and renal tissues, with an increase in urinary microalbuminuria, an indicator of renal dysfunction (Supowit et al., 2005). A follow-up study from this group showed an increase in vascular inflammatory markers within the kidneys of CGRP KO in this model (Bowers, 2005). Recently, this group has addressed whether the inflammatory phenotype they see in this model is pressure-dependent or –independent, by normalising elevations in blood pressure using hydralazine. Whilst hydralazine delivery did improve blood pressure in the hypertensive animals, it had no effect on the renal inflammation seen in the CGRP KO. This was measured in terms of macrophage infiltration and quantification of renal cytokine expression (Li et al., 2013a). Thus, whilst one must be cautious of the conclusions these studies make, owing to the use of the CALC I KO, it is possible that CGRP has an important role in protecting the kidney during hypertension. To provide clarity, we have recently shown that in an ATII infusion model of experimental hypertension, mice *solely* lacking αCGRP had exacerbated hypertension *versus* WT. This phenotype was coupled with increased adverse vascular remodeling and when hypertension was extended from 14 days to 28 days, we observed a loss of vascular eNOS expression, an indicator of endothelial and vascular dysfunction (Smillie et al., 2014).

Others have manipulated RAMP1 expression in mice to determine the relevance of CGRP in cardiovascular health. Owing to the ability of AM to bind to RAMP1-CLR receptors, this is not an ideal model to use to interrogate CGRP-dependent responses and this must be kept in mind, though RAMP1-CLR is typically thought of as the CGRP receptor. The first study to examine the role of RAMP1 in cardiovascular regulation was published in 2007, where the

RAMP1 gene was deleted in mice. These animals were found to be spontaneously hypertensive, similar to the phenotype observed in CALC I KO mice (Tsujikawa et al., 2007). Conversely, others have studied the role of RAMP1 *via* its global overexpression. At baseline, the RAMP1 overexpressers were not found to differ in terms of cardiovascular phenotype versus WT animals. However, once rendered hypertensive *via* ATII infusion, the RAMP1 transgenic animals were dramatically protected from the induction of hypertension, only becoming very mildly hypertensive at around 2 weeks of ATII infusion (WTs developed hypertension on day 3). This protection could be removed by supplementation of the CGRP receptor antagonist, $CGRP_{8-37}$. Furthermore, RAMP1 overexpressers appeared to have improved baroreceptor function and thus were better able to regulate their blood pressure in face of a vasopressor stimulus (Sabharwal et al., 2010). In a separate study, control mice were found to develop endothelial dysfunction following ATII infusion, measured in terms of vasodilatory responses in isolated arteries to acetylcholine. However, RAMP1 transgenic mice were not found to have any degree of endothelial dysfunction (Zhang and Russo, 2010). These results help bolster the key role CGRP may play in hypertension.

1.2.8 CGRP Receptors

1.2.8.1 History of the CGRP receptor

Early evidence for the existence of a receptor for CGRP was largely accumulated *via* radioligand binding studies in a wide range of tissue preparations. Research at this stage was led by evidence showing that CGRP peptide expression was highest in regions of the central nervous system. Studies using rat tissue showed moderate expression of "specific" CGRP-binding substances (bearing in mind current knowledge of CGRP's affinity for many related receptor subtypes), with a notably high binding capacity in the spinal cord and pituitary gland (Goltzman and Mitchell, 1985). Binding of CGRP to regions of the central nervous system was further demonstrated in human tissue, where a similar high level of binding was shown in regions of the spinal cord, particularly in the dorsal horn (Tschopp et al., 1985). Headway was also made into probing the binding of CGRP to receptors expressed by cells of the cardiovascular system. Hirata *et al* showed in 1988 that radiolabeled CGRP had the capacity to bind to VSMCs isolated from rat and also ECs derived from bovine aortae. In addition to binding assays, the authors of this paper further characterized the significance of CGRP binding to a receptor by showing that the binding event elicited the stimulation of adenylyl cyclase activity in both cell types (Hirata et al., 1988). Following from this, Coupe *et al* demonstrated that both guinea pig and human heart tissue had the capacity to specifically bind CGRP, with the highest binding density concentrated within the coronary arteries (Coupe et al., 1990).

Whilst autoradiographic studies can be informative of where a ligand is binding and therefore where a receptor for such a ligand is likely to be located, they shed little to no light on the nature of the receptor itself. Thorough characterization of a receptor can only be properly carried out through the deployment of a "pharmacological toolkit", encompassing specific agonists and, more importantly, antagonists of the receptor in question. Much of the early quantitative knowledge of the CGRP receptor stemmed from the use of two distinct biological preparations: the guinea pig atria and rat vas deferens. In both of these preparations, full length human CGRP was demonstrated to produce a positive inotropic response in a concentration-dependent manner (Dennis et al., 1989). However, the use of CGRP fragments and linearised/cyclised derivatives of the CGRP peptide revealed discrepancies in activity between tissues. The linearised CGRP analogue [Cys(ACM)2,7]hCGRP was found to be a potent agonist in the rat vas deferens bioassay, but not guinea pig atrial preparations (Dennis et al., 1989). Later discovery of a truncated form

of the peptide sequence, $CGRP_{8-37}$, helped advance the field of CGRP receptor pharmacology. It was shown to be capable of binding to CGRP receptors present in rat liver (Yamaguchi et al., 1988) and could concentration-dependently inhibit receptor binding of full length CGRP and its ability to generate a response, as measured by adenylyl cyclase activity (Chiba et al., 1989). Thus, $CGRP_{8-37}$ was validated as a useful tool in probing the CGRP receptor and its utilization by Dennis *et al* in a subsequent study was pivotal in helping to describe CGRP pharmacology. $CGRP_{8-37}$ was found to function as an antagonist to the effects of full-length CGRP only in the guinea pig atria and not in the rat vas deferens (Dennis et al., 1990). This pointed to the existence of two (or more) distinct populations of CGRP receptor, subsequently defined as '$CGRP_1$' and '$CGRP_2$'. $CGRP_1$ receptors were classified according to their ability to be antagonised with high potency to $CGRP_{8-37}$ ($pK_b > 7.0$) whilst $CGRP_2$ receptors were characterised by low potency antagonism from $CGRP_{8-37}$ ($pK_b < 6.0$). Whilst the existence of such receptor heterogeneity has been debated for many years, it is now accepted that only one true CGRP receptor exists and the apparent existence of additional receptors may be explained by the peptide having affinity for other related receptors of distinct molecular composition, such as the amylin receptor AMY_1 (Hay et al., 2008).

Evidence supporting the finer details of the CGRP receptor, in particular its molecular composition, was more difficult to come by due to the complex nature of the receptor itself. Previous knowledge of the calcitonin receptor gene sequence informed early knowledge of the CGRP receptor. Initial work by Chang *et al* identified a novel cDNA that encoded a seven transmembrane domain protein that coupled to adenylyl cyclase but was unresponsive to CGRP exposure (Chang et al., 1993). Soon after this, Njuki *et al* isolated two rat clones that possessed high sequence homology to known calcitonin receptor sequences yet did not possess the tissue distribution fingerprint expected of the calcitonin receptor. Instead, these sequences were highly expressed in regions of the lung, in particular in close association with the pulmonary vessels. This discovery led to the christening of the term 'calcitonin receptor-like receptor' or 'CLR' (Njuki et al., 1993). Subsequent work performed by Flühmann *et al* uncovered a homologous structure to the rat CLR sequence in human cerebellum. Transfection of this cDNA into a cellular expression system and stimulation of cells by CGRP resulted in no measurable signal in terms of adenylyl cyclase activity (Flühmann et al., 1995). The resultant protein was found to be 461 amino acids in length,with seven transmembrane domains as part of its structure. The protein was found to share 96% homology with the previously identified rat protein and around 56% homology with human calcitonin receptor (Flühmann et al., 1995). Frustratingly, whilst this receptor

was initially shown to be unresponsive to CGRP stimulation, researchers were in fact looking at the correct protein but were unaware of the complexity of the receptor as a whole.

This oversight only became apparent in 1996 when Aiyar and co-workers discovered that the same receptor cloned by Chang and Flühmann, when expressed in HEK293 cells (as opposed to OK and COS-7 cell lines), produced a sixty-fold increase in cyclic AMP (cAMP) generation over baseline following CGRP stimulation, which was blocked by $CGRP_{8-37}$(Aiyar et al., 1996). This gain of function was clearly attributable to a factor endogenously expressed by HEK293 but not in COS-7 that, when co-expressed with the CLR, could generate a signal in response to CGRP. The identity of this protein was dissected in a seminal paper by McLatchie *et al* in 1998. By generating a cDNA library from SK-N-MC neuroblastoma cells (known to bind and respond to CGRP), the authors transfected the clones into a *Xenopus* oocyte cystic fibrosis transmembrane regulator assay and measured cAMP generation secondary to agonist stimulation as an inward cystic fibrosis channel current, as this is known to be regulated by cAMP activity (McLatchie et al., 1998). They identified a single cDNA that encoded a 148 amino acid protein that was named 'receptor activity modifying protein 1', or RAMP1. This key paper then described how RAMP1 was an obligate binding partner of CLR and was essential to signal transduction from the CGRP receptor. The manner by which RAMP1 conferred CGRP responsiveness was due to its role as a molecular chaperone, facilitating the terminal glycosylation of CLR and allowing for its export from the endoplasmic reticulum. In cells that do not express RAMPs, the CLR is retained by the endoplasmic reticulum and does not reach the cell surface, thereby explaining how cell lines that do not endogenously express RAMP1 cannot respond to CGRP stimulation (McLatchie et al., 1998). Where RAMPs are expressed in isolation, they are retained by the Golgi apparatus in the form of a disulphide-linked homodimer (Hilairet et al., 2001b). However, when the RAMP translocates to the cell membrane, it is stabilized in a heterodimeric complex with the CLR, facilitated by noncovalent interactions. Screening of public databases identified two other proteins with high homology to RAMP1, which were christened as RAMP family members RAMP2 and RAMP3 (McLatchie et al., 1998). RAMP2 and RAMP3 are important for the formation of functional AM receptors, AM_1 and AM_2. The authors suggested that differences in glycosylation states of the CLR induced by differential RAMP interactions was critical in conferring ligand specificity. For instance, terminally-glycosylated CLR allowed for binding of CGRP whilst core-glycosylated CLR allowed for AM binding. This was later contested by Hilairet *et al*, who showed that whilst glycosylation was an essential step in trafficking the receptor to the plasma membrane, it had no bearing on ligand specificity (Hilairet et al.,

2001b). They therefore concluded that differences in ligand binding were likely to be attributed to differences in the steric conformation of a binding pocket produced by the CLR:RAMP interface.

What remains unclear with regards to receptor subunit stoichiometry is how the formation of receptors is directed in cells that express more than one RAMP isoform, endogenously or experimentally. Experiments performed in rabbit aortic ECs showed that transfection of RAMP1 (not endogenously expressed by this cell type) produced a receptor responsive to CGRP stimulation. This response was not affected by co-transfection with RAMP2 (already expressed in rabbit aortic ECs) but was significantly attenuated by co-transfection with RAMP3 cDNA (Muff et al., 1998). From this, the authors concluded that a level of competition exists between RAMP family members, where the co-expression of alternative isoforms might radically change receptor pharmacology. This hypothesis was tested by Bühlmann *et al*, who showed that overexpression of RAMP1 in cells that also express RAMP2 leads to the loss of AM-mediated signalling in favour of the formation of a CGRP receptor (Bühlmann et al., 1999). Thus, CLR may preferentially couple to RAMP1 in the presence of other RAMPs, though the mechanism by which this might happen is not known.

To facilitate research and discussion, the nomenclature of the calcitonin family of peptide receptors has been finalised. Dimerisation of CLR and RAMP1 leads to the formation of the CGRP receptor. Dimerisation of CLR and RAMP2 forms a receptor that is responsive to AM (the AM_1 receptor) and interactions between CLR and RAMP3 form an additional receptor for AM, the AM_2 receptor (Alexander et al., 2011). Rank potencies for typical agonists used to probe calcitonin receptor family pharmacology are listed in Table 1.

Receptor	Composition	Rank order agonist potency
CGRP	CLR + RAMP1	αCGRP > AM ≥ AM2/IMD > AMY ≥ CT
AM_1	CLR + RAMP2	AM > αCGRP, AM2/IMD > AMY > CT
AM_2	CLR + RAMP3	AM = AM2/IMD ≥ αCGRP > AMY > CT

Table 1: Composition and pharmacology of the calcitonin family of receptors, adapted from (Alexander et al., 2011).

1.2.8.2 Calcitonin receptor-like receptor (CLR)

The CLR belongs to the Class B "secretin-like" family of G protein-coupled receptors (GPCR), which also includes receptors for calcitonin, vasoactive intestinal polypeptide, pituitary adenylate cyclase activating polypeptide and parathyroid hormone. It constitutes the typical seven transmembrane component of the receptor and shares approximately 55% sequence homology with the related calcitonin receptor (Flühmann et al., 1995). Human and mouse CLR are around 89% homologous, with the murine protein slightly longer in length (463 versus 461 amino acid residues). As previously mentioned, expression of this protein in isolation is not sufficient to form a functional CGRP receptor as it requires the co-expression of a RAMP in order to be exported from the endoplasmic reticulum and inserted into the plasma membrane. In the absence of RAMPs, the endoplasmic reticulum retains the CLR. Therefore, it is the dimerisation with RAMPs that dictates the complexity of this unusual receptor.

Owing to its requirement to form receptors for both CGRP and AM and also intermedin (AM2), CLR has a wide tissue expression profile, with a high level of expression within regions of the central nervous system associated with higher autonomic control, feeding and thirst (Hagner et al., 2002). With respect to cells of the cardiovascular system, CLR is expressed by both vascular endothelial and smooth muscle cells from a variety of species and tissues and from both conduit and resistance vessels (Kamitani et al., 1999a; Frayon et al., 2000; Jansen-Olesen et al., 2003; Nagoshi et al., 2004; Nikitenko et al., 2006). There is also a strong body of evidence supporting the expression of CLR by cardiac cells, particularly in reference to the AM receptor. In the heart, CLR is expressed throughout development and into adulthood (Dvorakova et al., 2003) within both cells of the endocardium and myocardium (Saetrum Opgaard et al., 2000).

1.2.8.3 Receptor activity modifying proteins (RAMP)

As previously mentioned, the RAMPs were first described in a seminal paper published by McLatchie and colleagues in 1998 (McLatchie et al., 1998). The three RAMP family members are critical in conferring ligand specificity to CLR protein to form receptors for CGRP and AM. Dimerisation of CLR with RAMP1 leads to the formation of a CGRP receptor, RAMP2 association forms the AM_1 receptor and RAMP3 forms the AM_2 receptor, which has appreciable affinity for CGRP ligand. Dimerisation of CLR with any of the three RAMP isoforms generates a nonselective receptor for AM2(Roh et al., 2004). Aside from the CLR, RAMPs may also associate with another Class B GPCR, the calcitonin receptor (CTR). These interactions promote the formation of the amylin receptors AMY1, AMY2 and AMY3. Where RAMPs do not interact with the CTR, the calcitonin receptor is formed (Muff et al., 1999). Despite only sharing around 30% sequence identity, all RAMPs possess a similar topology, consisting of ~150 amino acid primary sequence arranged into three major domains. They possess a short intracellular C-terminus of around 10 amino acids, a transmembrane domain of roughly 20 amino acids and an extensive extracellular N-terminus. Of the three family members, RAMP2 is the least conserved member of the family, containing a 26 amino acid insert into the extracellular domain (Parameswaran and Spielman, 2006).

Perhaps unsurprisingly, owing to their general promiscuity with respect to forming a diverse range of receptors, the RAMPs are expressed somewhat ubiquitously throughout the body. However, the relative abundance of their expression varies depending on the tissue in question and also whether samples are extracted from healthy or diseased models/patients as RAMPs are differentially expressed in a wide range of pathophysiological conditions. The initial McLatchie publication described RAMP mRNA expression in human tissues. Here, RAMP1 expression was highest in heart, skeletal muscle and pancreas. RAMP2 was also high in these tissues, but was also highly expressed in placenta and lung, whilst RAMP3 seemed to exhibit consistently high mRNA expression in all tissues observed (McLatchie et al., 1998). A subsequent publication by Husmann *et al* attempted to map the expression of murine RAMP isoforms. They identified a high expression of RAMP1 in brain, lung and skeletal muscle extracts. RAMP2 had an even wider range of expression, with highest levels in heart, lung, skeletal muscle and brain. RAMP3 appeared to be less widely-expressed, with mRNA being expressed mainly in embryonic and adult brain lysates (Husmann et al., 2000).

With reference to cells and tissues of the cardiovascular system, RAMP expression seems to vary greatly depending on the resident tissue of the cell. Early studies in human cells showed

no expression of RAMP1 or RAMP3 mRNA in aortic smooth muscle cells, HUVEC and aortic ECs but high RAMP2 (and CLR) expression in all three cell types (Kamitani et al., 1999b). Conversely, brain-derived microvascular smooth muscle and ECs appear to highly express RAMP1 message and the other RAMPs to a much lesser extent (Moreno et al., 2002; Jansen-Olesen et al., 2003). Such a discrepancy in expression may explain why CGRP is such a key player in the vascular nociceptive response in migraine. With respect to human dermal microvascular ECs RAMP1 expression is a little less clear. In 2006, Nikitenko *et al* showed that RAMP1 mRNA did not exist in this cell type, whilst signal for the other RAMP family members could be detected (Nikitenko et al., 2006). However, a more recent study performed by Huang *et al* in 2011 showed that human dermal microvascular cells and the HMEC-1 endothelial cell line expressed all RAMP isoforms (Huang et al., 2011). With these findings in mind, it is important for the researcher to be clear in defining which receptor their phenotype is being mediated by, if any, as a response alone does not indicate expression of a particular RAMP, given the potential for the calcitonin family of peptides to bind to multiple receptor subtypes.

1.2.8.4 Receptor component protein (RCP)

It is now known that an additional third protein is required to form an optimally functional CGRP receptor, termed the CGRP receptor component protein (RCP). The RCP is a small, hydrophilic, membrane-associated protein that has little homology to other known protein sequences. RCP was first identified in guinea pig organ of Corti, where an isolated cDNA was shown to encode for a protein that conferred specific pharmacological sensitivity to CGRP stimulation of *Xenopus* oocytes, as measured by a cystic fibrosis transmembrane conductance regulator assay. Stimulation of cells with ligands known to stimulate other $G_{\alpha s}$-coupled receptors did not result in a positive response, indicating that the 17 kDa protein identified in this study was directly involved with CGRP receptor stimulation (Luebke et al., 1996). Indeed, when antisense RNA was constructed against RCP and transfected into NIH3T3 cells, cAMP generation subsequent to CGRP receptor stimulation was lost. Disruption of RCP protein synthesis had no effect on the ability of CGRP to bind to its receptor, whilst significantly attenuating cAMP generation (Evans et al., 2000). This meant that the role of this particular protein was likely to be involved with the modulation of the second messenger response following receptor stimulation, rather than serving a structural role in stabilising the receptor. In support of this finding, the RCP was shown to co-immunoprecipitate with the CLR protein, confirming that association of RCP with the CGRP receptor is important for optimal signal transduction (Evans et al., 2000). In addition to its role in driving the CGRP response, RCP also appears to be important in regulating signal generated by AM receptor stimulation, though potentially to a lesser extent than CGRP (Prado et al., 2001). This is perhaps unsurprising given this protein's ability to associate with the CLR, which is also a critical subunit of AM receptors. Interestingly, it has recently been shown that in a mouse model of experimental autoimmune encephalomyelitis, the RCP is capable of translocating from the neuronal plasma membrane to the nuclear compartment. Although this has only been shown *via* immunofluorescent techniques, this may point to the description of an exciting new feature in RCP function, whereby it may regulate gene transcription in conjunction with its role in regulation cyclic nucleotide signal amplification (Sardi et al., 2014).

Despite being discovered more than a decade ago, little is known about the function of RCP in driving the CGRP-mediated response and its relevance in health and disease. Shortly after the discovery of the protein, its expression profile was mapped in the guinea pig nervous system, with a particularly abundant gene expression within the hippocampus and

cerebellum, reflecting the early autoradiographical studies concerning radioactive CGRP binding in the brain (Oliver et al., 1999). Later studies performed in rats showed that RCP expression was high in other regions of the nervous system, including regions of the lumbosacral spinal cord and also the DRG(Pokabla et al., 2002). The authors of this study also highlighted an association between RCP tissue immunoreactivity and innervation of CGRP-ergic neurons, indicating a close relationship between receptor expression and the site of ligand release. Other notable sites of RCP expression include the ocular ciliary body (Rosenblatt et al., 2000), myometrium (Naghashpour et al., 1997) and testis (Balkan et al., 1999). A potentially important role for the RCP *in vivo* has recently been uncovered in the setting of hypertension. Rats rendered hypertensive *via* subtotal nephrectomy and saline consumption were found to have an augmented depressor response to intravenous CGRP than sham-operated animals and isolated resistance vessels from these animals were found to be more sensitive to exogenous CGRP application (Supowit et al., 2011). This was an effect that was suggested to be directly attributable to an increased expression of RCP protein within the vasculature of the hypertensive rats, whereas expression of other CGRP receptor proteins was found to be unchanged. It is therefore conceivable that the fine-tuning of RCP expression in disease conditions may drastically change CGRP-mediated responses. An illustration of CGRP receptor stoichiometry, basic pharmacology and associated signallingpathways is depicted in Figure 5, below.

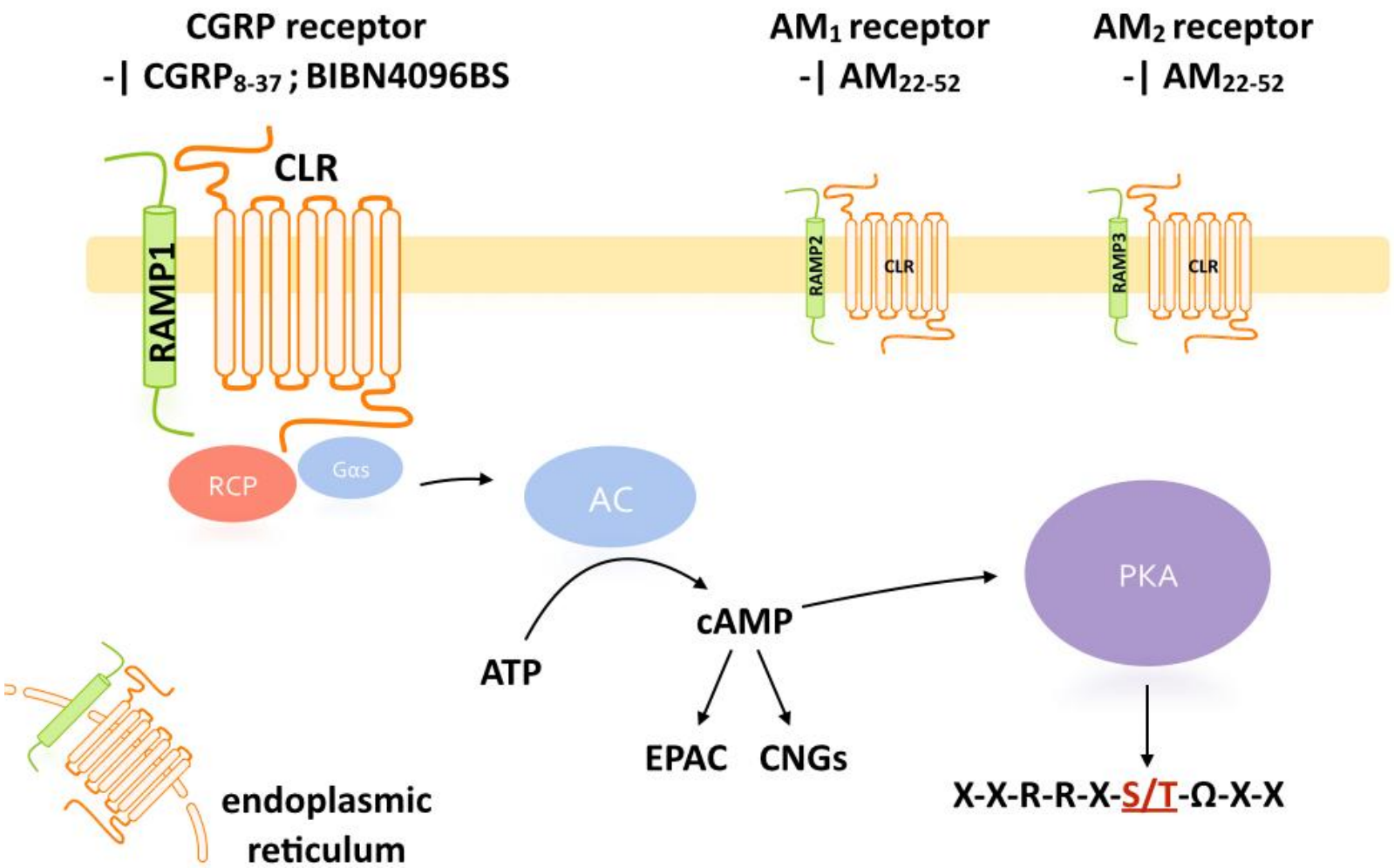

Figure 5: Diagram depicting CGRP-family receptor stoichiometries, pharmacology and commonly associated signalling pathways. CGRP; calcitonin gene-related peptide, AM; adrenomedullin, CLR; calcitonin receptor-like receptor, RAMP; receptor activity-modifying protein, RCP; receptor component protein, AC; adenylate cyclase, ATP; adenosine triphosphate, cAMP; cyclic AMP, EPAC; exchange protein activated by cAMP, CNG; cyclic nucleotide-gated ion channel, PKA; protein kinase A. Amino acid shorthand: X; any amino acid, R; arginine, S; serine, T; threonine, Ω; hydrophobic residue.

1.2.9 Molecular Pharmacology and Ligand Binding

1.2.9.1 Agonist binding and receptor activation

Much effort has been put into attempting to understand the molecular determinants of agonist/antagonist binding to CGRP receptors. Given the complex heterodimeric nature of the receptor, advances in this area of CGRP biology are laudable but we are far from comprehensively understanding the precise mechanisms involved in ligand binding. Reasonably soon after researchers began turning their focus onto the molecular pharmacology of Class B GPCRs, Hoare proposed a global model by which this family of receptors might bind ligands in order to activate the receptor and generate a signal. This generalised mechanism was termed the "two-domain model" (Hoare, 2005). In this, the C-terminal region of the peptide agonist is 'captured' by the large extracellular N-terminus of the Class B receptor with high affinity. This high-affinity capture results in the increased local availability of agonist and in particular an increased local concentration of the unbound N-terminus of the ligand. This greatly increases the probability of weak interactions between the ligand and the juxtamembrane domain of the receptor, leading to subsequent receptor activation (Hoare, 2005). This model if strengthened by the observation that truncated CGRP peptides (e.g. $CGRP_{8-37}$) are capable of binding to the receptor with low affinity but do not activate it and therefore act as competitive antagonists. Conversely, the two-domain model appears to explain why small molecule antagonists designed against the CGRP receptor have high potency, as they are mostly believed to bind at the N-terminus of the receptor, thereby blocking high-affinity capturing interactions between endogenous ligand and its receptor. Mechanisms related to antagonist binding will be discussed later in this section.

It has been known for some time that, despite being a relatively small peptide, there are distinct regions within the molecular structure of CGRP that appear to be critical for receptor binding and activation. Initial evidence for this emerged as early as 1989 from Chiba *et al* who found that truncation of the first seven amino acid residues from the N-terminus of the peptide to form $CGRP_{8-37}$ resulted in a CGRP receptor antagonist that had the capacity to bind receptor but not activate it (Chiba et al., 1989). Clearly, this pointed to a critical role of at least one of these residues in mediating receptor activation. To support this hypothesis, Maggi *et al* produced N-terminal fragments of the peptide and tested their hypotensive effect in the anaesthetized rat, where they were shown to be bioactive (Maggi et al., 1990). The importance of the N-terminus of CGRP in mediating receptor activation is in keeping with other peptide agonists of Class B GPCRs that have a common N-terminal motif required for activation. In the case of CGRP, this motif is formed by a disulphide-bonded ring between $cysteine^2$ and $cysteine^7$(Neumann et al., 2008). Comparative

molecular biology has revealed that there is a high-degree of sequence identity up to position 14 in species from mammals to bony fish, indicating that the N-terminus of the peptide appears to be evolutionarily conserved in order to retain its efficacy. Though there is little experimental evidence to inform which particular N-terminal residues are critical in mediating receptor activation, a recent paper from the Hay group has shown through site-directed mutagenesis of CGRP that Ala5Cys mutants possessed a 270-fold reduction in affinity and a reduced efficacy of cAMP generation and β-arrestin recruitment. In addition to this, Thr6Cys mutants possessed no agonist properties (Hay et al., 2013). Whilst the effects of these mutations are quite clear, it is less clear why the authors chose to mutate their focused residues to cysteine. Whilst this residue is small and hydrophobic and apparently innocuous, the inclusion of a thiol group as part of the sidechain introduces a highly reactive group to the peptide backbone. This residue may have seemed like a suitable replacement for Ala5 but it is less clear why the authors did not choose to mutate Thr6 to alanine to test the loss-of-function effect. For a further review of the structure-activity relationship of CGRP interacting at its receptor, see Watkins *et al* 2012 (Watkins et al., 2012).

To date there are limited data available concerning the three dimensional structure of the CGRP receptor, as determined by crystallographic techniques. In 2010, ter Haar *et al* solved the structure of the CLR/RAMP1 ectodomain in the unbound state and also when bound with small molecule antagonists of the receptor (Haar et al., 2010). However, what is still lacking is the resolution of a structure, ectodomain or otherwise, with CGRP bound to it. Thus, our knowledge of the molecular determinants of agonist binding is perhaps best informed by site-directed mutagenesis studies and comparing modes of binding of other ligands to Class B GPCRs. Current thinking is that CGRP binds to its receptor within a binding cleft at the interface between CLR and RAMP1. Within this environment, tryptophan-84 of RAMP1 appears to be critical in allowing ligand binding (Moore et al., 2010) whilst multiple residues present within the CLR N-terminus appear to be also quite important. Directed mutagenesis of Leu41, Gln45, Cys48 and Tyr49 of the CLR to alanine resulted in diminished potency of CGRP at its receptor, as determined by cAMP assay. Furthermore, Leu41Ala, Ala44Leu, Cys48Ala and Tyr49Ala mutagenesis led to complete inhibition of agonist binding (Barwell et al., 2010). It is thought that this interface between RAMP1 and CLR is only important in ligand anchoring rather than activation of the receptor itself.

1.2.9.2 CGRP Receptor Antagonists

Critical to the advancement of our understanding of receptor pharmacology, it is essential to have at hand a specific pharmacological toolkit comprised of specific agonists and antagonists for the receptor. As previously discussed, the first antagonist for the CGRP receptor was developed in 1989 by Chiba *et al*.(Chiba et al., 1989). Other peptide antagonists have also been investigated (e.g. $CGRP_{27-37}$, which is the minimal fragment required for receptor binding; (Yan et al., 2011)) Whilst an invaluable tool, the peptide nature of $CGRP_{8-37}$ meant that it exhibited low potency and was an undesirable drug for *in vivo* use and a clear need was established to synthesise a stable and selective small molecule antagonist of the CGRP receptor. Development of selective non-peptide antagonists of the CGRP receptor (and, indeed, other Class B GPCRs) has been met with some difficulty owing to the fact that the endogenous ligand of the receptor is a peptide. Therefore, a low molecular weight antagonist working at the orthosteric site would have to prevent the binding of a much larger molecule that is likely to have a complex repertoire of interactions with its receptor. Furthermore, the heterogeneity of the CGRP family of receptors begets further complexity, where the design of novel antagonists must overcome structural differences between CLR/RAMP heteromers. However, the need was met in 2000 when Boehringer Ingelheim developed the new chemical entity BIBN4096BS, or olcegepant,: 1-piperidinecarboxamide,*N*-[2-[[5-amino-1-[[4-)4-pyridinyl)-1-piperazinyl]carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)-methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2H)-quinazolinyl)-,[*R*-(*R**,*S**)]. This novel non-competitive antagonist of the CGRP receptor was shown to be capable of blocking binding of endogenous ligand to receptor in both cells and tissue preparations from rodent and human and also produced a rightwards shift in cAMP accumulation curves (Doods et al., 2000). It displayed an affinity of 14.4 ± 6.3 pM for human CGRP receptors in SK-N-MC cells and 3.4 ± 0.5 nM for CGRP receptors found in spleen (Edvinsson et al., 2001). This is a striking difference in affinity in comparison to $CGRP_{8-37}$. *In vivo* preparations demonstrated that the antagonist, although not orally-active, was further capable of inhibiting increases in dermal blood flow following trigeminal ganglion stimulation in the marmoset (Doods et al., 2000). Whilst this antagonist has been shown to have increased potency over the peptide antagonist, it is considered to have 200-fold increased potency in humans and non-human primates than in rodent tissue (Doods et al., 2000).

Based on the promising pharmacology of olcegepant, an additional member of the 'gepant' family was identified by Merck & Co, *via* high-throughput screening. Originally termed 'compound 2', the benzodiazepine-like lead structure was shown to possess affinity for the human CGRP receptor, with a K_i of 4.8μM. Optimisation of this structure led to the development of the more potent telcagepant;

N-[(3*R*,6*S*)-6-(2,3-Difluorophenyl)-2-oxo-1-(2,2,2-trifluoroethyl)azepan-3-yl]-4-(2-oxo-2,3-dihydro-1*H*-imidazo[4,5-*b*]pyridine-1-yl)piperidine-1-carboxamide. This compound was shown to have good oral bioavailability (Paone et al., 2007). Early characterization studies showed that telcagepant had a K_i of 1.1nM at CGRP receptors expressed in HEK293 cells and could inhibit capsaicin-induced increases in blood flow in the rhesus forearm (Salvatore et al., 2008). Merck continued with the success of telcagepant with the development of a more potent antagonist, MK-3207. This compound displayed a K_i of 0.024nM at human and rhesus CGRP receptors, but again this high potency was not conserved in species other than human and primate (Salvatore et al., 2010).

Other less well-characterised antagonists have also been developed to block CGRP activity. A second compound was filed under the Boehringer patent WO98/11128, 'compound 1'. Compound 1 was shown to have pK_i values of 7.8 in SK-N-MC cells (compared to that of around 8.9 for $CGRP_{8-37}$). It was shown to be a weak antagonist of CGRP responses in human cerebral and guinea pig basilar arteries (Edvinsson et al., 2001), but not in porcine coronary arteries (Hasbak et al., 2001). Interestingly, Compound 1 has been shown to antagonise the effects of CGRP in the same vascular bed, albeit from humans (Hasbak et al., 2003), demonstrating further the species difference for CGRP antagonists. There has also been a range of cyclic non-peptide antagonists based on $CGRP_{27-37}$ with potencies in the nanomolar range (Yan et al., 2011).

More recently, Bristol-Myers Squibb have published data on a novel set of non-peptide antagonists of the CGRP receptor. With accumulating evidence for many small molecule antagonists unfortunately being potent inhibitors of hepatic cytochrome P3A4 (which led to the withdrawal of telcagepant from clinical trials for migraine), BMS have developed compounds that possess modified benzothiophene side chains in order to circumvent these issues, including BMS-694153, a compound with high affinity for the human CGRP receptor (K_i = 13pM). Significantly, this compound was shown to have excellent intranasal bioavailability in the rabbit, a physicochemical characteristic that may suit the treatment of an acute migraine attack (Degnan et al., 2008). For a further review of all major CGRP receptor antagonists, including their pharmacological characterization, please see Moore and Salvatore, 2012 (Moore and Salvatore, 2012).

1.2.9.3 Antagonist binding and molecular determinants of species-dependent potency

A great stimulus in initiating work concerning the molecular pharmacology of antagonists binding to the CGRP receptor was the finding that these antagonists exhibited substantial species selectivity with respect to their potency. In brief, both of the 'gepant' series of CGRP receptor antagonists display roughly a thousandfold increase in potency at human and primate CGRP receptors in comparison to rodent and canine receptors (Doods et al., 2000; Salvatore et al., 2008). Adding further evidence to support differences in receptor composition between species, MK-3207, a CGRP receptor antagonist that is structurally-distinct from the gepants still appears to possess a potency around 400 times as high at human and primate receptors versus rat and dog (Salvatore et al., 2010). Clearly, these differences in antagonist potency point to differences in the molecular composition of the CGRP receptor between species.

An initial study by Mallee *et al* attempted to explore this problem more fully. This study focused on the clear difference in antagonist potency between human and rat CGRP receptors. They confirmed that the small molecule antagonists were more potent when acting at the human receptor and constructed receptor chimeras to address whether the CLR or RAMP1 portion of the receptor mediated this selectivity. They found that receptors comprised of human RAMP1 and rat CLR could be antagonised in a similar manner to that of hRAMP1/hCLR constructs, whilst rat RAMP1/human CLR constructs mimicked that of the native rat receptor (Mallee et al., 2002). Strikingly, this dramatic shift in potency was governed by a single amino acid mutation. Site-directed mutagenesis of rat RAMP1 lysine 74 to tryptophan (the homologous amino acid of the human receptor) resulted in a regain of function, indicating that antagonist potency is largely governed by a single amino acid residue found within the extracellular domain of RAMP1 (Mallee et al., 2002). Kusano *et* al made progress in resolving the crystal structure of the extracellular domains of human RAMP1 in 2008, which uncovered four additional residues that potentially could be important for antagonist binding: Arg67, Asp71, Glu78 and Trp84 (Kusano et al., 2008). Validation of these key residues in conferring antagonist affinity uncovered for the first time a key role for Trp84 in the regulation of not only affinity for small molecules, but also affinity for peptide fragment antagonists and peptide agonists alike (Moore et al., 2010). This residue exists as part of the binding interface between RAMP1 and the CLR, indicating that the ligand-binding domain of the CGRP receptor is formed of a hydrophobic pocket located between both structures. Interestingly, mutation of Trp84 to alanine results in a lowered membrane expression of the receptor, bolstering the role for RAMP1 as a molecular chaperone for CLR to reach the plasma membrane and supporting the role of Trp84 in mediating RAMP1:CLR interactions.

Subsequent studies also turned their attention to the potential role of the CLR in governing antagonist binding. The rationale for investigating the role of the CLR in antagonist binding came from the observation that CLR:RAMP1 and calcitonin receptor CTR:RAMP1 heterodimers possessed different affinities for CGRP receptor antagonists, indicating a role for the CLR. Salvatore *et al* constructed CLR/CTR chimeras and showed that residues 37-63 of the CLR were critical in determining antagonist binding, in particular those belonging to the 'gepant' class of drugs (Salvatore et al., 2006). However, aspects of transmembrane domain 7 appeared to be important for a second class of antagonists, such as compound 4 (Salvatore et al., 2006). Having antagonists that are dependent on binding to a site that is so far removed from the CLR:RAMP1 interface may be indicative of a site of allosteric modulation. The pivotal involvement of Trp74 of RAMP1 and residues present within the extreme N-terminus of the CLR was confirmed by Miller *et al* in 2010 (Miller et al., 2010). Indeed, they expanded on Salvatore *et al*'s finding and showed that a further key residue within the CLR sequence for antagonist binding was methionine-42 (Miller et al., 2010).

Whilst molecular determinants of both agonist and antagonist binding to the CGRP receptor have been identified, it has been difficult to determine the crystal structure of this family of receptors. Early *ab initio* modeling of RAMP1 provided evidence and predicted a structure composed of three alpha helices (Simms et al., 2006). Kusano and colleagues developed this prediction by scrutinising the soluble extracellular domanis of the RAMP1 molecule and indeed they confirmed that the structure was trihelical (Kusano et al., 2008). As previously mentioned, Trp74 was found to be an important residue as part of this structure and formed part of a hydrophobic cleft that existed between the CLR and RAMP1 that was essential for forming a ligand-binding domain. Alongside Arg67, Asp71 and Glue78, Trp74 was found to exist within the structure of the second alpha helix of RAMP1 whilst the aforementioned Trp84 was found to exist on the loop adjoining helix 2 and 3, with its side-chain oriented in the same direction as Trp74, towards the solvent-exposed side of the structure.

However, these isolated resolutions of the RAMP1 extracellular domain shed no light on how both the CLR and RAMP1 complex together and this complexing is clearly critical for function. In order to address this issue, Koth and colleagues created a construct consisting of both extracellular regions of RAMP1 and CLR in order to act as a surrogate for studying drug interactions with the shared ligand-binding domain between both molecules. Indeed, this construct was validated by its ability to compete with wild type CGRP receptors for binding CGRP in SK-N-MC cells. However, the construct displayed lower affinity for CGRP than the full-length receptor (IC_{50} = 12μM) but was shown to retain high-affinity binding for each of the small molecule antagonists (Koth et al., 2010). Resolution of the

crystal structure of the extracellular domain in the liganded and unliganded state may pave the way for rational drug design. Whilst difficulties do exist in resolving complex GPCR crystal structures, only with the full structure will we be able to gain a full understanding of how CGRP receptors interact with their endogenous ligands and exogenous drug molecules.

1.2.10 Intracellular signalling pathways

As is typical with all GPCRs, binding of CGRP to its receptor results in a conformational change in the three-dimensional shape of the receptor, which facilitates coupling to intracellular signalling pathways. As is realised with other better-understood GPCRs, receptors do not have to be exclusively coupled to one pathway or another and there may be great diversity in signalling mechanisms stemming from one single receptor in order to bring about biological complexity (see Figure 5). This also appears to be the case with the CGRP receptor.

The classical paradigm of CGRP receptor signalling involves coupling positively to adenylate cyclase, which generates the formation of cAMP from ATP (as seen in Figure [X]). As a consequence of this, CGRP receptor activity has commonly been characterised by measuring cAMP responses (Walker et al., 2010a). Local rises in cAMP can lead to the activation of three main intracellular effectors, as governed by the spatial distribution and compartmentalization of signal generators (such as adenylate cyclases), terminators (phosphodiesterase family enzymes) and effectors. These effectors may take the form of cyclic nucleotide-gated ion channels, exchange proteins activated by cAMP (EPACs) or the prototypical cAMP effector, PKA (Zaccolo, 2009). Activated PKA is capable of phosphorylating a multitude of serine/threonine-containing protein candidates that possess the consensus motif R-R-X-S/T-Ω, where X denotes any amino acid residue and Ωdenotes hydrophobic residues (Ubersax and Ferrell, 2007). With respect to the biological action of CGRP, activation of PKA may be incredibly important with respect to mediating smooth muscle relaxation, vascular or otherwise. This vasorelaxation appears to be strongly influenced by PKA's ability to phosphorylate K^+_{ATP}channels to induce hyperpolarisation of the cell, thereby causing relaxation (Nelson et al., 1990; Quayle et al., 1994). There is also a substantial body of evidence supporting the coupling of CGRP receptors to mitogen-activated protein kinase (MAPK) cascades in various cell types. This recruitment of kinase cascades may be dependent or independent of upstream PKA activation (Kawase et al., 2005).

Finally, raised intracellular levels of cAMP can influence long-term changes in cellular function by inducing *de novo* gene transcription by virtue of activation of the cAMP response element-binding protein (CREB)(Kawase et al., 2005; Vause and Durham, 2010), a transcription factor that may bind to sequences of DNA containing cAMP response elements within the promoter region of particular genes (Sassone-Corsi, 1998). In fact, the CGRP gene promoter itself is regulated by CREB, meaning elevation in neuronal cAMP can induce CGRP gene transcription (Freeland et al., 2000).

Other than signalling through the cAMP axis, it is possible that activation of the CGRP receptor results in activation of alternative signalling pathways. These may comprise $G\alpha_{i/o}$, $G\alpha_{q/11}$, and β-

arrestin-dependent pathways (Walker et al., 2010a), although these are not very well understood. Interestingly, a role for G-protein $\beta\gamma$ subunits has been uncovered and these subunits appear to be important in terminating the long-lasting pressor response of arteries in response to ET(Meens et al., 2012).

1.2.11 Receptor tachyphylaxis

An important feature in GPCR signalling is that of tachyphylaxis, or desensitisation. This process serves to attenuate hyperstimulation of a particular receptor in response to prolonged exposure of a particular agonist and it may occur in an agonist-dependent (homologous) or –independent (heterologous) manner. Whilst receptor desensitisation has been famously and extensively studied in typical Class A GPCRs, such as the β_2-adrenoceptor, there is a growing body of evidence investigating the kinetics and behaviour of Class B receptor tachyphylaxis, including the CGRP family of receptors.

Early investigations into CGRP receptor desensitisation in SK-N-MC cells were led by the hypothesis that desensitisation mechanisms were mediated by the CLR and, as such, repeated exposure to either CGRP or AM should lead to a similar reduction in response as both peptides share the requirement of the CLR. Drake *et al* showed in a short communication that pre-exposure of cells to CGRP led to a subsequent diminished response to CGRP *and* AM, which was prevented by the use of H-89 to block PKA. Conversely, it was shown that pre-treatment of cells with AM and subsequent stimulation with either CGRP or AM led to no loss in signal. These results hinted at agonist-dependent differences in the desensitisation response (Drake et al., 1999). The same group expanded on this novel finding by investigating rat aortic VSMCs, managing to recapitulate their previous findings (Drake et al., 2000). Interestingly, however, the authors also showed that pre-exposure of cells to isoproterenol (pan-β-adrenergic receptor agonist, Gα_s-coupled) *or* ATII (pan-angiotensin receptor agonist, Gα_q-coupled) also induced a diminished response to CGRP exposure, hinting at the possibility of heterologous desensitisation of the receptor by different receptor systems (Drake et al., 2000). Whilst these findings supported the idea that CGRP receptors do desensitise, they lacked mechanistic insight into precisely how this phenomenon was achieved. Furthermore, whilst a role for PKA was established within this system, this has since been contested and an additional role for protein kinase C (PKC) has been hypothesised (Pin and Bahr, 2008).

In order to attain more information regarding the mechanisms of CGRP receptor tachyphylaxis, Kuwasako *et al* published a study in 2000 to shed light on this field. HEK293 cells stably transfected with fluorescently-labeled CLR and RAMPs were found to be responsive to stimulation by agonist and also underwent tachyphylaxis. The receptors appeared to internalise *via* their localisation to clathrin-coated pits, where a significant proportion of the internalised receptors were subsequently targeted to the lysosomal compartment for degradation, hinting at a prolonged desensitisation due to inefficient vesicular recycling of the receptor back to the plasma membrane (Kuwasako et al., 2000). This result was quickly confirmed by Hilairet *et al*, who showed again that clathrin-coated pits

were required for receptor internalisation though this was downstream of receptor phosphorylation following activation and translocation of β-arrestin from the cytosol to the activated receptor, a phenomenon typical of Class A GPCR desensitisation (Hilairet et al., 2001a). The finding that an initial receptor phosphorylation event is involved complements early publications that showed an inhibition of receptor desensitisation following PKA inhibition (Drake et al., 1999; 2000). Furthermore, CLR, RAMP1 and β-arrestin were shown to remain complexed within the endosome after internalization. Subsequent work building on this result has shown that CGRP actually internalises to the endosomal compartment whilst remaining bound to the receptor itself (Cottrell et al., 2005).

Experiments examining typical Class A GPCRs have shown that the fate of the desensitised receptor is not fixed following internalisation and this also appears to be the case with the CGRP receptor. Initial CGRP receptor recycling studies showed that duration of receptor stimulation could greatly affect the fate of the internalised receptor. Transient stimulation of the CGRP receptor with agonist for one hour could induce internalisation of receptor and recycling of the receptor back to the plasma membrane within 2-6 hours. This process was not affected by inhibition of *de novo* protein synthesis by cycloheximide but was inhibited by vacuolar disruption, implicating a role for the endosomes. Conversely, sustained stimulation of the receptor with CGRP resulted in a prolonged state of desensitisation where the receptor was targeted towards the lysosomal network for degradation. A role for ubiquitin-dependent proteolysis was excluded (Cottrell et al., 2007). A further layer of complexity in determining receptor fate was introduced with the discovery that the ECE-1 plays a role in CGRP receptor regulation. Specific splice variants of ECE-1 are localised to the endosomal compartment where they cleave CGRP ligand from the internalised receptor and degrade it. This removal of ligand from receptor encourages the dissociation of β-arrestins and their translocation to the cytosol. This results in a more rapid recycling of the CGRP receptor back to the plasma membrane for further stimulation (Padilla et al., 2007). These initial findings were demonstrated HEK-293 and SK-N-MC cell lines but a recent paper has been published to show that this phenomenon is present in isolated rat mesenteric arteries and explanted VSMCs (McNeish et al., 2012). In this study, inhibition of ECE isoforms resulted in a desensitisation of the vasodilator response to CGRP, an effect linked to a decrease in cyclic nucleotide generation. This regulation of signal by ECEs has been shown to occur with other neuropeptides, such as SP (Roosterman et al., 2007), but not with other peptide ligands such as ATII(Padilla et al., 2007).

1.3 Aims and Hypotheses

As previously mentioned, there is an active and clear body of research investigating CGRP-mediated vascular protection in a range of CVD models, including hypertension. There is a growing body of knowledge related to the ageing vasculature but to date no research has been performed on how CGRP regulates the ageing cardiovascular system in terms of gross physiological parameters or how it may influence subtle cellular processes that may predispose the cardiovascular system to disease. Based on current knowledge on the protective nature of CGRP within the cardiovascular system, the hypothesis of this PhD is that CGRP will be protective against age-associated vascular dysfunction and will possess a further protective nature in an animal model of geriatric hypertension. The aims of this project are threefold:

1. To characterise the *in vivo* and *ex vivo* cardiovascular phenotype of the CGRP KO mouse in a model of natural ageing.
2. To study how CGRP influences inflammation-associated leukocyte-EC interactions *in vitro* and to determine molecular mechanisms that may govern this process.
3. To establish a model of geriatric hypertension and to characterise this model and investigate potential protective mechanisms therein.

2. | Method and materials

2.1 Animals

Male WT and KO αCGRP C57BL/6 mice were bred in-house and used throughout. All animals were maintained on standard chow, with access to food and water *ad libitum*. Mice were maintained in a climatically controlled, SPF environment on a 12-hour light/dark cycle. KO mice were age-matched with respective WT counterparts. All experiments were performed in line with the Animals (Scientific Procedures) Act 1986. Experiments were performed under PIL 60/12706 and PPL 70/7959. Juvenile mice were used at three months of age and aged mice were used at fifteen months of age.

2.2 Generation of αCGRP KO mice

WT and KO αCGRP mice were gifted by Dr. A.M. Salmon, Institut Pasteur, Paris, France. KO mice were generated by the targeted disruption of exon 5 of the CALC I gene *via* the insertion of a LacZ/CMV/Neo transgene (Salmon et al., 1999). This results in the removal of CGRP gene transcription, leaving calcitonin expression unaltered. Mice were bred in-house from both homozygous and heterozygous pairs. In the case of homozygous pairing, mice were backcrossed every ten generations to limit genetic convergence. Pairs produced litters with ratios in accordance with traditional Mendelian genetics. Further, KO mice displayed normal growth and behavioural characteristics and were reproductively viable.

2.3 Genotyping

Genotyping of WT and KO αCGRP mice was performed by end-point PCR. Ear notches were taken under 2% isoflurane anaesthesia. Subsequent isolation of genomic DNA was performed using a commercially-available kit (ExtractRED-n-Amp Tissue PCR Kit, Sigma, UK), in accordance with the manufacturer's instructions and PCR was performed on these samples using the following primers (Eurofins Scientific, Europe) and reaction conditions (Table 2), using a thermal cycler (PTC-225 Peltier Thermal Cycler, MJ Research, USA).

Primer A: CCCCTAATGGCCTTGTGATTG

Primer B: ACCTCCTGATCTGCTCAGCAG

Primer D: GATGGGCGCATCGTAACCCG

Temperature (°C)	Time (min)	
94	5	
94	5	
56	1	
72	2	(x30 cycles)
72	7	

Table 2: Table illustrating thermocycling conditions necessary to genotype αCGRP WT and KO mice.

The amplified genomic DNA was then visualised *via* agarose gel electrophoresis (1.8% w/v agarose, Sigma Aldrich) in tris/borate/EDTA acid (TBE, Biorad, UK). Electrophoresis was performed at 90V for 20 minutes to allow for resolution of DNA fragments. The gel was then visualised under ultraviolet light (G-Box, Syngene, USA). A single DNA band at 420bp denoted a KO mouse whilst a single DNA band at 290bp indicated the presence of a WT. A band at both locations denoted a heterozygote mouse. Figure 6 shows a representative PCR reaction showing WT and KO genomic DNA samples.

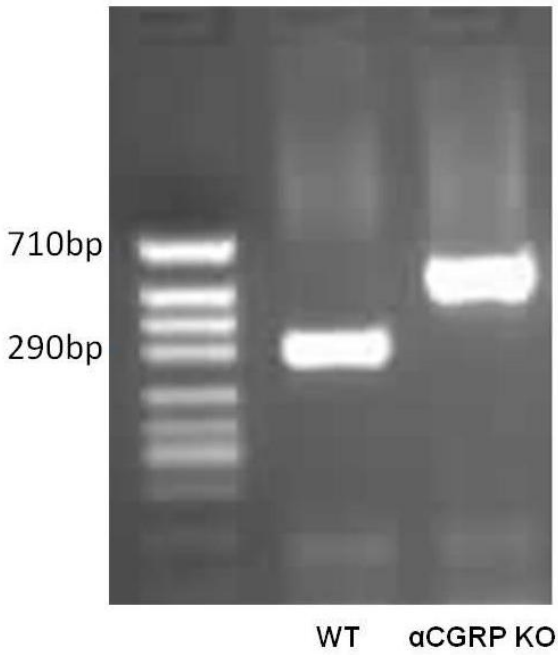

Figure 6: Representative gDNA PCR reaction for αCGRP mouse genotyping. WT band appears at ~290bp; KO band appears at ~420bp.

2.4 Anaesthesia

For the majority of studies presented in this thesis, anaesthesia was not necessary. However, in the case of implantation of osmotic minipumps, surgical anaesthesia was required. Mice were anaesthetised with isoflurane. 4% isoflurane in oxygen carrier was used to induce anaesthesia at a flow rate of 4L/minute. Anaesthesia was then maintained at 2-3% isoflurane (3L/minute oxygen). Deep anaesthesia was assessed by a lack of reflex to a light pinch of the hindpaw. Animals were single-housed and returned to clean cages post-surgery, where they were allowed to recover in a thermal incubator (27°C) for half an hour before returning to holding rooms.

Anaesthesia for non-recovery procedures was induced with 4% isoflurane (4L/minute oxygen) until reflexes were lost. Typically, exsanguination occurred *via* cardiac puncture, prior to cervical dislocation.

2.5 Ageing

For experiments examining the influence of αCGRP gene deletion on ageing processes, male αCGRP WT and KO littermate mice were used. Juvenile mice were used at three months of age and aged animals were fifteen months. The mice used were bred onto a C56BL/6J background, which have been shown to live to 815 ± 16 days (~25 months) in standard laboratory conditions (Jackson Laboratories, 2008). Thus, from this, we deemed our mice to be “middle-aged”. We thought this an appropriate time at which to study age-induced vascular inflammation, as middle-age is generally the time at which humans tend to manifest CVD.Mice were maintained on a standard diet throughout and group-housed until *in vivo* experiments commenced, where they were then singly housed for approximately a two-week period.

2.6 Angiotensin II-Induced Hypertension

For the induction of experimental hypertension, male αCGRP WT and KO littermate mice were used at fifteen months of age and a small pilot experiment was performed using male αCGRP WT and KO littermates at three months of age. Mice were group-housed until *in vivo* experiments commenced, where they were then singly housed for approximately one month.

To induce hypertension, Alzet osmotic minipumps (Charles River, UK) were implanted into mice. Minipumps had a 14-day pumping capacity with a drug infusion rate of 0.25 µL/hour. Pumps were filled with either vehicle (0.9% saline) or ATII (Sigma Aldrich, UK) at a concentration adjusted to the body weight of the animal to produce a continuous pumping dose of 1.1 mg/kg/day. Minipumps were implanted subcutaneously in the mid-scapular region *via* a small dorsal incision. Implantation was carried out under isoflurane anaesthesia as described above. Post-implantation, 1 mL saline was administered to retain moisture of the wound and the incision was closed using vicryl-coated sutures (5.0, Ethicon, Johnson and Johnson, UK) in a discontinuous pattern. Post-operative analgesia was administered *via* intramuscular injection of 50 µg/kg buprenorphine (Vetergesic, Alstoe Animal Health, UK). Mice were allowed to recover and then were returned to holding rooms. Hypertension was monitored over a two-week period, followed by termination of the animal.

2.7 Metabolic Caging

Some animals were housed in metabolic cages for the measurement of food and water intake as part of the ATII-induced hypertension model. Mice were singly housed to metabolic cages for 18 hours overnight on the day before minipump surgery and on day 13 of experimental hypertension. Food and water intake was monitored over this 18-hour period and urine excretion measured.

2.8 Tail Cuff Volume-Pressure Recording

Mouse blood pressure was measured using tail cuff volume-pressure recording (VPR) using the CODA 6 acquisition system (Kent Scientific, Torrington, CT, USA). This is a non-invasive system that has previously been validated within our group (Smillie et al., 2014). Furthermore, there is good correlation between results obtained from VPR and those obtained from the gold-standard technique in blood pressure monitoring, radiotelemetry(Feng et al., 2008).

Prior to collecting blood pressure measurements, mice were trained for a period of one to two weeks in order to grow accustomed to the protocol to generate a stable baseline. Once a stable baseline was recorded, experimental data were collected. Mice were restrained in Perspex tubes and held in position with the introduction of a nose cone. Mice were then placed on a heated platform to induce vasodilatation of the peripheral vasculature to promote blood flow, as data collection is dependent on sufficient blood flow. An occlusion cuff was then placed around the base of the tail, followed by the placement of the VPR cuff below it. Measurements then commenced by inflation of the occlusion cuff followed by its gradual deflation over a period of fifteen seconds. The VPR cuff senses changes in tail blood flow associated with changes in occlusion cuff pressure and calculates blood pressure as a result of this. This measurement cycle was performed 20 times on each day of recording, where the first five measurements were excluded to allow for animal acclimatisation. Furthermore, on cycles where the animal showed visible signs of stress (movement or tail flinching), data were excluded. The mean systolic, diastolic and mean arterial pressures of the remaining replicates were calculated for each mouse on each day of recording.

2.9 Pulse Oximetry

Mouse heart rate was determined in a subset of aged animals using peripheral pulse oximetry (Kent Scientific, Torrinton, CT, USA). Mice were restrained in Perspex tubes and held in position with the introduction of a nose cone. An oximetry device was then introduced around the base of the tail to measure heart rate by measuring changes in peripheral skin colouration associated with pulse. Heart rate was measured every 30 seconds over a five minute period and these values were averaged to provide the mean heart rate for each mouse.

2.10 Histology

2.10.1 Preparation of Samples

Mouse aortae were dissected from the animal with perivascular fat attached and fixed in 4% paraformaldehyde (PFA, Sigma Aldrich, UK) for three days. Following fixation, samples were processed into paraffin blocks using an automated tissue processor (Tissue-Tek VIP Vacuum Infiltration processorm Sakura Tissue Tek VIP 1000). Paraffin-embedded tissues were cut into 5 µm sections using a microtome (Reichert-Jung 2030 Biocut microtome). Sections were then floated on water and subsequently mounted onto poly-l-lysine-coated microscope slides (Sigma Aldrich, UK) before leaving to dry overnight.

For staining, sections were deparaffinised in 100% xylene (VWR BDH Prolab International Ltd) for 10 minutes, followed by immersion in 100% ethanol (VWR BDG Prolab International Ltd) for 5 minutes, 95% ethanol for 5 minutes and 70% ethanol for a further 5 minutes. Slides were immersed in distilled water for 5 minutes.

2.10.2 Masson's Trichrome Staining

Aortae were labelled with Masson's Trichrome stain. This is a three-colour stain that identifies cellular cytoplasms as red/pink, cell nuclei as purple and connective tissues as blue. Following deparaffinisation, slides were immersed in Bouin's Solution (Sigma Aldrich, UK) heated to 65°C for 15 minutes. After rinsing with water, slides were then immersed in haemotoxylin for 1 minute, Biebrich scarlet for 1 minute (0.09% Biebrich scarlet, 0.1% fuschin and 1% acetic acid, Sigma Aldrich, UK) and aniline blue for 1 minute (2.4% aniline blue, 2% acetic acid, Sigma Aldrich, UK). The resulting stained sections were then dehydrated through an ethanol gradient (70%, 95% and 100% ethanol for 5 minutes each) and re-immersed in 100% xylene. Slides were then allowed to air-dry prior to addition of DPX mountant (Sigma Aldrich, UK) and a glass coverslip. Slides were then allowed to dry overnight prior to visualization and quantification.

2.10.3 Visualisation and Quantification of Aortic Histology

Aortae were visualised by microscopy, using a colour video camera (Olympus U-CAMD 3 soft imaging system) connected to a light microscope (Olympus BX51). Quantification of aortic morphology was performed using specialised morphometry software (Cell P software, 1986-2007, Imaging Solutions, GmbH). Parameters were measured in four replicate sections per animal and averaged to give the mean measurement for each experimental subject. Smooth muscle and collagen width were measured by taking eight measurements per section at roughly 45-degree angles to each other and then an average of these measurements was taken. Smooth muscle and collagen areas were measured by tracing the internal and external circumferences of the medial and adventitial layers using a free-hand polygon-drawing tool and subsequent calculation of the area by the software from these parameters.

2.11 Myography

Second-order mesenteric arteries (approximately 30-40 µm diameter at rest, 100 µm under tension) were isolated from juvenile and aged male αCGRP WT and KO mice and transferred immediately to ice cold Krebs-Henseleit buffer (118 mM NaCl, 24 mM $NaHCO_3$, 1 mM $MgSO_4$, 4 mM KCl, 0.5mM NaH_2PO_4, 5.5 mM glucose and 2.5 mM $CaCl_2$, Sigma Aldrich, UK). Arteries were cleaned of perivascular fat and mounted under a microscope in a Mulvany-Halpern wire myograph (DMT 610M), using 0.025 mm tungsten wire. Arteries were then stretched to their optimal internal circumference $IC_1 = 0.9xIC_{100}$, where IC_{100} is an estimate of the internal circumference of the vessel under a passive transmural pressure of 100 mmHg (13.3 kPa), in order to obtain optimal conditions for active tension development following pharmacological stimulation. Vessels were maintained throughout the experiment in 37°C Krebs solution, gassed with 5% CO_2 in air.

Tissue viability was assessed by magnitude of peak contractions induced by three 45-second incubations in high-potassium Krebs (38 mM NaCl, 24 mM $NaHCO_3$, 1mM $MgSO_4$, 80 mM KCl and 0.5mM NaH_2PO_4, 5.5 mM glucose and 2.5 mM $CaCl_2$). Contractions of < 1.5 mN were discarded owing to poor tissue viability. Endothelial function was tested using 10 µM carbachol (Sigma Aldrich, UK), where a relaxation of >60% over 1 minute was deemed to indicate preservation of endothelial function. No tissue was found to have endothelial function, by this metric, which was ascribed to handling of the tissue by the experimenter. U46619 (Sigma Aldrich, UK) was used to submaximally preconstrict the artery prior to assessment of endothelial function, using a concentration that induced 80% maximal response, as assayed by a full concentration-response curve. U46619 was chosen as a preconstrictor agonist over another commonly used agonist, phenylephrine, owing to the stable level of tone it produces. Phenylephrine, in my hands, was found to induce quite transient constrictions that were not amenable to interrogating vasodilator function. U46619 produced vasoconstrictions that were very stable over a 20-minute time period. Typically, 10 nM was sufficient to induce 80% maximal constriction.

Cumulative concentration-response curves were constructed to various vasoconstrictor and vasodilator agonists. Vasoconstrictors were added to the bath at half log concentrations every two minutes. Phenylephrine (Sigma Aldrich, UK) was assayed between 100 pM and 10 mM, until a plateau of the response was achieved. U46619 was assayed between 100 pM and 3 µM. Where vasodilators were assayed, vessels were preconstricted submaximally with U46619 and allowed to achieve prolonged tone over a 5-minute period. Following this,

increasing concentrations of either αCGRP (Cambridge Bioscences, UK) or sodium nitroprusside (SNP; Sigma Aldrich) were added to the bath at half log increments for one minute. CGRP was assayed between 100 pM and 1 μM. SNP was assayed between 1 pM and 30 mM. Vehicle controls (water) were used for each drug and were not found to significantly alter vascular tone. Vasoconstrictor responses were either expressed as raw force generated in millinewtons (mN) or as a percentage of the maximal force generated by high potassium Krebs solution. Vasodilator responses were expressed as percentage change in preconstrictor-induced vasoconstriction.

2.12 Plasma CGRP ELISA

Plasma CGRP was measured using an ELISA kit (rat/mouse, Phoenix Pharmaceuticals Inc., USA). This kit detects 0.16-100 ng/mL CGRP. Plasma peptides were extracted using SEP-COLUMNs (Phoenix Pharmaceuticals, USA) before use in the ELISA. Briefly, plasma was acidified with an equal volume of citrate 'Buffer A' (Phoenix Pharmaceuticals, USA). SEP-COLUMNs were then equilibrated with one wash of 'Buffer B' (Phoenix Pharmaceuticals, USA), followed by three washes of Buffer A. Acidified plasma was then loaded onto the column and allowed to filter through the column. Columns were then washed twice with Buffer A and peptide was eluted by washing columns with Buffer B. Peptide eluates were then concentrated by lyophilisation. In this assay, a pre-coated plate contained secondary antibodies raised against an anti-CGRP primary antibody. This antibody was competitively bound by CGRP in the sample/standard or by biotinylated peptides. Where biotinylated peptides were bound, interaction with streptavidin-HRP allowed production of a yellow product from substrate. The intensity of colouration was directly proportional to the amount of CGRP bound to the primary antibody, and thus the concentration found in the extracted plasma sample. This kit displayed 100% cross-reactivity with β-CGRP and thus could not discriminate between both isoforms, leading to observable signal in KO mice. There was no cross-reactivity with other CGRP family peptides and this kit was capable of detecting between 0-100 ng/mL CGRP in plasma.

2.13 Multiplex Cytokine ELISA

Plasma cytokines were assayed in a small subset of aged male αCGRP WT and KO mice using a seven spot multiplex mouse pro-inflammatory assay kit (Meso-Scale Discovery, Gaithersburg, MD, USA). Standard manufacturer's instructions were followed and reagents supplied. Results were expressed as picograms cytokine per mL of sample, calculated against a supplied standard curve. This assay employs a standard sandwich immunoassay format, where specific cytokine capture antibodies are pre-fixed on the specific spots on the base of the well. Cytokine levels were quantified using a cytokine-specific detection antibody labeled with a tag that emits light following electrochemical stimulation. All detection antibodies exhibited less than 1% cross-reactivity with other analytes.

2.14 Cell Culture

Cell culture experiments made use of either HUVEC (HUVEC) or THP-1 monocytic cells. HUVEC were purchased from Lonza, USA; THP-1 cells from ATCC, USA. All cells were maintained in either complete M199 or RPMI-1640 medium at 37°C in a 5% CO_2 humidified incubator. Subculture of HUVEC was performed on 85% confluent cells by washing twice with pre-warmed phospho-buffered saline (PBS) and incubating at 37°C with trypsin-EDTA (1 mL/75 cm^2; PAA Laboratories, L11-004) until complete detachment of cells was achieved (1-2 minutes). Cells were then resuspended in complete M199 and replated. HUVEC were initially expanded to third passage in T175 (175 cm^2) flasks, pre-coated with 10 µg/mL bovine fibronectin (Sigma Aldrich, UK). HUVEC were grown in complete M199 medium, supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin and 2 mM L-glutamine. Further EC supplements were included, as follows: 1 ng/mL EC growth factor (Sigma Aldrich), 3 µg/mL EC growth supplement from bovine neural tissue (Sigma Aldrich), 10 U/mL heparin (Sigma Aldrich) and 1.25 µg/mL thymidine (Sigma Aldrich). All experiments using HUVEC were performed at fourth passage.

Subculture of THP-1 cells in suspension was performed by splitting cells once the initial culture had reached a density of 800,000 cells/mL and reseeding in complete RPMI at a density of 50,000 cells/mL. THP-1 were grown in complete RPMI-1640 medium, supplemented with 10% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine and 0.05 mM β-mercaptoethanol.

2.15 Parallel Plate Flow Chamber Assay

For parallel plate flow chamber experiments, HUVEC were grown in 6-well plastic dishes and were allowed to form a confluent monolayer. Prior to flow assay, HUVEC were stimulated with 10 ng/mL human recombinant TNFα (R&D Systems, UK) or vehicle (ddH_2O) for six hours to induce endothelial activation. Where the effect of CGRP on the leukocyte was being assessed, THP-1 cells were stimulated for 5 minutes with 300 nM CGRP or vehicle (Protocol 1, Figure 7). Where the effect of CGRP on EC biology was being assessed, HUVEC were also stimulated with 300 nM human αCGRP (Bachem, Switzerland) or vehicle (ddH_2O) for 1, 3 or 6 hours (Protocol 2, Figure 8). Following the six-hour incubation, HUVEC medium was supplemented with 25 nM HEPES in order to act as a pH buffer for the duration of the experiment taking place outside the cell culture incubator. Prior to the perfusion experiment, THP-1 cells were labeled with 10 nM CellTracker Green (Molecular Probes, Invitrogen, Paisley, UK) in order to visualise their interactions with the endothelium. THP-1s were then harvested by centrifugation and resuspended in perfusion medium (complete RPMI-1640) at a density of 0.5 million cells/mL.

Figure 7: Timeline of protocol related to stimulation of THP-1 cells with 300 nM CGRP prior to perfusion.

Figure 8: Timeline of protocol related to stimulation of HUVEC with 300 nM CGRP prior to perfusion.

All flow chamber experiments were performed inside an environmental chamber at 37°C and 5% CO_2 (SolentScientific). A Harvard syringe pump (Harvard Apparatus, USA) was used to generate a physiological flow rate of 0.25 mL/minute, which corresponds to a shear stress on the endothelium of 1.24 dyn/cm^2, a value comparable to microvascular shear stress. Leukocyte-EC interactions were visualised using an Olympus IX81 time-lapse inverted fluorescence microscope, connected to a Hamamatsu C10600 ORCA-R2 digital video camera. Images were acquired as video files from a CCD camera with video recorder, using Volocity Imaging Software, Perkin Elmer. The parallel plate flow chamber assay was assembled and performed as follows, and illustrated in Figure 9:

1. A silicon gasket was attached to the plastic flow chamber, comprised of inlet/outlet ports and a vacuum port to allow for firm attachment to the cell culture dish.
2. Inlet/outlet and vacuum tubes were attached to their respective ports and the vacuum pump was switched on.
3. Perfusion medium was washed through the inlet tube and drawn through the outlet tube to remove air bubbles. The flow chamber was then lowered onto the dish and the chamber was fixed onto the culture *via* vacuum. The syringe pump was then switched on and medium was allowed to perfuse the endothelium.
4. Using phase-contrast microscopy, three regions of the endothelial monolayer were pre-selected as suitable platforms for leukocyte recruitment, based on a confluency of >90%. This led to unbiased quantification of leukocyte adhesion as regions were selected prior to perfusion of white blood cells.
5. The inlet tube was then switched to a bijou containing the THP-1 cells and this was considered time zero of the flow experiment. Leukocyte-EC interactions were recorded from this time using a x4 objective. Three frame-views per minute were acquired for the phase contrast and GFP channels to visualise the endothelium and THP-1s, respectively. Adherent cells were then counted manually throughout the course of the experiment, up to a period of fifteen minutes perfusion.

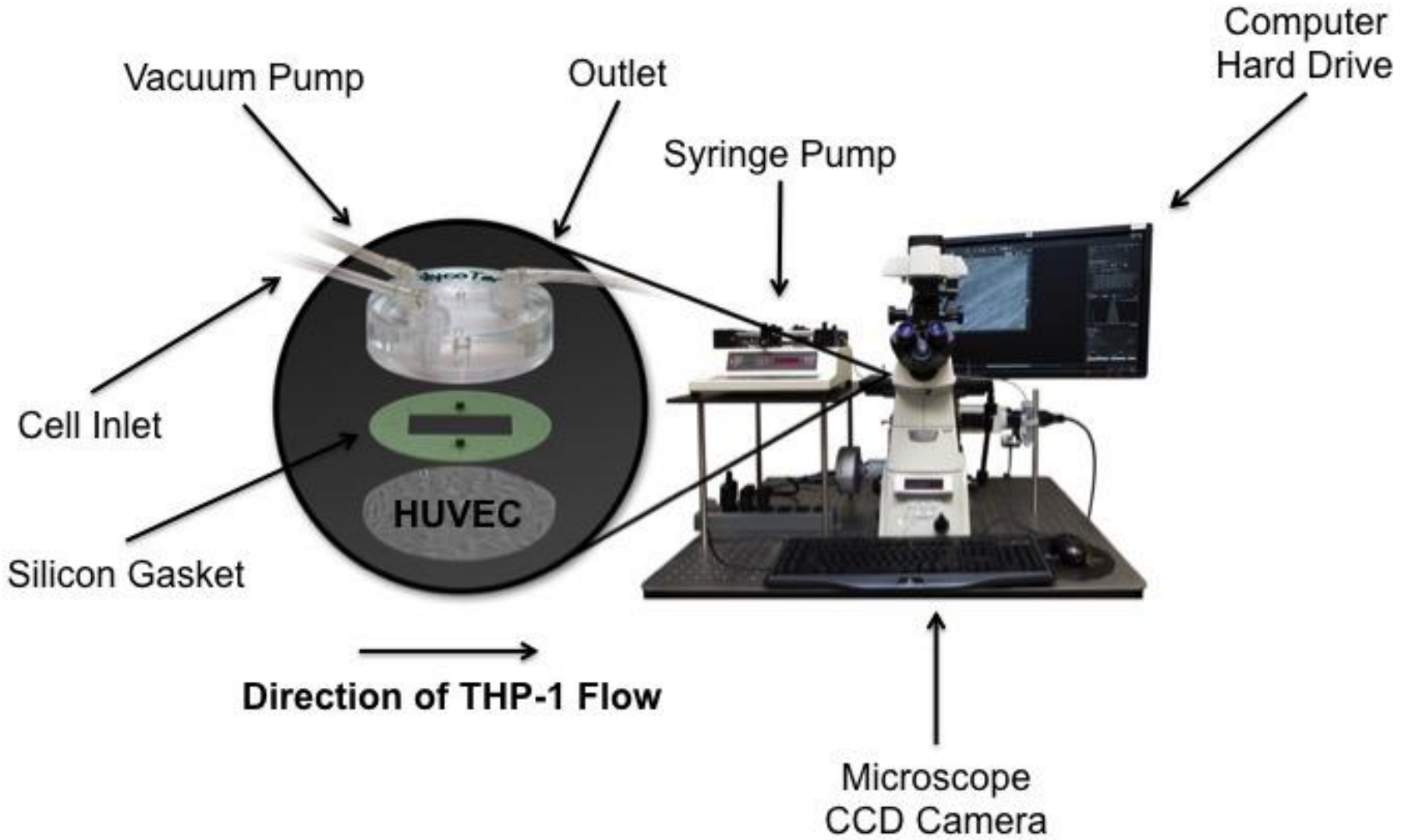

Figure 9: Figure illustrating flow chamber assay setup. Top part of the chamber containing inlet/outlet ports and vacuum pump tube was attached to the silicon gasket. Inlet/outlet tubes and vacuum pump tube were then attached to their respective ports. Vacuum force was then applied and the chamber was then placed onto the HUVEC monolayer until a vacuum was achieved. The attached chamber was then was placed over the objective of an inverted fluorescence microscope in an environmental chamber heated to 37°C and maintained with 5% CO_2. A syringe pump applied suction force that drew the THP-1 cell suspension through the chamber at a specified flow rate. The flow-through waste medium containing unbound cells was collected in an effluent syringe. Video footage of the microscope image was recorded by a CCD camera with video recorder, connected to the computer, acquired with Volocity visualization and analysis software.

2.16 Analysis of gene expression using real-time quantitative polymerase chain reaction (RTqPCR)

2.16.1 RNA isolation and purification

Total tissue RNA was extracted and purified from samples using Qiagen Microarray RNA extraction kits (Qiagen, UK), in accordance with the manufacturer's instructions. Samples to be processed for RNA extraction were dissected with clean tools and placed immediately into 500 µL RNA*later* solution (Applied Biosystems, Life Technologies Ltd, Paisley, UK) for a minimum of 24h at 4°C, followed by long-term storage at -80°C until required.

During RNA extraction, tissue samples were removed from RNA*later* and placed into a microcentrifuge tube containing 700 µL QIAzol reagent and a 5mm stainless steel bead. Samples were then homogenised using a Tissue Lyser II LT for 2 minutes at 20Hz. 200 µL chloroform was then added to each sample, followed by centrifugation at 12,000 rpm for 15 minutes at 4°C to induce organic phase separation. The upper aqueous phase containing nucleic acids was then sequentially purified and eluted with silica-based spin columns for RNA extraction using ethanol (70% in RNAse/DNAse-free water) and wash buffers (phosphate buffers: RW1 and RPE which facilitate binding of nucleic acid to silica columns and allow excess ethanol and unwanted cellular components to be eluted and discarded). 40 µL RNA was then eluted in nuclease-free water and stored at -80°C until required.

2.16.2 Quantification of RNA yield

RNA concentration and purity were determined by use of a spectrophotometer (Nanodrop 1000, Thermoscientific, UK). 1 µL sample was loaded onto the spectrophotometer and absorbance was measured at 260nm, allowing RNA concentration to be calculated on the basis that 1 unit of absorbance corresponds to 40 µg/mL RNA. The ratio of absorbance at 260/280nm gave an estimate of RNA purity, with regards to contamination by protein (typically giving an absorption peak at 280nm). The ratio of absorbance at 260/230nm gave an estimate of organic solvent contamination (i.e. ethanol, absorbing at 230nm). Where RNA did not seem 'clean', it was cleaned up *via* ethanol precipitation. If samples still had evidence of impurity following ethanol cleanup, samples were not used for further analysis.

2.16.3 Reverse Transcription

RNA (500ng) was reverse-transcribed into complimentary DNA (cDNA) using the High Capacity RNA to cDNA kit (Applied Biosystems, UK), in accordance with manufacturer's instructions. Total RNA was aliquoted in nuclease-free water to produce the required concentration. RNA was then reverse-transcribed using a thermal cycler (DNA Engine Tetrad 2 Peltier Thermal Cycler) at 37°C for 60 minutes, 95°C for 5 minutes and then held at 4°C for a minimum of 5 minutes. cDNA was then stored at -20°C before use in RTqPCR experiments. Samples that underwent the reverse transcription reaction without active enzyme were used as negative controls, to prove that signal observed in later experiments was dependent on the formation of cDNA.

2.16.4 RTqPCR

Real time quantitative PCR (RTqPCR) was performed using aforementioned cDNA samples using a SybrGreen-based PCR mix (Sensi-Mix, SYBR-green no ROX, Bioline, UK). Briefly, a 10 µL reaction mix was created using 5 µL SYBR Green, 0.5 µL of forward and reverse primers (stock concentration 10 µM), 2 µL nuclease-free water and 2 µL of sample cDNA.

Target genes were amplified using a three-step program in a PCR thermocycler (Rotor-Gene 6000, Qiagen) under the following conditions: (1) the polymerase enzymes were first activated by heating samples to 95°C for 10 minutes, (2) then subjected to 40 cycles of 95°C for 10s to denature strands, 57°C for 15 seconds to anneal primers to target cDNA and 72°C for 10 seconds to amplify product. (3) Samples were then finally melted at 68-90°C. Details of primers used and their respective PCR products are summarised in Table 3. Target gene expression was expressed as copies/µL sample and standardised to the internal reference genes GAPDH, β-actin and PLA_2. Efficiency of the RTqPCR reaction was assessed by examination of the product melt curve to ensure only one PCR product was formed. Using GeNorm v1.2 software, a normalisation factor for each sample was obtained, based on calculation of the geometric mean of the reference genes analysed (Vandesompele et al., 2002). RTqPCR was performed on all samples simultaneously to minimise experimental artefacts related to sample/internal standard degradation, variations in sample preparation and efficiency of PCR reactions.

	Forward Sequence	Reverse Sequence	Amplicon Size (bp)
αCGRP	AGCAGGAGGAAGAGCAGGA	CAGATTCCCACACCGCTTAG	71
βCGRP	CCTGCAGGCCTGAGTCAC	GGCATGGTGAGTTCAACTTTATG	64
CLR	CTCCTGAGACTATTCCCACAGAA	CAAGATGTTGCTGTATCATCATAGG	72
eNOS	GACCCTCACCGCTACAACAT	GTCCTGGTGTCCAGATCCAT	62
F4/80	CAACCTGCCACAACACTCTC	AGTCCTGAGTTGCACGTACA	52
HO-1	GGTCAGGTGTCCAGAGAAGG	CTTCCAGGGCCGTGTAGATA	70
HPRT-1	TCCTCCTCAGACCGCTTTT	CCTGGTTCATCATCGCTAATC	90
MCP-1	ACTGAAGCCAGCTCTCTCTTCCTC	TTCCTTCTTGGGGTCAGCACAGAC	274
MPO	CCTGGAGTCAATCGCAATGG	TCCTTGGTCAGCTGATCGTT	92
NOX2	TGCCAACTTCCTCAGCTACA	GTGCACAGCAAAGTGATTGG	73
NOX4	GAACCCAAGTTCCAAGCTCA	AAGGCACAAAGGTCCAGAAA	63
PLA2	TGGATATAAACCATCTCCACCA	GGGAAGGGATACCTATGTTCAGA	77
p16	CCCGCCTTTTTCTTCTTAGC	TTCTCATGCCATTCCTTTCC	172
p53	ACAGTCGGATATCAGCCTCG	GCTTCACTTGGGCCTTCAAA	159
RAMP1	TGACTATGGGACTCTCATCCAG	CCTGCTTGGTGCAGTAAGTG	137
RAMP2	CTGAGGACAGCCTTGTTGTCA	GGCATCGCTGTCTTTACTCC	374
SDHA	CCCTGAGCATTGCAGAATC	TCTTCTTCCAGCATTTGCCTTA	70
TRPV1	CAACAAGAAGGGGCTTACACC	TCTGGAGAATGTAGGCCAAGAC	77
VCAM-1	TGGTGAAATGGAATCTGAACC	CCCAGATGGTGGTTTCCTT	86

Table 3: List of primer sequences used in RTqPCR experiments, including forward and reverse sequences and target amplicon size.

2.17 Analysis of gene expression using transcriptome array

Microarray experiments were performed in collaboration with Dr. Matthew Arno of the Genomics Centre, King's College London. RNA extraction from HUVEC cultures was done personally and Dr. Matthew Arno performed subsequent experimental steps leading up to data analysis. We also thank Dr. Matthew Arno for assistance in data analysis.

Briefly, total RNA was extracted from HUVEC treated in a similar manner to those used in flow chamber assays, with respect to CGRP and TNFα, using a Qiagen RNeasy Plus Mini Kit extraction kit. HUVEC monolayers were washed twice with ice-cold PBS and then lysed directly using 350 µL Buffer RLT plus to cover the surface of the dish. The lysate was then passed through a 20-gauge needle fitted to an RNase-free syringe to shear genomic DNA. The lysate was then transferred to a gDNA eliminator spin column and centrifuged for 30 seconds at 10,000 rpm prior to the addition of an equal volume of 70% ethanol. The sample was then transferred to an RNeasy spin column and centrifuged for 15 seconds at 10,000 rpm. Following subsequent washes with Buffer RW1 and Buffer RPE, RNA was eluted from the spin column in 40 µL RNase-free water and stored at -80°C prior to use.

Total RNA integrity was analysed using Agilent RNA 6000 Nano Kit and Agilent 2100 Bioanalyser (Agilent Technologies, USA), in accordance with the manufacturer's protocol. RIN values representing RNA integrity were obtained and samples with RIN values >8 were deemed acceptable for use in microarray experiments. 500 ng RNA was then reverse-transcribed into cDNA using the High Capacity RNA to cDNA kit (Applied Biosystems, UK), in accordance with manufacturer's instructions. Total RNA was aliquoted in nuclease-free water to produce the required concentration. RNA was then reverse-transcribed using a thermal cycler (DNA Engine Tetrad 2 Peltier Thermal Cycler) at 37°C for 60 minutes, 95°C for 5 minutes and then held at 4°C for a minimum of 5 minutes. cDNA was then stored at -20°C before use in microarray experiments. HUVEC cDNA was then labeled and fragmented and then used to hybridise Affymetrix GeneChip Human Transcriptome Array 2.0. Chips were then washed and stained using the GeneChip Fluidics Station 450, in accordance with manufacturer's instructions. Following staining, the Affymetrix GeneChip Scanner (GCS3000) was used to scan the chips. Data generated by chip scanning was normalised, summarized and background corrected using the standard gene-level Robust Multi-array Average (RMA) method, as included in the Expression Console package from Affymetrix. For analysis of array data, two-tailed student's t-test was applied, using the Benjamini and Hochberg false discovery rate (FDR) multiple testing correction, in order to limit the detection of false

positives from multiple testing. For this, a FDR threshold of q = 0.05 was used. Here, the p-values of differences in expression of each gene are ranked from smallest to largest. The largest p-value remains as it is, whilst the second largest p-value is multiplied by the total number of genes identified, divided by its rank in the list and so on throughout the ranked list. If this value is less than 0.05, it is considered to be a statistically significant effect. Differentially-expressed genes were then enriched into known biological pathways using MetaCore GeneGo software (Thomson Reuters), in order to understand better how observed changes related to biologically-meaningful phenomena. Enrichment pathways were ranked according to the likelihood that genes identified in microarray analysis appeared in pre-defined networks. The hypergeometric mean was used to take into account the number of objects appearing in the microarray dataset, the number of objects in the intersecting networks and the number of objects in the entire network database. This returned a p-value that showed the likelihood that the intersection between the microarray dataset and a particular network was obtained purely by chance. As some p-values were classed as being very significant, these values were multipled by $-\log_{10}$ for ease of visualisation in a histogram. The top 10 most significantly enriched pathways were then ranked and graphed.

2.18 Western Blotting

2.18.1 Sample Preparation

Tissue samples were dissected from mice and snap frozen in liquid nitrogen, prior to storage at -80°C. Tissues were then homogenised in 700 µL sodium dodecyl sulphate (SDS) lysis buffer (50 mM Tris base pH 6.8, 10% glycerol and 2% SDS) supplemented with 0.02% protease inhibitor cocktail (4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, bestatin, E-64, leupeptin and aprotinin) and phosphatase inhibitor (1 tablet/10 mL PhosSTOP, Roche, UK) in a microcentrifuge tube containing a 5 mm stainless steel bead, using a Tissue Lyser II LT (2-5 minutes, 30Hz). Tissue homogenates were centrifuged at 13,000rpm for 15 minutes at 4°C and supernatants were then collected and stored at -20°C. Cell culture samples were collected on ice by the addition of 200 µL lysis buffer, followed by mechanical disruption before transferring to a microcentrifuge tube.

2.18.2 Determination of protein concentration

To determine sample protein content, the BCA assay was performed using the BioRad Reagent A/Reagent B kit (Bio-Rad, UK). To generate a standard curve to calculate sample concentration, bovine serum albumin (BSA, 5 µL of 0.1-2 mg/mL) was dissolved in double-distilled water. Samples were diluted 1:10 in ddH_2O if found to be too concentrated. Both standards and samples were loaded in duplicate into clear wells of a 96-well microplate and incubated with 25 µL Reagent A and 200 µL Reagent B. The plate was placed on a microplate shaker and incubated for 15 minutes at room temperature. Following this, the plate was read at an absorbance spectrum of 750nm on a plate spectrophotometer (SpectraMAX 190, SOFTmaxPRO software v3.13, Molecular Devices Corporation, California, USA). Protein standard curves were generated by expressing the mean optical density values obtained at 750nm relative to the known protein concentration of the BSA standard. Standard curves with correlation coefficients of > 0.90 were acceptable for use. Sample protein concentration was calculated by interpolation of OD against the standard curve.

2.18.3 SDS-PAGE

Following quantification of protein concentration, 30 µg of protein was reduced with 10% 2-mercaptoethanol and 0.2% bromophenol blue used as a sample marker. Protein samples were then boiled at 95°C for 5 minutes to denature. Samples were then loaded alongside a molecular weight marker into lanes of a pre-set SDS-PAGE gel prior to electrophoresis. The gel was comprised of a lower resolving gel (10% polyacrylamide of composition: 3 mL 30% acrylamide (National Diagnostics, UK); 2.25 mL 0.5M tris-HCl (pH 8.8); 3.75 mL of ddH_2O; 45 µL 10% ammonium persulphate and 15 µL tetramethyethyldiamine) and an upper stacking gel (5% polyacrylamide of composition: 1.75 mL ddH_2O; 750 µL Tris-HCl pH 6.8; 500 µL of 30% acrylamide, 25 µL 10% ammonium persulphate and 10 µL tetramethylethyldiamine). The final concentration of acrylamide in the resolving gel was altered depending on the mass of the protein of interest. 12% gels were run for proteins of mass 20-60 kDa, 10% gels were run for proteins of 60-100 kDa and 8% gels were run for proteins of >100 kDa. SDS PAGE was performed in running buffer (10% tris-glycine buffer (BioRad, USA), 0.5% 20% SDS pH 7.2 in ddH_2O) at 150V for approximately 1.5-2 hours.

2.18.4 Immunoblotting

Following SDS-PAGE, gels were allowed to equilibrate in transfer buffer (10% Tris-glycine buffer (BioRad, USA), supplemented with 20% methanol (Fisher Scientific, UK) in ddH_2O). Polyvinyldiene difluoride (PVDF) membranes were activated in methanol for one minute and then equilibrated in transfer buffer. Gels and membranes were assembled into a sandwich and transferred to a semi-dry electrophoretic transfer apparatus (BioRad, USA). The separated proteins were then electro-transferred onto the PVDF membrane at 20V for 2 hours whilst bathed in transfer buffer.

Following transfer of proteins to PVDF, the membrane was rinsed in PBS to remove excess transfer buffer. Then, the membrane was blocked with 5% skimmed milk powder in phospho-buffered saline (PBS) plus 0.01% Tween-20 (PBST) for 1 hour at room temperature, in order to block non-specific binding sites present on the membrane. Membranes were then washed for 15 minutes in PBS followed by alternating 5-minute washes of PBS/PBST/PBS. Primary antibodies were diluted in 5% milk in PBS and were either incubated for 1h at room temperature or overnight at 4°C. Membranes were then washed in alternations of PBS/PBST in accordance with the aforementioned washing protocol.

Subsequently, horseradish peroxidase-conjugated secondary antibodies were incubated with the blot for 1h at room temperature, followed by another 30 minute wash procedure to remove excess. A list of primary and secondary antibodies used throughout this thesis is shown in Table 4.

For protein detection, the membrane was incubated with 1 mL enhanced chemiluminescence solution (Millipore, UK) for 60 seconds and then exposed to photographic film. Photographic films were scanned into a computer and densitometric image analysis was performed using ImageJ software (v1.32, NIH, USA). Proteins were usually expressed as arbitrary densitometric units, relative to an internal housekeeping protein, such as β-actin. Where phospho-specific antibodies were used, these proteins were normalised to the total expression of the protein in question.

Antibody	Manufacturer	Catalogue No	Species	Dilution
Anti-β-actin	Sigma Aldrich	A1978	Mouse	1 : 2000
Anti-4HNE	Abcam	ab46545	Rabbit	1 : 2000
Anti-VCAM-1	Santa Cruz	sc-8304	Rabbit	1 : 1000
Anti-eNOS	Santa Cruz	sc-654	Rabbit	1 : 500
Anti-phospho-eNOS	Cell Signalling	95715	Rabbit	1 : 500
Anti-JAM-A	Abcam	ab52647	Rabbit	1 : 1000
Anti-JAM-C	Abcam	ab89665	Mouse	1 : 1000
Anti-Nitrotyrosine	Abcam	ab61392	Mouse	1 : 500
Anti-PECAM-1	Santa Cruz	sc-1506	Goat	1 : 1000
Anti-Goat	Dako	P0449	Rabbit	1 : 2000
Anti-Mouse	Dako	P0260	Rabbit	1 : 2000
Anti-Rabbit	Dako	P0448	Goat	1 : 2000

Table 4: Table detailing primary and secondary antibodies used throughout this thesis, including target protein, manufacturer, catalogue number, species and working dilution used.

2.19 Data Analysis

Data were analysed in Microsoft Excel and GraphPad Prism 5.0, expressed as mean ± standard error of the mean (SEM). Where groups had smallsample sizes (i.e. 3-5), the SEM is used as a predictive value to illustrate data variation only.In this case, SEM is shown for these groups in order to allow illustration of data variability in a similar manner to the rest of the data shown in this thesis. All data sets were tested for normality using the Shapiro-Wilks test. Where they conformed to Gaussian distribution, data were analysed using the appropriate parametric test. Where data were found to be non-normally distributed, non-parametric tests were used. Specific statistical tests used for each data set are specified in each figure legend.

3. | Chapter Three: Characterisation and Phenotyping of the Ageing αCGRP WT and KO Mouse

3.1 Introduction

As discussed in the general introduction, relatively few papers have been published concerning the role of CGRP in regulating (patho)physiological processes as part of the ageing process. Of these, none have used the CGRP KO mouse to address this research question. As a result, there is little information concerning how αCGRP gene deletion affects parameters of growth and development in the mouse. However, in a major study by Walker *et al.,* data suggest that by 3 months of age, αCGRP KO mice begin to be protected against diet-induced obesity when compared to their WT counterparts. This was attributable to a lowered degree of general adiposity and improved overall metabolic health (Walker et al., 2010b). However, this study only followed the growth of these mice until around 8 months of age, which may not be considered as truly geriatric. Development of other organs essential to cardiovascular regulation has not been studied in the ageing αCGRP mouse.

There is a larger body of evidence that considers the changing dynamics of CGRP at the mRNA, protein and functional level as the organism ages. Many of these studies use the SHR model of hypertension and thus it is unclear as to whether CGRP is altered as a result of ageing, hypertension or a combination of both. The general consensus here, however, appears to be that CGRP expression within its principle site of synthesis (i.e. the DRG) declines as the SHR ages (Yamaga et al., 2001). As the animal becomes more hypertensive, the innervation of CGRP-positive nerve fibres in the vascular environment is also thought to decrease (Kawasaki et al., 1990a; 1991). Thus, less releasable CGRP is present (Kawasaki and Takasaki, 1992; Sun et al., 1998). This reduction in bioavailability may be reflected in the bloodplasma compartment, although this is currently unknown. Accompanying alterations in CGRP bioavailability with age may also be coupled with changes in functionality. Indeed, Chan & Fiscus have shown that caudal arteries and aortae isolated from elderly rats are significantly less capable of mounting a vasorelaxant response to exogenous CGRP both *in vitro* and *in vivo* (Chan and Fiscus, 2002). Despite being an interesting phenotype, this study has yet to be reproduced and the mechanism governing this attenuation in bioactivity is hitherto unknown. It is known that vascular reactivity to CGRP can be altered in CVD states, such as in a model of subtotal nephrectomy-salt induced hypertension. In this, the vascular response to CGRP is enhanced due to increased vascular expression of the RCP protein (Supowit et al., 2011). Thus, it is conceivable that changes in CGRP bioactivity may be related to alterations in receptor expression at the vascular level. At this stage, it is unknown how CGRP expression and functionality

is regulated by ageing in isolation and, indeed, how it is regulated over time in the mouse. Secondary to this, there is no current information on how CGRP receptor expression is altered as part of the ageing process.

3.2 Hypothesis

Deletion of αCGRP will not affect gross parameters of growth and development as mice age from 3 months to 15 months. We hypothesise that expression of CGRP at the gene and protein level will decline with age and this will lead to a change in vascular reactivity to known vasoconstrictors and dilators.

3.3 Aims

1. To investigate the role of αCGRP gene deletion on mouse growth and development in young and aged αCGRP WT and KO mice.
2. To investigate how the dynamics of αCGRP gene and protein expression are regulated in young and aged αCGRP WT and KO mice.
3. To investigate how vascular reactivity is altered in resistance vessels obtained from young and aged αCGRP WT and KO mice and how expression of CGRP receptor components may contribute to vasoactive responses.

3.4 Results

3.4.1 Developmental characteristics of the ageing αCGRP WT and KO mouse

In order to evaluate the effect of αCGRP gene deletion on gross developmental parameters with advancing age, mice were weighed at 3 and 15 months of age. Figure 10A shows that total body weights of juvenile WT and KO mice were comparable (27.80 ± 0.58 g *vs* 26.95 ± 0.40 g, respectively) and body weight increased significantly as both WT and KO mice aged to 15 months, although no differences were detected between WT and KO (42.91 ± 3.73 g *vs* 49.30 ± 3.22 g, respectively). We also sought to determine how body fat composition changed with age by weighing of the epididymal adipose tissue. Figure 10B demonstrates that epididymal fat content is similar between juvenile WT and KO mice (241.90 ± 31.23 mg *vs* 265.75 ± 27.04 mg, respectively) and significantly increased in mass with advancing age in both genotypes, with no significant differences between aged WT and KO (2436.71 ± 339.99 mg *vs* 2771.66 ± 711.92 mg, respectively). In order to exclude increased fat content as a function of increased body weight, Figure 10C illustrates how fat content changes when expressed as a ratio to body weight. In this case, fat content is significantly increased in both WT and KO mice as they age to 15 months. This indicates that animals have more fat as they get older, regardless of body size. Again, no differences were detected between WT and KO mice at either timepoint.

Aside from body weight/fat content analysis, we examined how the growth of some organs was affected by CGRP gene deletion, both at 3 and 15 months of age. We found juvenile WT and KO animals had similar wet heart weights (136.40 ± 6.27 mg *vs* 141.92 ± 4.96 mg, respectively) that increased significantly as the animals aged (203.54 ± 18.35 mg *vs* 201.66 ± 11.22 mg, respectively) (Figure 11A). When heart weight was expressed as a ratio to body weight, this difference was not apparent, indicating that hearts grew in line with increases in body weight (Figure 11B). Similar results were observed for left andright kidney weights (Figures 12A-D) and spleen weights (Figures 13A & 13B).

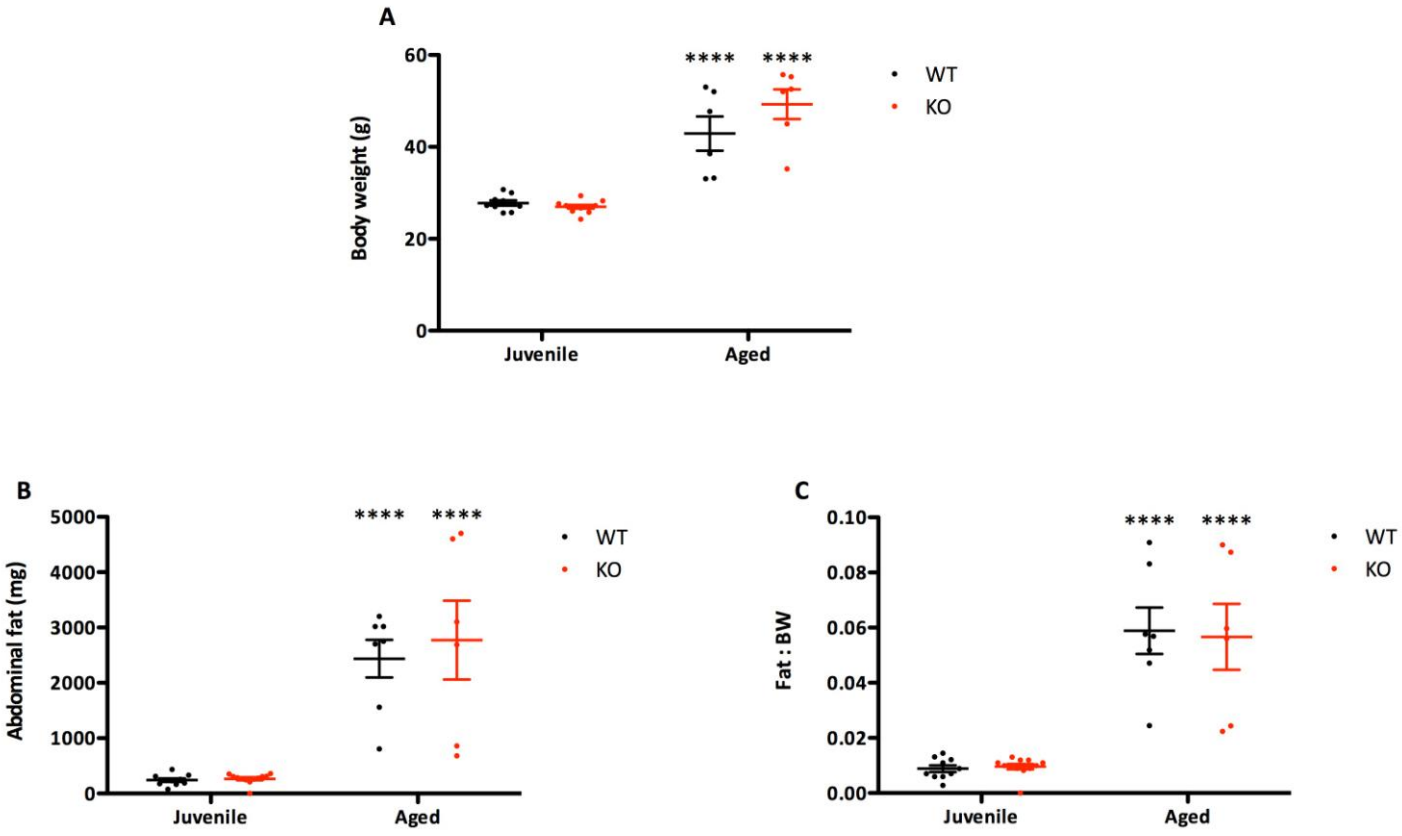

Figure 10: Growth parameters of juvenile (3 months) and aged (15 months) αCGRP male WT and KO mice. Results show A) total body weights of male juvenile and aged αCGRP WT and KO mice; B) total epididymal fat weight of male juvenile and aged αCGRP WT and KO mice; C) epididymal weight expressed as a function of body weight of male juvenile and aged αCGRP WT and KO mice. Data expressed as mean ± SEM, n = 6-12. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. ****p < 0.0001 where juvenile and aged animals of the same genotype have been compared.

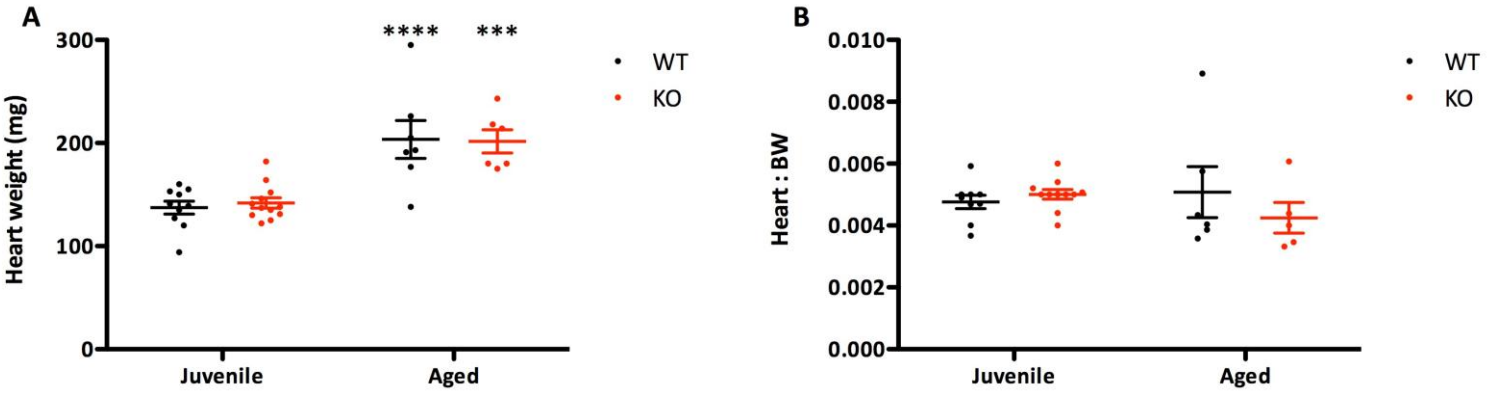

Figure 11: Heart weights of juvenile (3 months) and aged (15 months) αCGRP male WT and KO mice. Results show A) total heart weights of male juvenile and aged αCGRP WT and KO mice; B) heart weight expressed as a function of body weight of male juvenile and aged αCGRP WT and KO mice. Data expressed as mean ± SEM, n = 6-12. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. ***p < 0.001; ****p < 0.0001 where juvenile and aged animals of the same genotype have been compared.

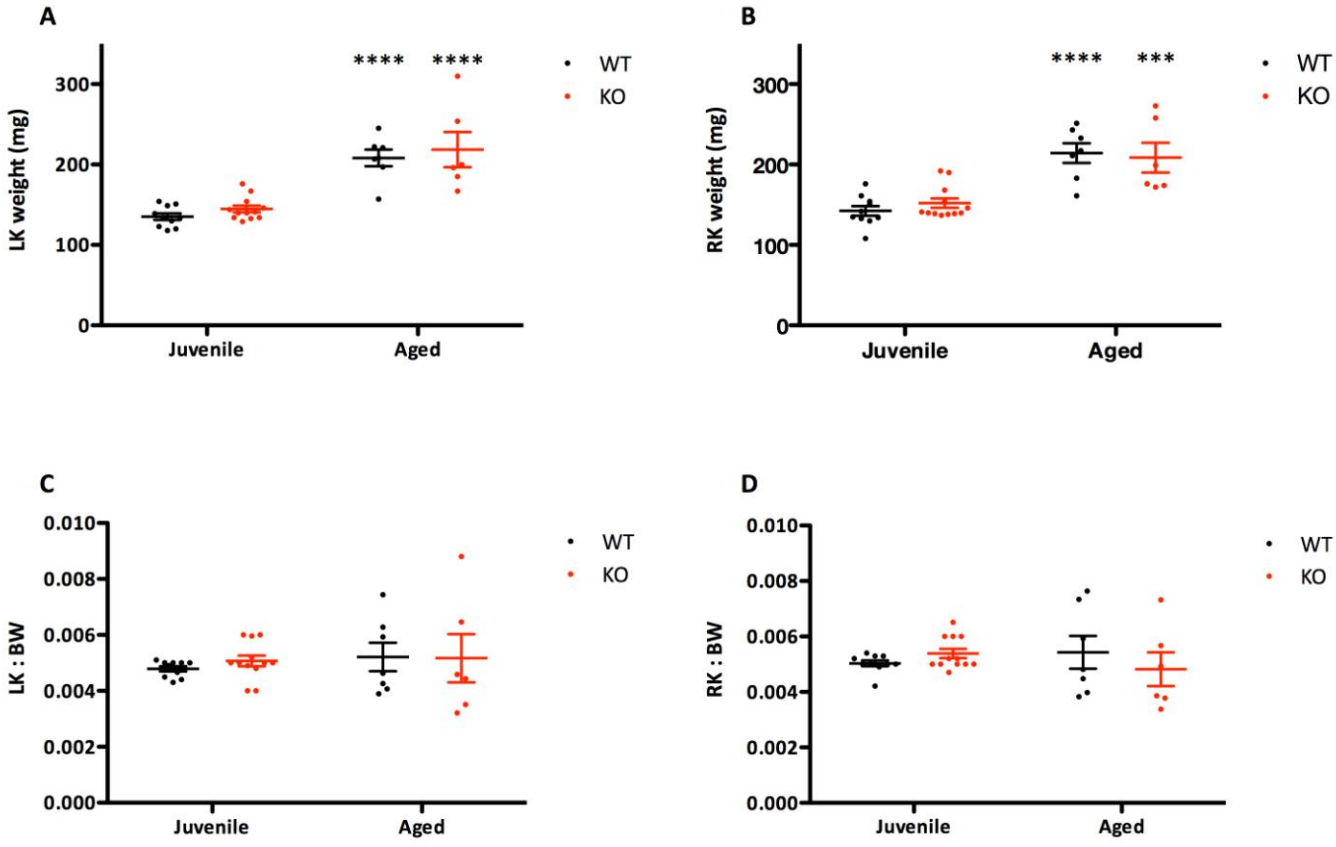

Figure 12: Left and right kidney weights of juvenile (3 months) and aged (15 months) male αCGRP WT and KO mice. Results show A) total left kidney weights of male juvenile and aged αCGRP WT and KO mice; B) left kidney weight expressed as a function of body weight of male juvenile and aged αCGRP WT and KO mice; C) total right kidney weights of male juvenile and aged αCGRP WT and KO mice; D) right kidney weight expressed as a function of body weight of male juvenile and aged αCGRP WT and KO mice. Data expressed as mean ± SEM, n = 6-12. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. ***p < 0.001; ****p < 0.0001 where juvenile and aged animals of the same genotype have been compared.

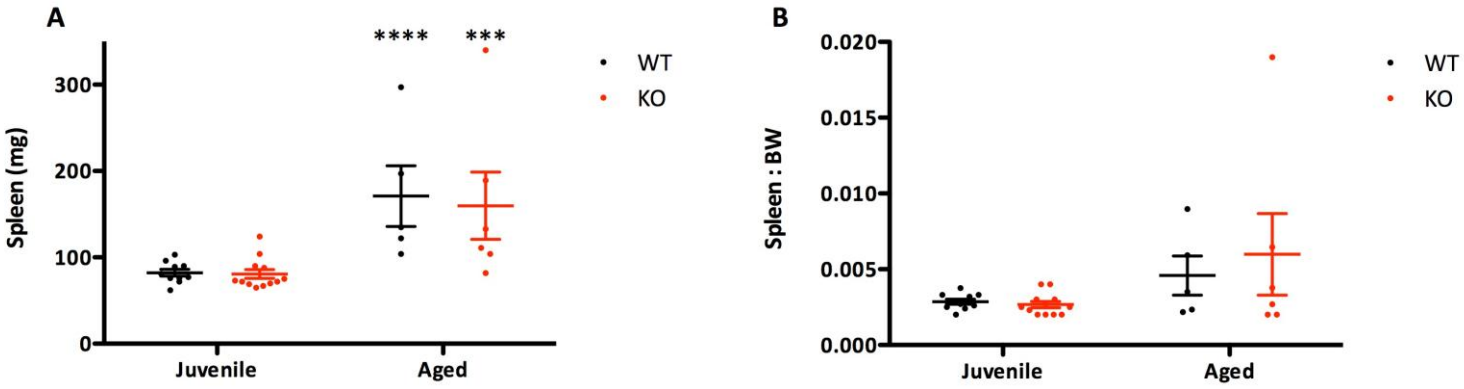

Figure 13: Spleen weights of juvenile (3 months) and aged (15 months) male αCGRP WT and KO mice. Results show A) total spleen weights of male juvenile and aged αCGRP WT and KO mice; B) spleen weight expressed as a function of body weight of male juvenile and aged αCGRP WT and KO mice. Data expressed as mean ± SEM, n = 6-12. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. ***p < 0.001; ****p < 0.0001 where juvenile and aged animals of the same genotype have been compared.

3.4.2 Temporal dynamics of CGRP mRNA and peptide expression in the ageing αCGRP WT and KO mouse

We investigated how CGRP gene and protein expression changed as a function of age. Figure 14 shows data obtained from a CGRP ELISA performed on plasma samples extracted from juvenile and aged αCGRP WT and KO mice. The antibody used in this assay cannot discriminate between CGRP isoforms and thus detects total CGRP circulating in the plasma. We found that circulating levels of CGRP are comparable in juvenile WT and KO animals (294.42 ± 81.34 ng/g *vs* 274.08 ± 77.87 ng/g, respectively). These levels did not change as animals grew older and there were no significant differences between genotypes (221.75 ± 61.21 ng/g *vs* 263.50 ± 70.65 ng/g, respectively).

Therefore, further experiments were carried out to examine the isoform-specific expression of CGRP at the mRNA level within DRG, which may have more relevance than measuring CGRP in the plasma. Figure 15A confirms that αCGRP KO mice do not express any mRNA for αCGRP within the DRG. However, we have shown that αCGRP gene expression within the DRG is significantly elevated in juvenile *vs* aged WTs (383.75 ± 65.23 copies/µl *vs* 3157.50 ± 1015.05 copies/µl). Figure 15B illustrates how βCGRP gene expression alters with age in αCGRP WT and KO mice. This figure shows that there is a significant compensatory upregulation of βCGRP DRG transcript in juvenile αCGRP KO mice (2172.50 ± 773.43 copies/µl in WT *vs* 13491.67 ± 2082.54 copies/µl in KO). βCGRP levels were high and similar in aged WT and KO mice (13491.00 ± 2082.53 copies/µl *vs* 10470.00 ± 4504.10 copies/µl, respectively), although they did not differ significantly from juveniles.

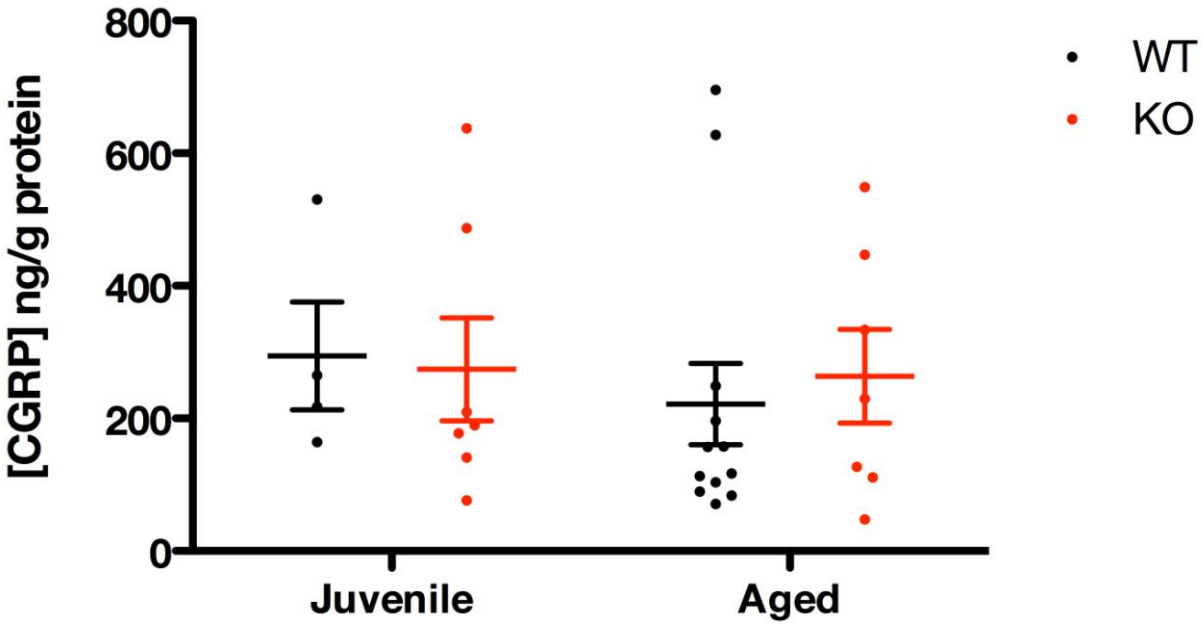

Figure 14: Total plasma immunoreactive CGRP obtained from juvenile (3 months) and aged (15 months) male αCGRP WT and KO mice. Data expressed as nanograms CGRP per gram of protein, mean ± SEM, n = 4-12. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. No significant differences were detected between experimental groups.

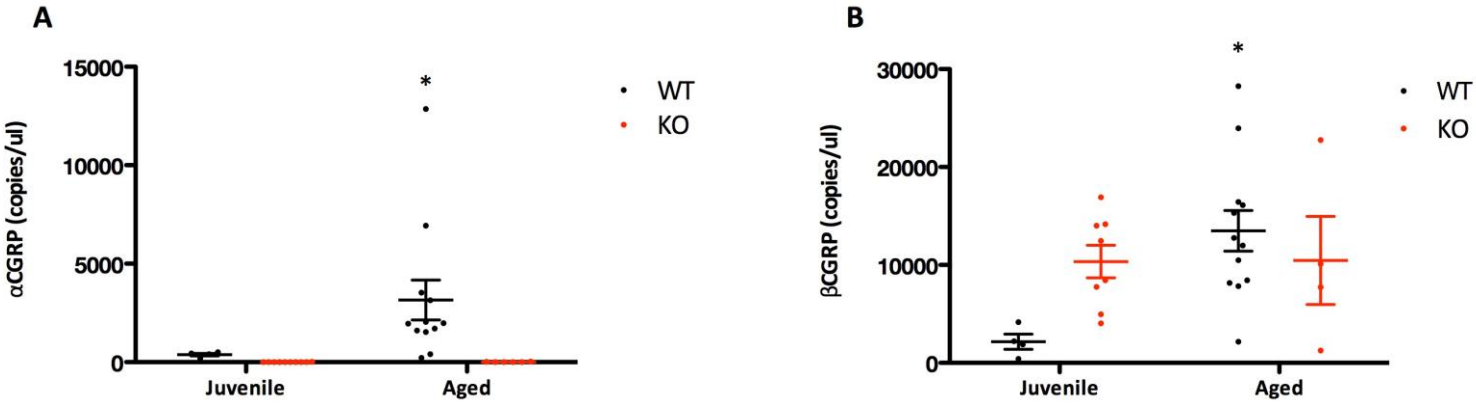

Figure 15: mRNA expression of α and βCGRP isoforms in DRG of juvenile (3 months) and aged (15 months) male αCGRP WT and KO mice. Results show A) αCGRP mRNA expression in DRG obtained from male juvenile and aged αCGRP WT and KO mice; B) βCGRP mRNA expression in DRG obtained from male juvenile and aged αCGRP WT and KO mice Data expressed as copies per microliter, mean ± SEM, n = 4-13. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. *p < 0.05 where juvenile and aged animals of the same genotype have been compared.

3.4.3 Evaluation of vascular reactivity of mesenteric resistance arteries in the ageing αCGRP WT and KO mouse

Phenotyping of vascular reactivity in juvenile and aged αCGRP WT and KO mice was performed *via* small vessel wire myography. Initial studies illustrated in Figure 16 show the differential vascular response to 80 mM K^+ stimulation. Juvenile WT and KO mice responded similarly to this stimulus, producing a force of 3.57 ± 0.41 mN *vs* 3.89 ± 0.34 mN, respectively. The response to high potassium was significantly increased in both WT and KO animals as they aged, with no difference between genotypes (5.36 ± 0.39 mN *vs* 5.04 ± 0.25 mN, respectively). Figure 17 shows endothelial-dependent vasodilatory responses to 10 μM carbachol, where we show that none of the vessels used in our experiments produced >60% vasodilatation to this agonist, indicating that the endothelium was likely mechanically ruptured during preparation and therefore was not a contributing factor throughout experiments.

Experiments then focused on the ability of vessels obtained from juvenile and aged αCGRP WT and KO mice to react to pharmacological agents known to produce vasoconstriction and vasodilatation. Figure 18 illustrates cumulative concentration-response curves constructed in response to phenylephrine (PE) stimulation. Figure 18A shows raw data expressed as force generated by PE whilst Figure 18 shows constriction expressed as total force generated by the vessel with 80 mM K^+ stimulation. Here the data show that vessels obtained from juvenile WT and KO mice respond to PE in a similar manner, as measured by E_{max} (108.60 ± 4.71 % HiK *vs* 110.60 ± 4.50 % HiK, respectively) and EC_{50} analysis ($logEC_{50}$ -5.89 ± 0.13 *vs* -6.08 ± 0.10, respectively). However, as animals aged to 15 months, sensitivity to PE was significantly increased whilst E_{max} was preserved. Aged WT vessels had a lowered $logEC_{50}$ of -7.53 ± 0.13 that was significantly different from their juvenile counterparts, whilst aged KO vessels had a $logEC_{50}$ of -6.95 ± 0.10. A significant difference in $logEC_{50}$ was detected in aged WT *vs* KO animals. A summary of pharmacological parameters measured is included in Table 5.

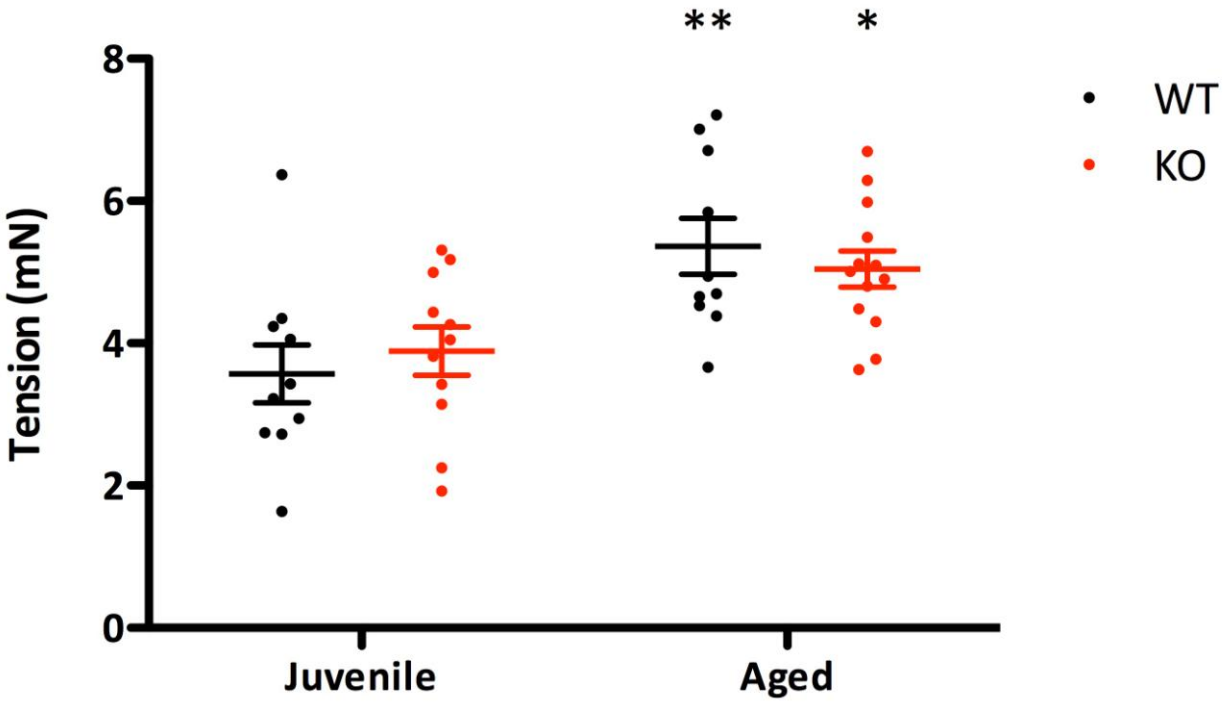

Figure 16: Myogenic response of isolated second order mesenteric arteries to high potassium stimulation (80mM K^+), obtained from juvenile (3 months) and aged (15 months) male αCGRP WT and KO mice. Data expressed as force in millinewtons (mN), mean ± SEM, n = 10-13. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. **p < 0.01 where juvenile and aged animals of the same genotype have been compared.

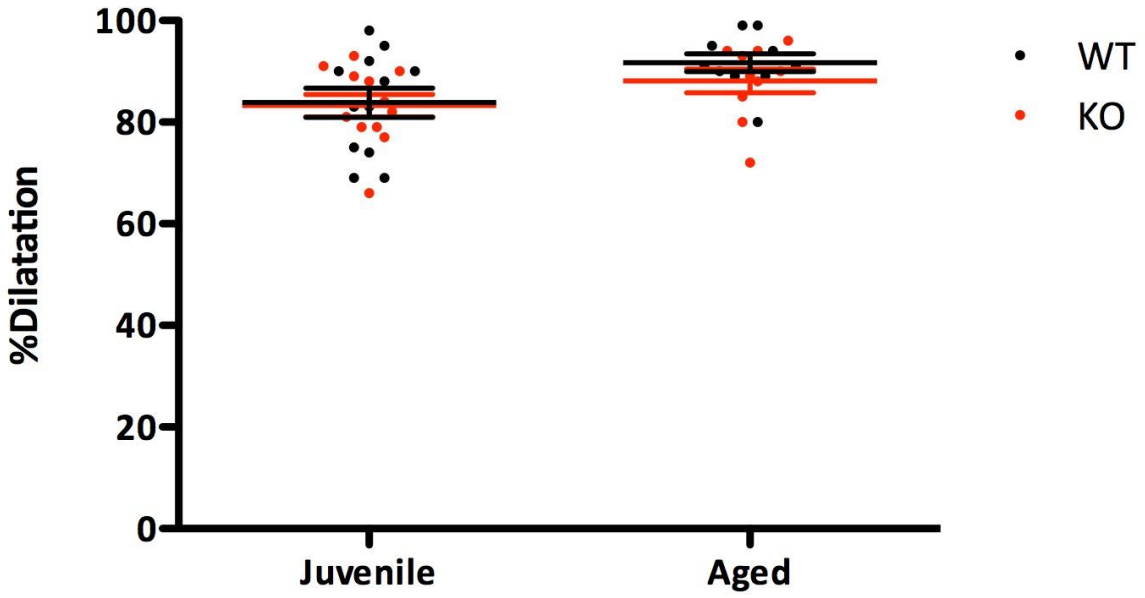

Figure 17: Assessment of endothelial viability in second order mesenteric arteries obtained from juvenile (3 months) and aged (15 months) male αCGRP WT and KO mice, stimulated with 10 µM carbachol. Data expressed as percentage change in force generated of vessels preconstricted with EC_{80} concentrations of U46619, mean ± SEM, n = 10-12.

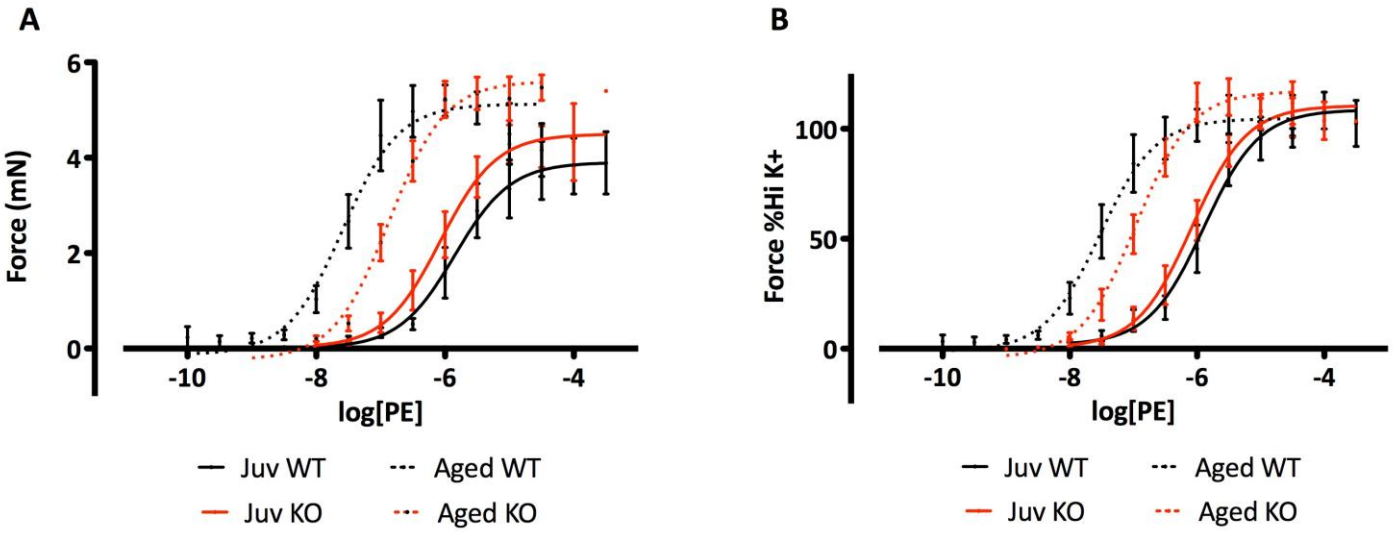

Figure 18: Myogenic response of isolated second order mesenteric arteries to increasing concentrations of phenylephrine (PE) stimulation, obtained from juvenile (3 months) and aged (15 months) male αCGRP WT and KO mice. Results show A) total force generated by arteries obtained from male juvenile and aged αCGRP WT and KO mice; B) force generated expressed as a percentage of the maximal response of the contraction generated to 80mM K^+. Data expressed as force in millinewtons (mN) or percentage of 80mM K^+, mean ± SEM, n = 8-11.

Phenylephrine	Juvenile WT	Juvenile KO	Aged WT	Aged KO
E_{max} (mN)	3.90 ± 0.27	4.49 ± 0.24	5.12 ± 0.24**	5.60 ± 0.19**
E_{max} (%Hi K^+)	108.60 ± 4.70	110.60 ± 4.50	104.60 ± 4.60	117.00 ± 4.46****##
logEC_{50} (M)	-5.84 ± 0.20	-6.07 ± 0.14	-7.58 ± 0.13****	-6.86 ± 0.09****##

Table 5: Data illustrating pharmacological analysis of CCRCs constructed to PE in mesenteric arteries obtained from juvenile and aged male αCGRP WT and KO mice. Results show E_{max} (mN), E_{max} (%Hi K response) and logEC_{50} (M), expressed as mean ± SEM, n = 8-11. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post-hoc* test. ****p < 0.0001, **p < 0.01 where juvenile and aged animals of the same genotype have been compared; ##p < 0.01 where age-matched animals were compared to counterparts of differing genotypes.

Subsequent experiments were undertaken to assess how vessels reacted to the thromboxane receptor agonist, U46619. Figure 19 illustrates concentration-response curves constructed in responseto U46619 stimulation. Figure 19A shows raw data expressed as force generated by U46619 whilst 19B shows constriction expressed as total force generated by the vessel with 80 mM K^+ stimulation. Here the data reveal that vessels obtained from juvenile WT and KO animals respond in a similar manner, in terms of both E_{max} (119.80 ± 8.50 %High K^+*vs* 133.60 ± 8.30 %High K^+, respectively) and $logEC_{50}$ (-8.27 ± 0.21 M vs -8.14 ± 0.18 M, respectively) values. However, as the animals aged, there was a significant shift to the right of $logEC_{50}$ values for both aged WT and KO mice (-6.79 ± 0.13 M *vs* -6.88 ± 0.19 M, respectively). E_{max} values were not found to change as the animals grew older. A summary of pharmacological parameters is included in Table 6.

Finally, a series of concentration-response curves were constructed to vasodilator compounds to assess the ability of the murine vasculature to relax. Figure 20 illustrates time control experiments, showing that vascular tone achieved by U46619 did decline over the typical fifteen-minute experimental window, but not significantly so. This indicates that any changes in vascular tone due to a vasodilator agonist is attributable to that compound and not from a natural loss of vascular tone. Figure 21A illustrates CCRCs obtained in response to vascular stimulation with αCGRP following preconstriction with sufficient U46619 to generate 80% maximal constriction of the vessel. We found that juvenile WT and KO vessels again responded in a similar manner, in terms of both E_{max} (24.33 ± 6.92 %U46619 tone *vs* 39.02 ± 4.27 %U46619 tone, respectively) and $logEC_{50}$ (-8.44 ± 0.26 M *vs* -8.80 ± 0.23 M, respectively). As WT animals aged, there was a trend towards a reduction in sensitivity to exogenous CGRP stimulation, as measured by $logEC_{50}$ that did not reach statistical significance. However, vessels obtained from aged KO animals produced a significant rightwards shift in $logEC_{50}$ (-7.66 ± 0.42 M) when compared to juvenile counterparts and they also differed significantly from their aged WT counterparts (-7.73 ± 0.31 M). There was a trend towards a decrease in E_{max} values as animals aged that did not reach statistical significance. A summary of pharmacological parameters is included in Table 7. To control for VSMC impairment with age, we constructed CCRCs for the NO donor, sodium nitroprusside (SNP), illustrated in Figure 21B. There existed no differences in $logEC_{50}$ or E_{max} values across groups for this agent. A summary of pharmacological parameters for this agent is included in Table 8.

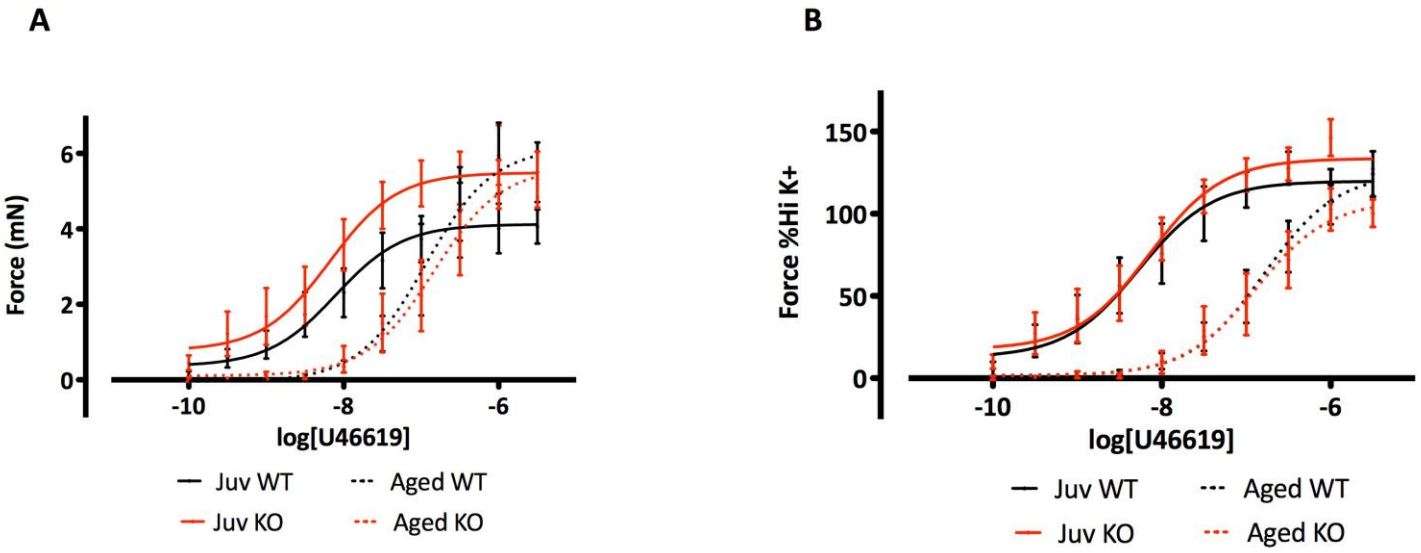

Figure 19: Myogenic response of isolated second order mesenteric arteries to increasing concentrations of U46619 stimulation, obtained from juvenile (3 months) and aged (15 months) male αCGRP WT and KO mice. Results show A) total force generated by arteries obtained from male juvenile and aged αCGRP WT and KO mice; B) force generated expressed as a percentage of the maximal response of the contraction generated to 80mM K^+. Data expressed as force in millinewtons (mN) or percentage of 80mM K^+, mean ± SEM, n = 6-10.

U46619	Juvenile WT	Juvenile KO	Aged WT	Aged KO
E_{max} (mN)	4.12 ± 0.35	5.49 ± 0.42	6.15 ± 0.65*	5.62 ± 0.59
E_{max} (%Hi K^+)	119.80 ± 8.50	133.60 ± 8.30	124.90 ± 10.20	108.10 ± 11.07
logEC_{50} (M)	-8.27 ± 0.21	-8.14 ± 0.18	-6.79 ± 0.13****	-6.88 ± 0.19****

Table 6: Data illustrating pharmacological analysis of CCRCs constructed to U46619 in mesenteric arteries obtained from juvenile and aged male αCGRP WT and KO mice. Results show E_{max} (mN), E_{max} (%Hi K response) and logEC_{50} (M), expressed as mean ± SEM, n = 6-10. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post-hoc* test. ****p < 0.0001, *p < 0.05 where juvenile and aged animals of the same genotype have been compared.

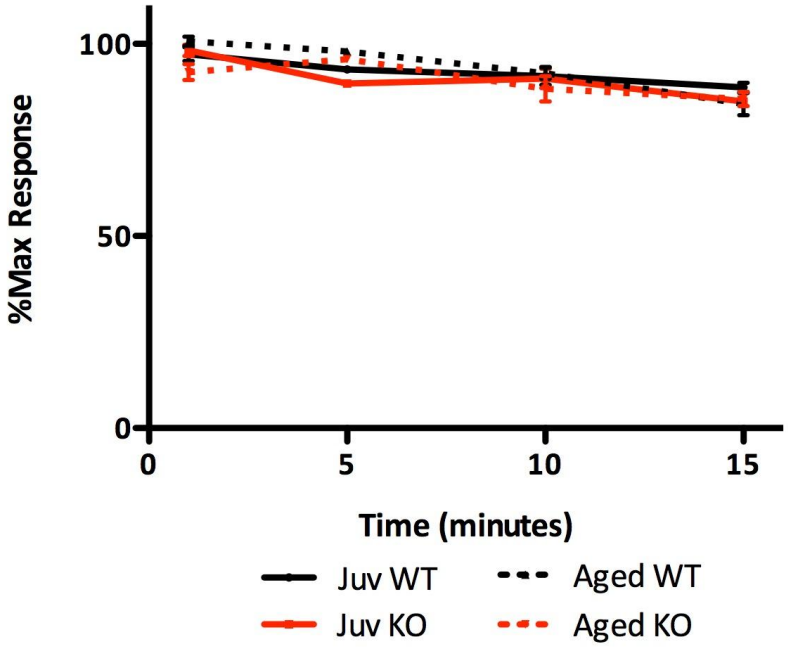

Figure 20: Myogenic response of isolated second order mesenteric arteries to a single concentration of U46619 sufficient to induce 80% maximal vessel constriction, over fifteen minutes. Data expressed as percentage change in U46619-mediated preconstricted tone over time, to give an impression of the tissue's inherent ability to relax, n = 3.

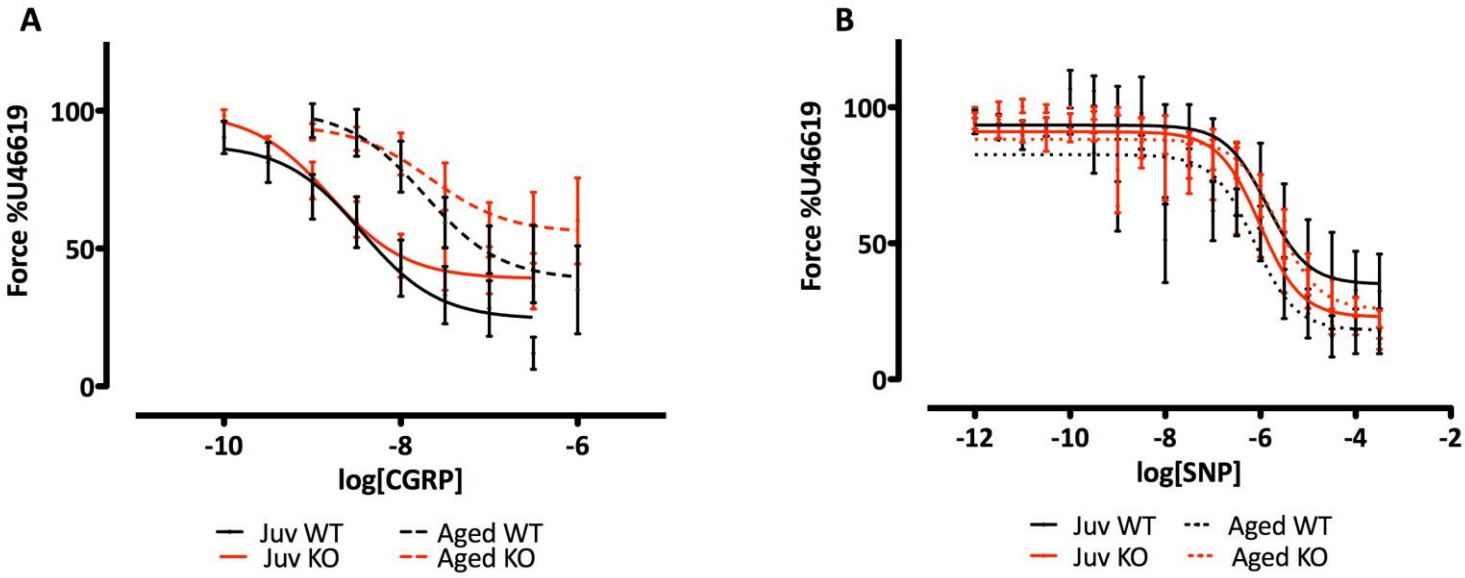

Figure 21: Myogenic response of isolated second order mesenteric arteries to increasing concentrations of vasodilators CGRP and SNP, obtained from juvenile (3 months) and aged (15 months) male αCGRP WT and KO mice. Results show A) change in force of vessels preconstricted with EC_{80} concentrations of U46619, followed by CGRP stimulation in arteries obtained from male juvenile and aged αCGRP WT and KO mice; B) change in force of vessels preconstricted with EC_{80} concentrations of U46619, followed by SNP stimulation in arteries obtained from male juvenile and aged αCGRP WT and KO mice. Data expressed as percentage change in U46619-mediated preconstricted tone, mean ± SEM, n = 10-12 for CGRP, n = 5-8 for SNP.

CGRP	Juvenile WT	Juvenile KO	Aged WT	Aged KO
E_{max} (%U46619)	24.33 ± 6.92	39.02 ± 4.27	38.62 ± 8.86	55.79 ± 7.04
$logEC_{50}$ (M)	-8.44 ± 0.26	-8.80 ± 0.23	-7.73 ± 0.31	-7.66 ± 0.42*#

Table 7: Data illustrating pharmacological analysis of CCRCs constructed to CGRP in mesenteric arteries obtained from juvenile and aged male αCGRP WT and KO mice, preconstricted with U46619. Results show E_{max} (%U46619 response) and $logEC_{50}$ (M), expressed as mean ± SEM, n = 10-12. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post-hoc* test. *p < 0.05 where juvenile and aged animals of the same genotype have been compared; #p < 0.05 where age-matched animals were compared to genodiscordant counterparts.

SNP	Juvenile WT	Juvenile KO	Aged WT	Aged KO
E_{max} (%U46619)	34.95 ± 7.80	22.89 ± 6.29	18.03 ± 5.16	25.61 ± 5.95
$logEC_{50}$ (M)	-5.87 ± 0.30	-6.01 ± 0.20	-6.12 ± 0.19	-5.63 ± 0.19

Table 8: Data illustrating pharmacological analysis of CCRCs constructed to SNP in mesenteric arteries obtained from juvenile and aged male αCGRP WT and KO mice, preconstricted with U46619. Results show E_{max} (%U46619 response) and $logEC_{50}$ (M), expressed as mean ± SEM, n = 5-8. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post-hoc* test.

3.4.4 Temporal dynamics of vascular CGRP receptor complex gene expression

Finally, we sought to examine the effect of αCGRP gene deletion on the regulation of CGRP receptor gene expression within the vasculature, both in juveniles and aged animals. Figure 22A demonstrates that aortic mRNA expression of the CLR is comparable between juvenile WT and KO animals (53.61 ± 27.02 copies/µl *vs* 15.92 ± 13.56 copies/µl, respectively). There is a significant increase in the expression of the CLR in aged WTs (187.70 ± 52.42 copies/µl) that was not different from aged KOs (99.66 ± 55.20 copies/µl). However, expression of the CLR did not significantly increase with age in the KO group. There were no other significant changes in aortic mRNA expression of the other CGRP receptor genes, RAMP1 and RCP, as detailed in Figure 22B and 22C, respectively.

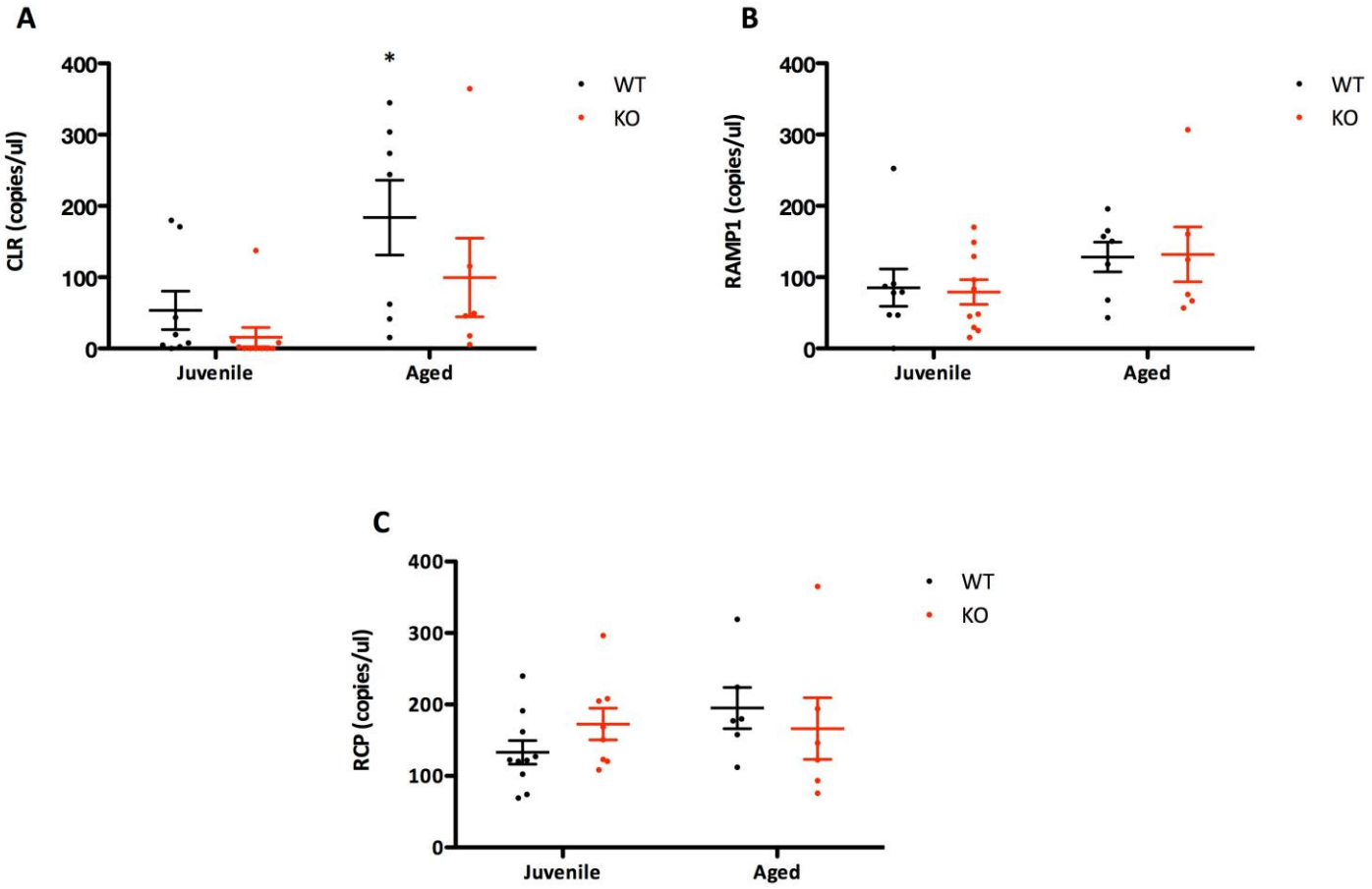

Figure 22: Gene expression analysis of CGRP receptor mRNA of aortic lysates obtained from juvenile (3 months) and aged (15 months) male αCGRP WT and KO mice. Results show A) mRNA expression of CLR in aortic lysates; B) mRNA expression of RAMP1 in aortic lysates; C) mRNA expression of RCP in aortic lysates. Data expressed as copies mRNA per microliter, mean ± SEM, n = 6-10. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. *p < 0.05 where juvenile and aged animals of the same genotype have been compared.

3.5 Discussion

In this Chapter, we aimed to characterise the response to ageing in male αCGRP WT and KO mice by examining gross aspects of their development, with respect to key cardiovascular organs (heart, kidney) and organs that may contribute pathologically to the metabolic syndrome (abdominal fat), including organs that may indicate immune system dysfunction (spleen). Further, we aimed to clarify how CGRP bioavailability alters as part of the ageing process and how this bioavailability may impinge upon bioreactivity and CGRP receptor expression. Alterations in these key processes may help us understand how CGRP contributes towards pathology, if it exists, in the ageing mouse.

3.5.1 Deletion of αCGRP does not adversely affect weight gain, adiposity or organ development in the ageing mouse

We initially attempted to characterise the growth and developmental characteristics of juvenile and aged αCGRP WT and KO mice. In brief, as expected, we discovered that animals increased their body weight as they aged to fifteen months. No differences were observed between genotypes for this parameter. We also witnessed a significant increase in epididymal fat content as animals aged. Interestingly, however, this increase did not appear to be directly linked to a general increase in body size. Instead, it would appear that mice aged fifteen months had a higher percentage of their body weight attributable to fat than their juvenile counterparts. Our results are in contrast to that of an important study published by Walker *et al.* in 2010. Here, the authors report that CGRP WT and KO mice have similar body weights when youngbut as the animals age to 250 days (~9 months), CGRP KO mice appear to have lower body weights (Walker et al., 2010b). Further, KOs protected against high fat diet-induced obesity, meaning one might expect differences in epididymal adiposity to be reflected in our results. It is possible, however, that differences in adiposity may only be revealed in a pathological model of obesity, rather than simply with increases in fat occurring as part of the natural ageing process. Our results also showed that organ weights (heart, kidney and spleen) progressively increased in line with body weight as the animals aged but CGRP gene deletion did not affect tissue weights. Overall, we concluded that CGRP gene deletion did not appear to adversely affect whole body growth or development of organs essential to cardiovascular (heart, kidney) or immunological (spleen) function.

3.5.2 Local expression of CGRP is increased in the ageing mouse: evidence for a compensatory role for βCGRP in the αCGRP KO mouse

We have shown for the first time how circulating levels and localised tissue expression of both CGRP isoforms change over time in the αCGRP KO mouse. Analysis of plasma CGRP provided surprising results where we found that levels of circulating CGRP were virtually indistinguishable when comparing juvenile WT and KO animals. The ELISA used to detect plasma CGRP makes use of an antibody with a paratope that is unable to discriminate between CGRP isoforms, presumably owing to their high sequence homology. Therefore, we assume that the signal observed in the KO mice is due to βCGRP expression. It would therefore appear that βCGRP expression is increased in αCGRP KO mouse as part of a compensatory mechanism to counteract the deletion of the primary isoform. We have previously shown a similar result in vehicle-treated αCGRP WT and KO mice as part of an ATII model of hypertension. Here, both genotypes had similar circulating levels of CGRP (Smillie et al., 2014). One novel and perhaps surprising finding of ours in this study was that circulating CGRP levels appeared to go unchanged as mice aged to fifteen months. Further to this, the degree of compensation from βCGRP appeared to be maintained throughout this time. It is curious that total levels of CGRP appear to be similar when comparing WT and KO mice. Even with a level of developmental compensation, one might expect KO mice to have slightly less circulating peptide. A potential reason the observed βCGRP signal is so high could be owing to its alleged restriction of expression to the enteric nervous system (Mulderry et al., 1988). Whether these nerve fibres originate from the DRG or from peripheral adventitial neurons located within the mesentery itself (Somasundaram et al., 2006; 2012) is unknown. Regardless, these nerves are in close proximity to one of the largest vascular beds in the body, meaning that any overspill has good access to the circulation, thus amplifying the signal of circulating peptide.

It could be argued that the plasma is not the most ideal depot to measure CGRP concentrations as it is often considered to be a 'spillover' from the site of release at the neurovascular junction. Therefore, it may be more relevant to consider expression of the peptide at its predominant site of synthesis, the DRG. As it is currently impossible to use antibodies to discriminate between αCGRP and βCGRP, the best-available technique to determine tissue expression is that of gene expression analysis *via* PCR. I have shown in this thesis that αCGRP gene expression is significantly increased within the DRG as mice age from three to fifteen months. This is somewhat surprising, given that rat studies pointed to a general decline in the expression of the peptide as they grew older (Kawasaki et al., 1990a; 1991; Kawasaki and Takasaki, 1992; Yamaga et al., 2001). However, to reiterate, these studies are often confounded by the use of SHRs and they investigate multiple and differing ageing timepoints, which might add further confusion. Additionally, it is possible that mice have very

different temporal expression patterns of these peptides, in comparison to rats. Whilst an intriguing result, it is unknown what this elevation in expression is in response to. One conjecture might be that increases in cytokine activity that are characteristic of the ageing process could be influencing CGRP gene expression. Of these in particular, TNFα has been shown to mobilise intracellular calcium in cultured DRG neurons, sufficient to produce activation of p38 MAPK and other stress-related kinases (Pollock et al., 2002; Durham and Russo, 2003). Activation of kinases such as these by TNFα is capable of inducing CGRP gene expression and subsequent release from the cell (Bowen et al., 2006). There is a longstanding train of thought that considers CGRP and TNFα to have a relationship akin to that of more traditional 'physiological antagonists', where neuronal CGRP counteracts the effects of immune cell-derived TNFα (Assas, 2014). With this in mind, our results might be explained by CGRP gene transcription being elevated as part of a feedback network, in order to work against a pro-inflammatory mediator, such as TNFα.

We also investigated how βCGRP gene expression was regulated both in juvenile and aged αCGRP WT and KO mice. We found that KO mice aged three months had significantly higher expression of the beta isoform when compared to age-matched WTs. This compliments our data obtained from plasma analysis of total CGRP, where we identified a potential compensatory role for βCGRP in the αCGRP KO mouse. This finding further strengthens the need to investigate the expression dynamics of each isoform at the tissue level, rather than solely investigating what is circulating in the plasma.

Others using the mixed calcitonin/αCGRP KO mouse have shown that βCGRP mRNA expression within the DRG was twofold lower in the KO as compared to the WT (Gangula et al., 2000). Groups using the pure αCGRP KO mouse, identical to those used in this thesis, have shown that there is no change in mRNA expression of the β isoform in multiple regions of the spinal cord (Schutz et al., 2004). Therefore, there is some discrepancy between results presented in this thesis and those in the published literature, the reason for which is somewhat unclear. However, an interpretation of our findings could potentially be that both genes for αCGRP and βCGRP are closely linked and therefore a negative feedback circuit could be established whereby the absence of one peptide stimulates the production of the other. Whilst failing to reach statistical significance, we also saw a strong trend towards an elevation in βCGRP transcript in DRG as both our WT and KO mice reached fifteen months of age. This result is in line with our observed increase in αCGRP expression with age.

Given that both CGRP isoforms are structurally very similar and possess almost identical pharmacology, these findings may point to problems with making use of the αCGRP KO mouse, particularly if the study does not make efforts to investigate how βCGRP may be behaving. Thus, potential interesting phenotypes in animal models of disease may be masked by the Janus-faced

nature of this particular KO mouse. To address this potential problem, it would be prudent to develop a double KO mouse that possesses deletion of both alpha and beta isoforms of the peptide. However, it would be difficult to generate such a mouse by traditional crossbreeding methods, as both genes for CGRP peptides are located closely together on the same chromosome. Alternatively, short-term studies employing the use of CGRP receptor antagonists may be of some benefit. In terms of understanding more the role of CGRP as part of the ageing process, the development of a double KO might be more practical.

3.5.3 Ageing differentially regulates contractile responses to α-adrenoceptor and thromboxane receptor agonists in mesenteric resistance arteries

Through functional vascular reactivity studies examining key pharmacological agents known to induce vasoconstriction, we have uncovered some interesting results pertaining to the ageing process. Little is known of age-related changes in the regulation of splanchnic vasomotor control by perivascular nerves. Given that the mesenteric arcade represents one of the most important vascular resistance beds in the regulation of blood pressure, any alteration to vasomotor properties of this bed could have significant consequences for systemic haemodynamics. We first examined the ability of the mesenteric resistance arteries isolated from juvenile and aged αCGRP WT and KO mice in response to stimulation with hyperkalaemic Krebs buffer. This allowed us to examine the endogenous contractile properties of the vessels that are independent of receptor stimulation. Results show that vasoconstriction produced by 80 mM potassium is significantly increased in mesenteric arteries obtained from fifteen-month-old mice *versus* those at three months. This increase in contractility to high concentrations of potassium has been shown by others in the rat (Lang et al., 1995). Further, studies in senescence-accelerated mice have shown enhanced potassium-dependent contractions, albeit in the aorta (Novella et al., 2011). Indeed, even the senescence-resistant mouse displayed an age-dependent increase in potassium-induced contractility. The reason for this is unclear, although authors of the aforementioned paper ascribed this phenotype to a direct action on voltage-gated calcium channels or distal mechanisms related to influxes of calcium. It is known that ageing can enhance the proliferation of VSMCs (Orlandi et al., 2006) and therefore older mesenteric arteries might be able to mount a larger potassium-dependent response as a result of simply having a larger VSMC population. However, we have no direct empirical evidence of this phenomenon. Alternatively, these results point to alterations to the activity of voltage-gated calcium channels or more distal mechanisms that act in response to calcium influx.

We also investigated how mesenteric resistance arteries isolated from juvenile and aged αCGRP WT and KO mice responded to stimulation with the α_1-adrenoceptor agonist, PE. There is a reported close regulatory relationship between sensory afferents and sympathetic nerves and this relationship can impinge upon the regulation of vascular tone (Kawasaki, 2002). Because sympathetic nerve activity increases with age throughout the body (Seals, 2004) and the mesenteric vasculature is richly innervated by these fibres, we hypothesised that vascular reactivity in response to α_1-adrenoceptor (α_1-AR) stimulation might become altered as the organism ages. We found that there was a significant improvement in vascular sensitivity to PE stimulation as both genotypes of mice aged to fifteen months. This finding is in stark contrast to a recent study published by the Segal

laboratory that showed that mesenteric arteries(Westcott and Segal, 2013) and gluteus maximus microvessels(Sinkler and Segal, 2014)obtained from C57BL/6 mice aged 24 months displayed diminished sensitivity and maximal contractions to PE. A simplistic explanation for increased reactivity to PE in juvenility would be an increase in adrenoceptor expression within the mesenteric vasculature. However, mRNA expression of α_1-AR subtypes within the mesentery has been shown to remain stable throughout twenty-four months in ageing rats (Xu et al., 1997). However, whilst receptor expression remains stable, it is possible that post-translational modifications to the receptor may alter functionality. Alternatively, it is known in humans that sympathetic nerve activity is increased with age (Sundlöf and Wallin, 1978), which presumably may result in a hyperstimulation of adrenoceptors that leads to their desensitisation, thereby reducing potency of agonists such as PE. Additionally, these receptors may have undergone changes to the intracellular signalling cascades they are coupled to, in order to augment vasoconstriction. For example, alterations to calcium-handling pathways within the VSMC(Rubio et al., 2002).

In contrast to our findings with stimulation of the vasculature with sympathomimetic compounds, we found stark differences in vascular reactivity with respect to stimulation with the thromboxane receptor agonist, U46619. Here, we have shown that mice aged 15 months display a significant reduction in pharmacological sensitivity to thromboxane receptor stimulation. Others have made use of a senescence-prone mouse model to show that female mice of this strain produce increased vasoconstriction in response to U46619, although these studies were conducted in the aorta. The authors ascribed this change to a reduction in endogenous NO release and a phenotypic switch from the production of vasodilator to vasoconstrictor prostanoids from the endothelium (Novella et al., 2011). It is known that vascular thromboxane receptor expression is increased in these senescence-accelerated mice (Novella et al., 2010). Further, in obese mice, thromboxane receptor expression in carotid artery has been shown to increase in order to promote U46619-mediated vasoconstriction (Traupe et al., 2002). Thus, with these findings in mind, our results are a little surprising.Table 9 reports a summary of findings related to vascular reactivity experiments.

Drug	Effect of Age	Effect of Genotype
80 mM K^+	Increased contractility with age	No effect
Phenylephrine	Increased contractility with age	No effect
U46619	Decreased contractility with age	No effect
CGRP	Decreased dilatation with age	Decreased contractility in aged KOs vs aged WTs
SNP	No effect	No effect

Table 9: Table summarising results obtained from vascular reactivity experiments, describing effects of ageing and mouse genotype on vasoconstrictor/vasodilator processes.

3.5.4 Ageing is associated with an impaired CGRP-dependent vasodilatation in mesenteric resistance arteries despite apparent increases in CGRP receptor gene expression

Whilst we have made efforts to examine how the expression of CGRP isoforms is regulated as part of the ageing process, this does not provide any indication of bioactivity. A maintenance or even upregulation of expression does not guarantee conserved or increased activity. Therefore, we sought to examine the bioactivity of exogenous CGRP on isolated mesenteric arteries from juvenile and aged αCGRP WT and KO mice. Here, we have shown that ageing significantly impairs sensitivity to exogenous CGRP, at least in the KO mouse. There is a clear trend for the same phenomenon in the WT mouse, though this difference did not reach statistical difference. This change in CGRP potency with age did not appear to be linked to a decrease in the capacity of the vasculature to mount a relaxant response, as our SNP responses remained intact. Thus, we concluded that the change could be ascribed to a CGRP receptor-dependent phenomenon. However, we do not know whether the loss of CGRP response is endothelial-dependent or –independent, owing to vessels from all experimental groups being unresponsive to acetylcholine stimulation. However, unpublished results from our laboratory have shown that CGRP responses in isolated mesenteric arteries are unaffected by mechanical removal of the endothelium. Indeed, many suggest that vasodilatation produced by CGRP in the smaller resistance arteries is more dependent on VSMC K^+_{ATP} channel-induced hyperpolarization (Quayle et al., 1994; Wellman et al., 1998), whereas the response in the larger conduit arteries appears to be more reliant on NO generation (Hao et al., 1994; Lu and Fiscus, 1999). Therefore, we might not expect the role of the endothelium in producing vasodilatation in this vascular bed to be particularly important.

Studies have shown that vascular sensitivity to CGRP both *in vivo* and *in vitro* becomes diminished with age in the rat (Chan and Fiscus, 2002). However, no mechanism has been proposed to date to explain this phenomenon. A study has been performed where CGRP potency has been seen to increase in hypoxic porcine intramyocardial arteries in the absence of alterations in RAMP1/CLR expression, at least at the mRNA level (Hasbak et al., 2005). The authors concluded that the increased potency arose from hypoxic conditions affecting the resting conformation of the receptor itself, thus altering agonist affinity. Others have reported increases in CGRP bioactivity during hypertension and this change was ascribed to increased vascular expression of the RCP (Supowit et al., 2011). In this study, we have seen a significant increase in aortic CLR mRNA expression as WT animals age and a trend towards a similar increase in CLR expression in juvenile *vs* aged KO mice. At first glance this may raise the expectation of increased potency for CGRP as more CLR mRNA might potentially be translated to protein to form a receptor. However, in the absence of elevations of RAMP1 transcript, it is possible that *functional* receptor expression levels are unchanged. Further to

this, unlike Supowit *et al*., we do not see any difference in aortic mRNA expression of the signal amplifying protein, RCP. Of course, one must concede that this gene expression analysis has been performed in a different vascular bed to that studied in the functional experiments and therefore expression might be regulated in a very different manner within this tissue. Another caveat of gene expression studies is that they do not necessarily represent what is happening at the protein level and therefore might not be indicative of how functional CGRP receptors are being regulated. Table 9 reports a summary of findings related to vascular reactivity experiments.

It is conceivable that ageing might affect receptor subcomponent expression in the opposite direction in order to decrease potency. I consider that regulation of CGRP receptor expression would represent one of the most rational explanations of how agonist potency may become altered, given what evidence currently exists in the literature. Doubtless, other mechanisms may be present, particularly those related to the development of a pro-inflammatory vascular environment with ageing. For instance, an increased expression and activity of peptidases responsible for metabolising CGRP within the vascular compartment, leading to decreased bioavailability and thus activity. Alternatively, increased ROS formation as part of the vascular inflammatory response may lead to oxidation of criticalcysteine residues present in the RAMP1 structure, such as Cys40, Cys57, Cys72 and Cys104. These four residues are critical for the heterodimerisation of RAMP1 with CLR to allow for subsequent export from the ER and insertion into the plasma membrane (Steiner et al., 2003). Thus, oxidised RAMP may be retained intracellularly and thus expression of the CGRP receptor at the plasma membrane may be significantly diminished, leading to decreased potency.

3.6 Conclusion

To conclude, this chapter has shown that αCGRP WT and KO mice do not differ in gross developmental parameters as they age from juveniles (3 months) to 'middle-aged' (15 months). Further to this, we have identified a potential compensatory mechanism present in αCGRP KOs where the removal of the alpha isoform of the peptide induces an upregulation of beta isoform gene expression within the DRG, which in turn produces a sizeable detectable peptide signal within the plasma of these mice. With respect to functional changes observed in these mice, this chapter has uncovered striking *ex vivo* results related to mesenteric vascular reactivity. We have shown that vasodilatation in response to stimulation by CGRP is markedly diminished in aged *versus* juvenile animals, despite apparent increases in CGRP receptor mRNA expression within vascular tissues. In addition, we witnessed pharmacological changes in vasoreactivity to the constrictor agonists phenylephrine and U46619, although the mechanisms behind these changes are unknown. In summary, deletion of αCGRP does not appear to promote a gross adverse phenotype as mice age but differences in vascular pharmacology become readily apparent.

4. |Chapter Four: Characterising the *In Vivo* Cardiovascular Phenotype and Vascular Inflammatory Response in the αCGRP WT and KO Mouse

4.1 Introduction

The role of CGRP in the regulation of baseline cardiovascular haemodynamics appears to be minimal. Mice that lack αCGRP usually have normal blood pressures (Lu et al., 1999; Smillie et al., 2014) and both laboratory animals and humans receiving acute administration of CGRP receptor antagonists or ligand-directed antibodies do not experience changes in baseline haemodynamics(Moore and Salvatore, 2012; Bigal et al., 2013; Walter et al., 2014). Conversely, however, there is a strong literature concerning the role of CGRP in various diseases of the cardiovascular system, including hypertension and inflammatory processes that accompany it. Animals that have had CGRP receptor activity disrupted by pharmacological antagonists have been shown to exhibit exacerbated elevations in blood pressure in comparison to control animals in a wide range of hypertensive models, accompanied with a greater degree of vascular inflammation (Gangula et al., 1997; Supowit et al., 1997; 1998). Similar phenotypes have been observed in CGRP KO mice (Oh-hashi et al., 2001; Kurihara et al., 2003) and we have recently published results showing enhanced hypertension, vascular remodeling and dysfunction in CGRP KO mice, within an ATII model of hypertension (Smillie et al., 2014). Conversely, hypertensive animals receiving the phytoalkaloid rutaecarpine (a reported TRPV1 agonist and thus releaser of CGRP) witnessed an improvement in disease parameters (Hu et al., 2003; Deng et al., 2004b; Qin et al., 2007; Li et al., 2008). Furthermore, mice engineered to overexpress RAMP1, a critical component of the CGRP receptor, are protected against hypertension-induced vascular dysfunction (Sabharwal et al., 2010). Thus, there is great evidence to implicate CGRP as a protective mediator within the setting of hypertension and also other diseases whereby the cardiovascular system becomes stressed (Russell et al., 2014).

However, as already discussed in the previous chapter, little is known about the role of CGRP within the setting of cardiovascular ageing. It is generally considered that ageing is associated with a pro-inflammatory environment and the cardiovascular system is not exempt from this inflammation, or “inflammaging”(Franceschi and Bonafè, 2003). Vascular wall ageing begins with chronic proinflammation, a form of sterile inflammation that occurs in the general absence of infection by microorganisms. Phenotypic shifts in ECs and VSMCs

promote this level of inflammation (Wang et al., 2013). This is why ageing is the single biggest independent risk factor for the development of CVD, as it generates a fertile substrate in which disease can take hold.

This subtle, subclinical proinflammation is often characterised by increased ROS generation and an inability to appropriately defend against this increased ROS burden(Puca et al., 2013). Vascular cells may have shifted their phenotype to one that is considered more pro-inflammatory and, indeed, some cells may have become senescent, further increasing the risk for pro-inflammatory factors such as cytokines being secreted by these cells. Such subclinical inflammatory processes may induce the expression of cell adhesion molecules, which in turn may positively influence the recruitment of circulating inflammatory cells (López-Otín et al., 2013). These inflammatory cells (monocytes, in particular) may be recruited by dysfunctional ECs in the early stages of atherosclerosis to form early vascular lesions, which in the human could progress to more severe vascular disease (Hopkins, 2013).

4.2 Hypothesis

Deletion of αCGRP in juvenile mice (3 months) will not affect blood pressure but as animals age (15 months), gene deletion will result in increased blood pressure. Elevated blood pressure will be accompanied by exacerbated vascular inflammation, leading to enhanced recruitment of circulating inflammatory cells into the vascular wall.

4.3 Aims

1. To investigate the role of αCGRP in the regulation of blood pressure and subsequent vascular remodeling in young and aged αCGRP WT and KO mice.
2. To investigate mechanisms by which CGRP might influence the genesis of vascular inflammation in young and aged αCGRP WT and KO mice.
3. To investigate how αCGRP affects vascular inflammatory processes related to leukocyte infiltration in young and aged αCGRP WT and KO mice.

4.4 Results

4.4.1 *In vivo* haemodynamic parameters and vascular morphologies of the ageing αCGRP WT and KO mouse

Following on from the initial growth and developmental characterisation of αCGRP WT and KO mice, we sought to discover how deletion of αCGRP might impinge upon *in vivo* cardiovascular function in these animals. To do so, we measured blood pressure and heart rate *via* non-invasive tail cuff volume pressure recording and peripheral pulse oximetry, respectively. Figure 23A shows systolic blood pressure recordings obtained from male αCGRP WT and KO mice, aged 3 or 15 months. Systolic blood pressures were comparable in our juvenile control groups (124.60 ± 4.17mmHg *vs* 132.20 ± 5.88 mmHg for WT and KO, respectively) and blood pressure was not found to change as the mice aged to 15 months (125.10 ± 3.96mmHg *vs* 123.60 ± 1.91 mmHg for WT and KO, respectively). Figures 23B and 23C detail diastolic and mean arterial pressures, respectively, showing similar trends. In addition to the measurement of blood pressure, we also assessed heart rate in a small sample of the aged cohort (Figure 23D). Again, we found there to be no differences in heart rate between WT and KO animals (566.80 ± 8.20 beats per minute *vs* 552.00 ± 35.10 beats per minute, respectively).

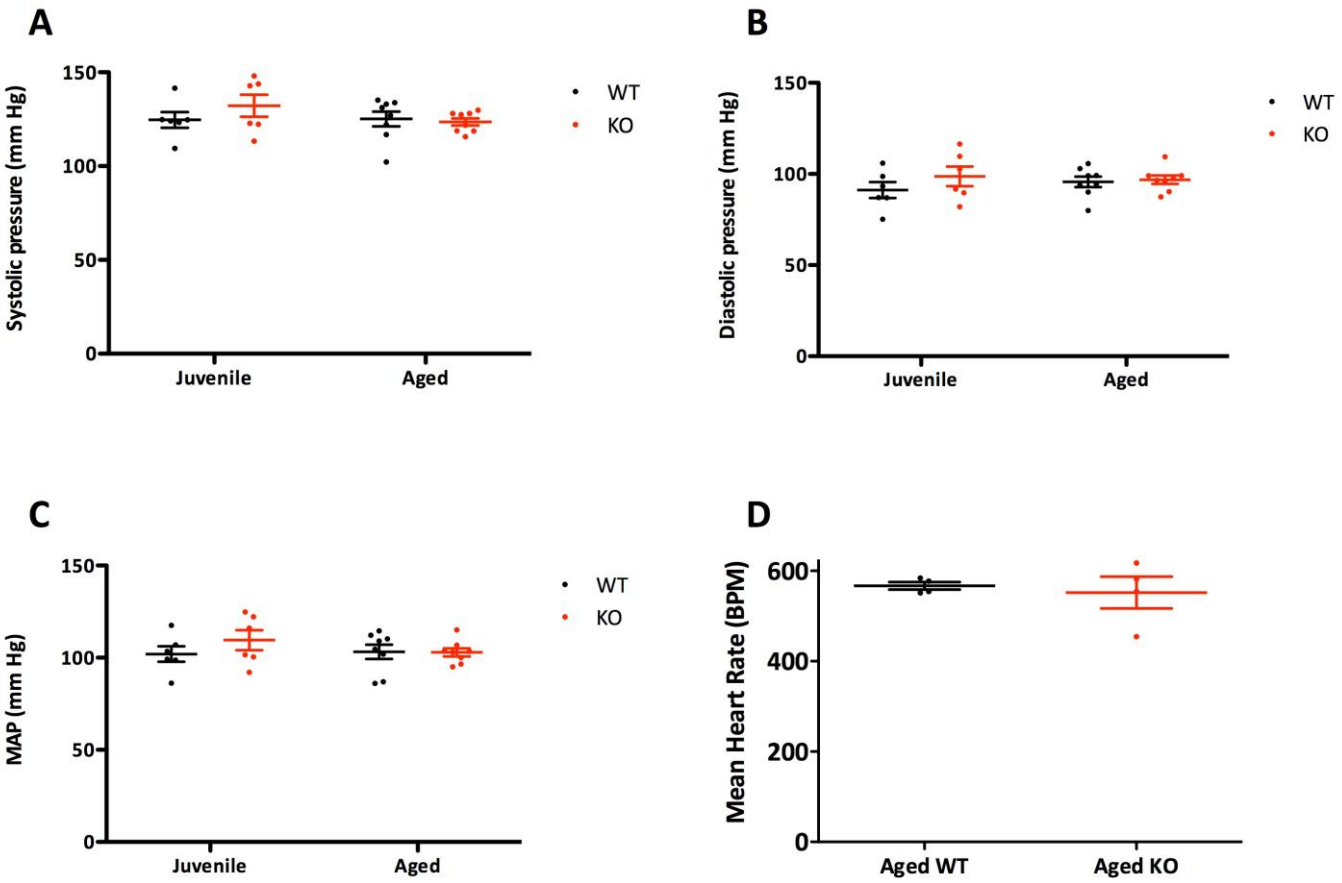

Figure 23: ***In vivo*** **cardiovascular parameters of juvenile (3 months) and aged (15 months) αCGRP male WT and KO mice. Results show A) systolic blood pressures of male juvenile and aged αCGRP WT and KO mice; B) diastolic blood pressures of male juvenile and aged αCGRP WT and KO mice; C) mean arterial blood pressures of male juvenile and aged αCGRP WT and KO mice; D) heart rates of male juvenile and aged αCGRP WT and KO mice. Data expressed as mm Hg, mean ± SEM, n=6-8 for blood pressure and beats per minute (BPM), mean ± SEM, n=4 for heart rate. Statistical significance was evaluated by two-way ANOVA plus Bonferroni** ***post hoc*** **test or unpaired student's t-test, where appropriate. No significant differences were detected between experimental groups.**

To complement our *in vivo* assessment of haemodynamics within our groups of juvenile and aged αCGRP WT and KO mice, we assessed the extent of large vessel remodeling that might reflect alterations in blood pressure *via* classical histological techniques. Figure 25 displays representative light photomicrographs of aortic sections obtained from juvenile and aged αCGRP WT and KO mice, stained with Masson's Trichrome. Here, cell nuclei are stained dark red/purple; the cytoplasm is stained pink and connective tissues are stained blue. From initial observation, it is clear that there are no gross differences from aortae obtained from different animals. However, it is possible that the degree of collagen fragmentation and disorganisation seen in older vessels is greater than that of their younger counterparts, although this has not been quantified. Morphological analysis of these sections was also performed, where we measured both smooth muscle and collagen width and total area, shown in Figure 24. Figure 24A shows the width of SMC populations within the aortae. Juvenile WT and KO mice possess similar SMC widths (69.88 ± 9.09 μm *vs* 72.25 ± 7.80 μm, respectively). This parameter does not change significantly as mice aged to fifteen months (75.21 ± 5.32 μm *vs* 89.47 ± 3.55 μm for aged WT and aged KO, respectively). When we measured the total area of aorta occupied by SMCs, we again found there to be no differences between groups (Figure 24B). Further to characterising SMC morphology within the aortae, we also sought to investigate evidence of altered collagen structuring in the tunicae adventitia of these vessels. Figure 24C represents analysis of collagen width within the vessel. Again, young animals possessed similar degrees of collagen width (16.60 ± 2.13 μm *vs* 21.67 ± 0.95 μm for juvenile WT and KO animals, respectively). However, we found that aged WT animals had a significantly higher collagen width within the aorta (27.29 ± 1.23 μm) when compared to their juvenile counterparts. Aged KO animals had similar collagen widths (25.63 ± 2.24 μm), though these were not found to differ significantly either from their juvenile counterparts or when compared to aged WTs. Total area of the adventitial region of the vessel occupied by collagenous structures is shown in Figure 24D. We found no statistically significant differences in collagen area across all groups. Therefore, we concluded that the extent of vascular remodeling occurring as part of the ageing process was minimal, in line with no observable changes in haemodynamic parameters. Further, there appeared to be no contribution from αCGRP gene deletion in either of these processes.

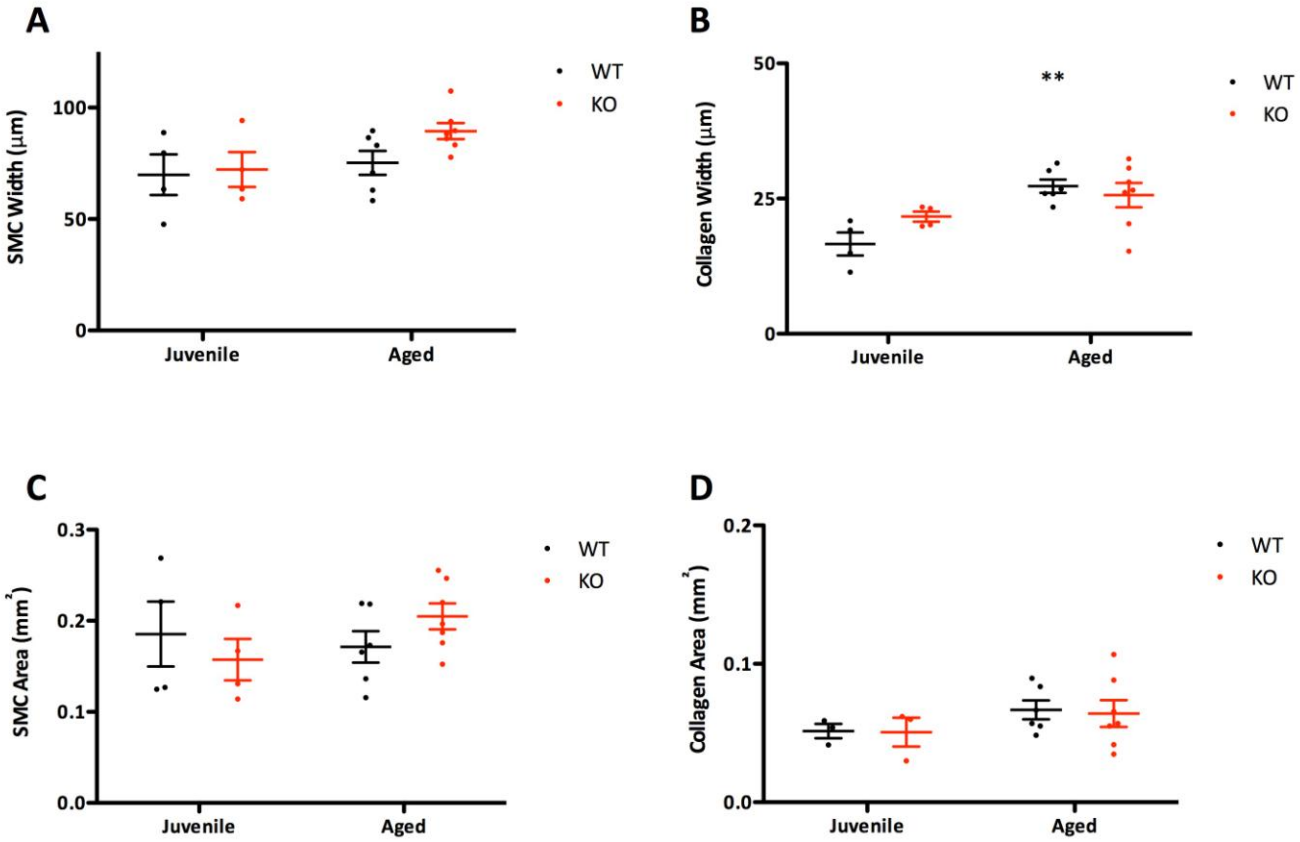

Figure 24: Morphological analysis of aortae obtained from juvenile (3 months) and aged (15 months) male αCGRP WT and KO mice. Results show A) smooth muscle cell width of aortae obtained from male juvenile and aged αCGRP WT and KO mice; B) smooth muscle cell area of aortae obtained from male juvenile and aged αCGRP WT and KO mice; C) collagen width of aortae obtained from male juvenile and aged αCGRP WT and KO mice; D) total collagen area of aortae obtained from male juvenile and aged αCGRP WT and KO mice. Data expressed as µm, mean ± SEM for width measurements and mm^2, mean ± SEM for area measurements, n=4-7. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. **p < 0.01 where juvenile and aged animals of the same genotype have been compared.

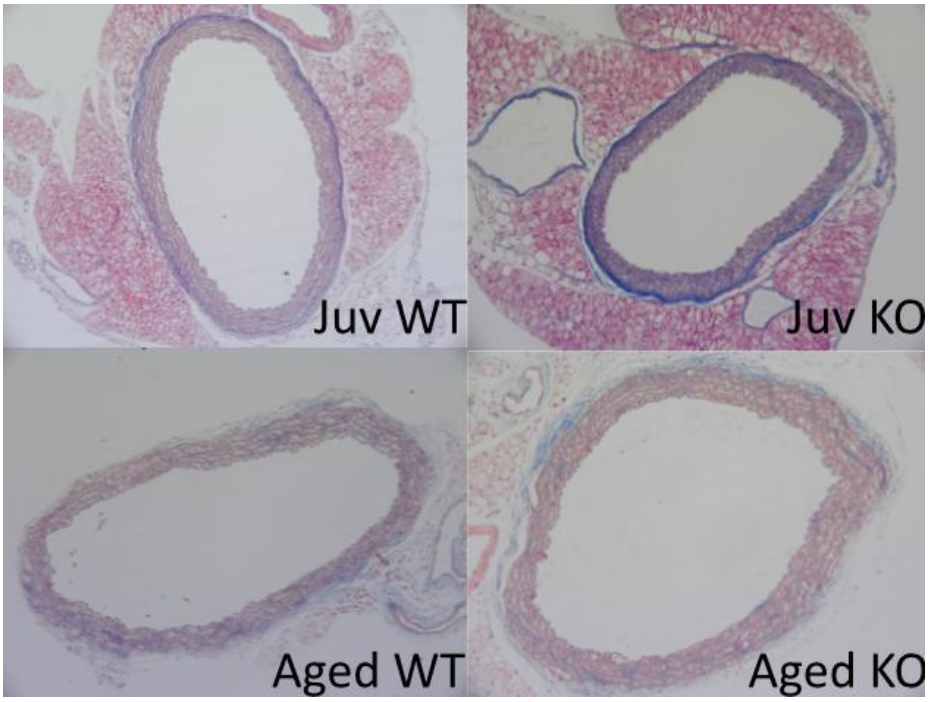

Figure 25: Representative light micrographs of upper thoracic aorta sections obtained from juvenile (3 months) and aged (15 months) male αCGRP WT and KO mice.

4.4.2 Characterisation of vascular eNOS expression and activity within the aortae of ageing αCGRP WT and KO mice

As NO is a critical signalling molecule involved in the regulation of both blood pressure and vascular inflammation, we sought to investigate how the processes involved in the generation of NO might differ during the ageing process, and how these processes may be influenced by CGRP. Initially, we probed total mRNA lysates obtained from the aortae of juvenile and aged αCGRP WT and KO mice to examine vascular eNOS gene expression. Figure 26 illustrates aortic eNOS mRNA expression. Here, we found that gene expression remained relatively stable in juvenile WT and KO animals (1487.37 ± 406.47 copies/µl *vs* 1204.80 ± 170.02 copies/µl, respectively). This level of expression did not appear to significantly change as animals aged to fifteen months, however one might argue that a trend towards a reduction in eNOS gene expression can be observed in the aged KO group, when compared to aged WT (1313.42 ± 267.22 copies/µl *vs* 811.83 ± 96.04 copies/µl for aged WT and aged KO, respectively). Thus, eNOS gene expression did not appear to change significantly as animals grew older and the effect of αCGRP gene deletion on this process was negligible.

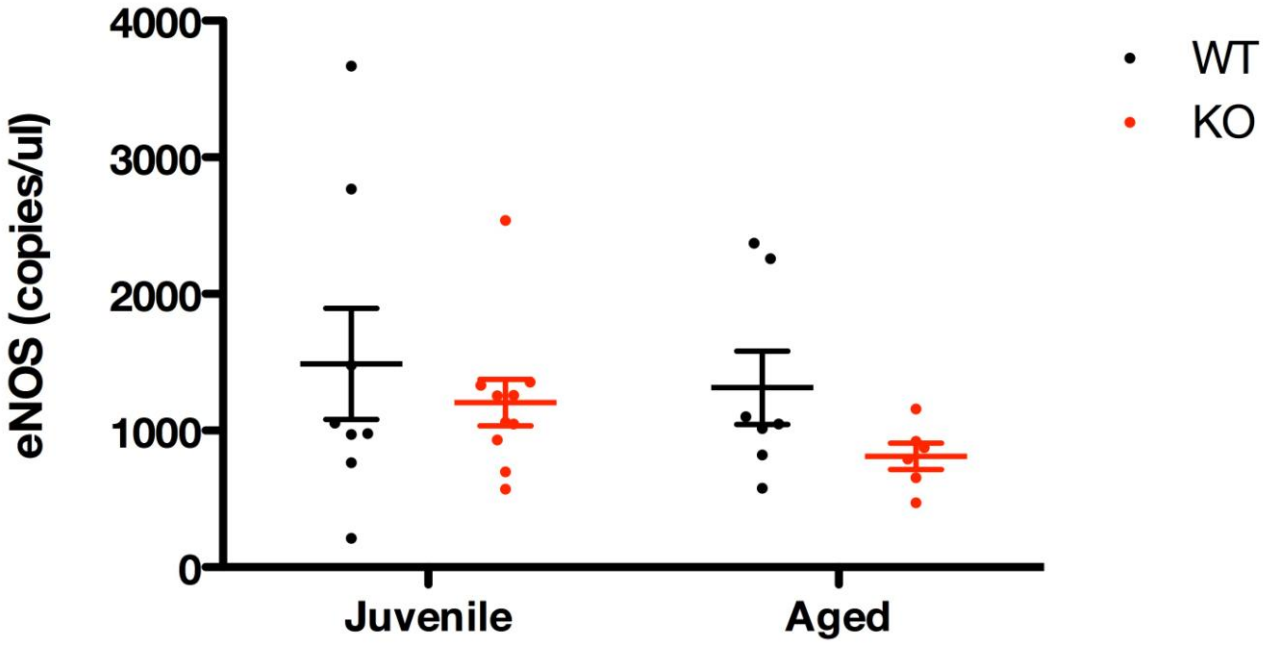

Figure 26: Gene expression analysis of eNOS in aortic lysates obtained from juvenile (3 months) and aged (15 months) male αCGRP WT and KO mice. Results show A) mRNA expression of eNOS in aortic lysates. Data expressed as copies mRNA per microliter, mean ± SEM, n=6-10. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. No statistically significant differences were found between groups.

Analysis of gene expression can only shed light on one aspect of the regulation of an entire biological process. Thus, we thought it prudent to analyse the expression of aortic eNOS at the protein level *via* immunoblotting. Figures 27 and 28 illustrate the data obtained from this experiment. Here, we have shown that eNOS protein expression is comparable in juvenile WT and KO tissues. Protein expression of eNOS did not appear to change as animals aged, indicating a preservation of eNOS expression throughout the ageing process that was unaffected by αCGRP gene deletion. Building on our knowledge of both eNOS gene and protein expression, we continued to probe this pathway by measuring the potential activity of eNOS enzyme, using a phospho-specific antibody that recognises a phosphorylated epitope within the eNOS protein sequence. This epitope is inclusive of a phosphorylated ser1177 residue, known to be important for enzyme activation. Figures 29 and 30 show results obtained from measuring the degree of phosphorylation of vascular eNOS at this critical residue. Here, we see that eNOS is significantly hyperphosphorylated at ser1177 in juvenile KO animals when compared to juvenile WTs (0.08 ± 0.02 AU for juvenile WTs *vs* 0.25 ± 0.06 AU for juvenile KOs), indicative of increased enzymatic activity. Further to this, WT animals experience a significant increase in the phosphorylation state of eNOS as they age to 15 months (0.08 ± 0.02 AU for juvenile WT *vs* 0.28 ± 0.10 AU for aged WT). Aged KO mice represented the group with the lowest degree of ser1177 phosphorylation (0.03 ± 0.03 AU), which was found to be significantly lower than the phosphorylation status of both juvenile KOs and aged WTs. This result indicates an initial hyperphosphorylation of eNOS in juvenile mice lacking αCGRP that is completely abolished as these animals age to fifteen months. This result is in stark contrast to that observed in WT animals, which significantly increase the level of ser1177 phosphorylation as they age. In summary, both ageing and the deletion of αCGRP can bring about both statistically and biologically significant changes in eNOS activity that may have consequences for cardiovascular processes.

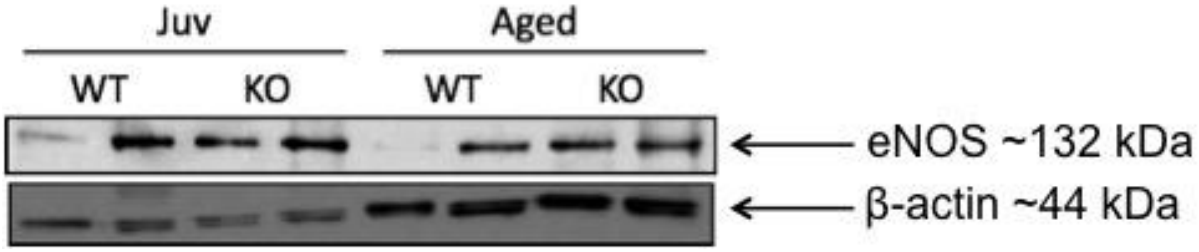

Figure 27: Representative immunoblot of aortic eNOS expression in male juvenile and aged αCGRP WT and KO mice, including β-actin loading control

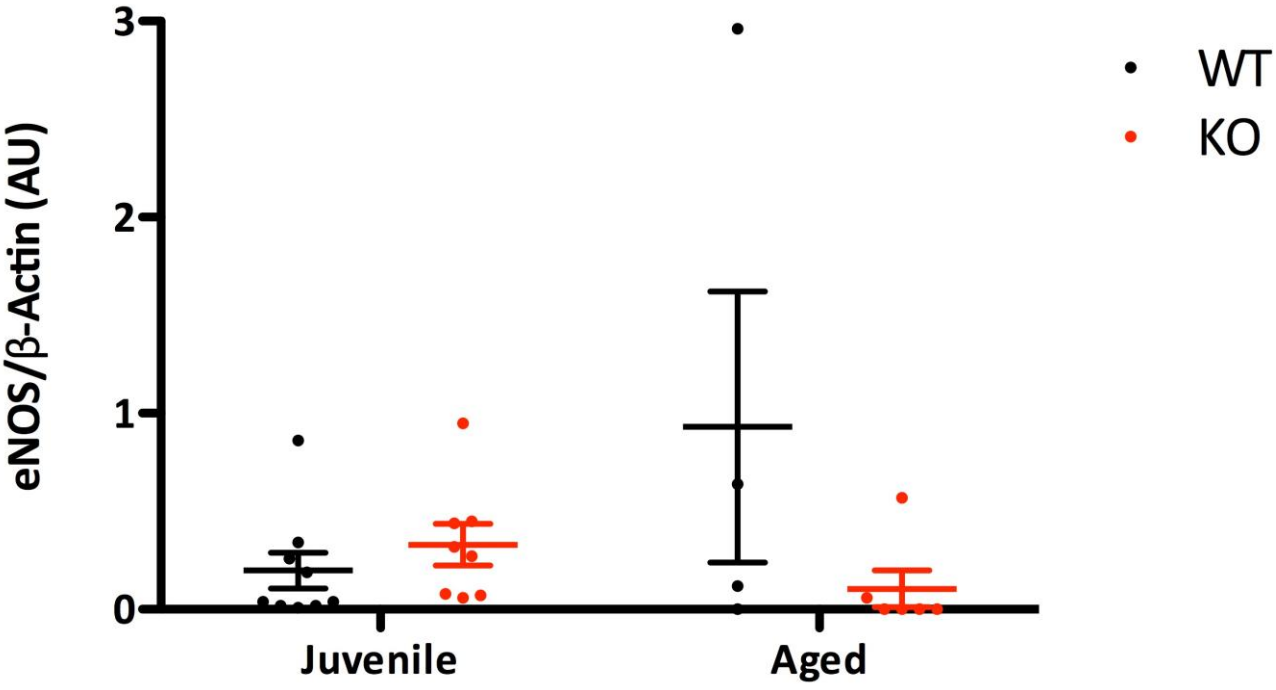

Figure 28: Immunoblot analysis of the expression of eNOS in aortic lysates obtained from juvenile (3 months) and aged (15 months) male αCGRP WT and KO mice. Data expressed as the densitometric ratio between eNOS and β-actin, mean ± SEM, n=4-9. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. No statistically significant differences were detected between groups.

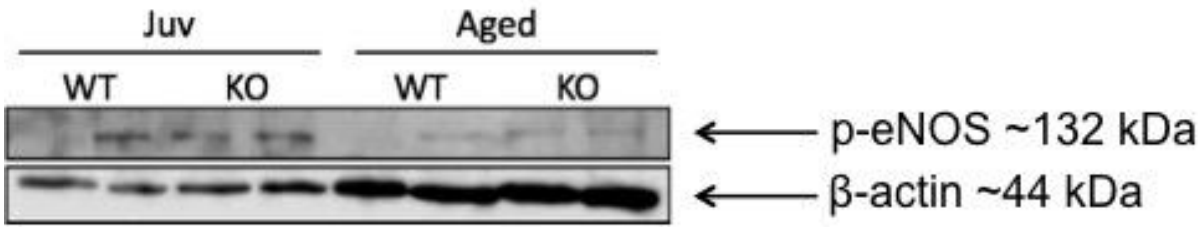

Figure 29: Representative immunoblot of aortic phospho-eNOS expression in male juvenile and aged αCGRP WT and KO mice, including β-actin loading control

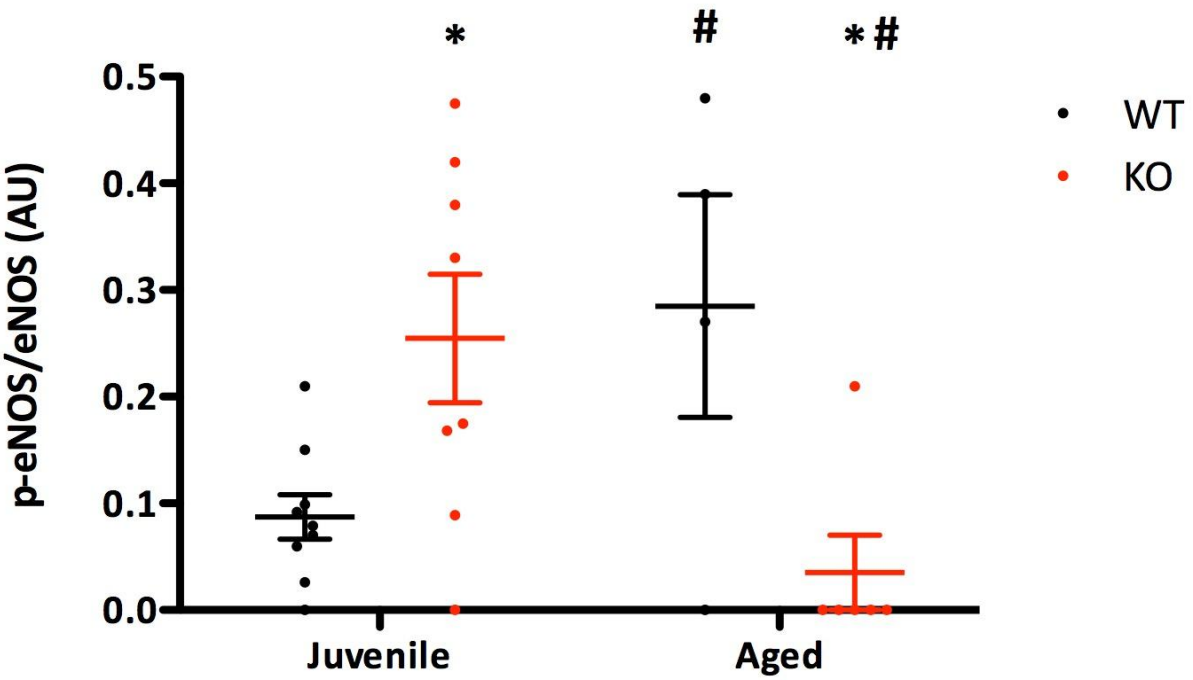

Figure 30: Immunoblot analysis of the expression of Ser1177 phospho-eNOS in aortic lysates obtained from juvenile (3 months) and aged (15 months) male αCGRP WT and KO mice. Data expressed as the densitometric ratio between p-eNOS and total eNOS, relative to β-actin, mean ± SEM, n=4-9. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. #p < 0.05 where juvenile and aged animals of the same genotype have been compared; *p < 0.05 where age-matched WT and KO animals have been compared.

4.4.3 Evaluation of the oxidative vascular environment of ageing αCGRP WT and KO mice

The regulation of NOsignalling is a complex process and is not solely dependent on the level of eNOS expression or indeed, its activation state. Its bioactivity is determined by its rate of removal, which is in part controlled by ROS. ROS are also involved in the genesis of vascular inflammation, so we considered that studying of the vascular environment with respect to oxidative stress an important area of research. To first address this area, we examined how gene expression of classical pro- and anti-oxidant enzymes differed in the aortae of juvenile and aged αCGRP WT and KO mice. Results obtained are illustrated in Figure 31. Figure 31A refers to aortic NOX2 gene expression, where we found that levels were comparable in juvenile WT and KO mice (137.87 ± 34.75 copies/µl *vs* 100.77 ± 16.11 copies/µl, respectively). NOX2 expression was significantly increased in aged WT animals (272.28 ± 54.97 copies/µl) when compared to their juvenile counterparts. Aged KO animals trended towards having higher expression than their juvenile counterparts (172.16 ± 21.53 copies/µl) although a statistically significant difference was not observed. Figure 31B shows a similar pattern of results for NOX4 expression. Gene expression levels were similar in juvenile WT and KO mice (3926.50 ± 941.09 copies/µl *vs* 3772.80 ± 616.21 copies/µl, respectively). NOX4 mRNA significantly increased in aged WTs (14180.29 ± 3212.27 copies/µl) when compared to their juvenile counterparts. NOX4 expression in the aged KOs appeared to increase (10796.00 ± 2505.78 copies/µl), though again this was not a statistically significant difference from juvenile animals. Aside from pro-oxidant enzyme gene expression, we also investigated the expression of anti-oxidant enzymes, specifically HO-1. Again, we found similar levels of expression in juvenile WT and KO mice, shown in Figure 31C (211370.40 ± 17635.86 copies/µl *vs* 181751.40 ± 24201.53 copies/µl). Aged WTs had significantly higher levels of HO-1 mRNA (471088.60 ± 114171.00 copies/µl) when compared to their juvenile counterparts. Aged KO animals did not differ significantly in HO-1 gene expression (264373.30 ± 49436.28 copies/µl).

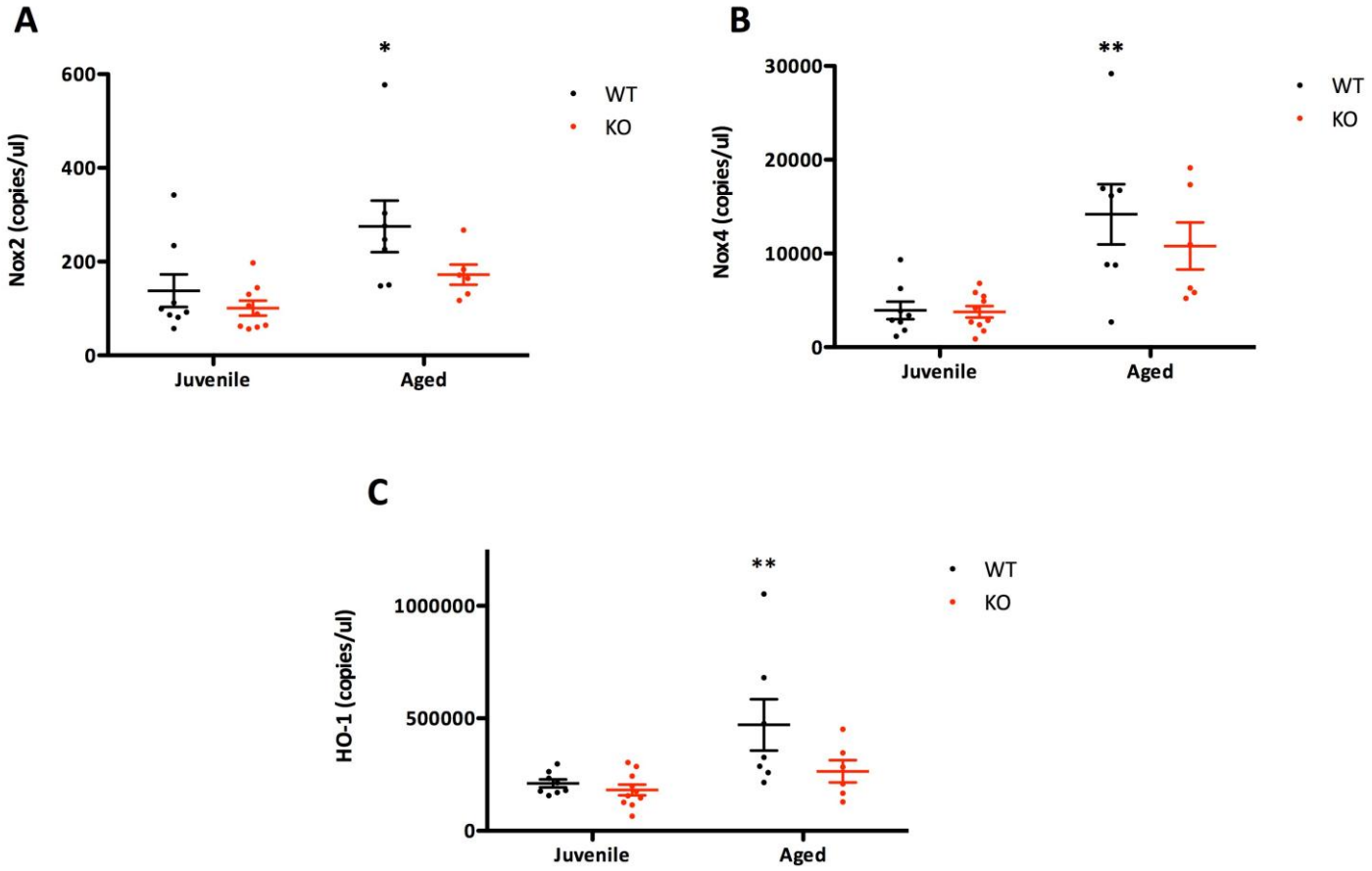

Figure 31: Gene expression analysis of vascular oxidative stress markers of aortic lysates obtained from juvenile (3 months) and aged (15 months) male αCGRP WT and KO mice. Results show A) mRNA expression of NOX2 in aortic lysates; B) mRNA expression of NOX4 in aortic lysates; C) mRNA expression of HO-1 in aortic lysates. Data expressed as copies mRNA per microliter, mean ± SEM, n=6-9. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. *p < 0.05; **p < 0.01 where juvenile and aged animals of the same genotype have been compared.

Further to the analysis of pro-/anti-oxidant gene expression within the aortae of juvenile and aged αCGRP WT and KO mice, we also examined macromolecular markers of oxidative damage, by looking for evidence of nitrosylated tyrosine residues present within vascular proteins and the presence of the lipid peroxidation product, 4-hydroxynonenal (4-HNE). Figures 32 and 33 show nitrotyrosine expression in aortic lysates, determined by immunoblotting. We show that there is a large degree of variability of nitrated tyrosine residues and expression was found to be comparable between all groups. In contrast to this, we found changes in the amount of peroxidised lipid products present within the aortic milieu of juvenile and aged αCGRP WT and KO mice. Figures 34 and 35 show that there was no difference in 4-HNE accumulation between juvenile WT and KO mice (0.61 ± 0.44 AU *vs* 0.65 ± 0.11 AU, respectively). However, levels were significantly increased in aged WT mice (1.45 ± 0.04 AU) when compared to their juvenile counterparts. Additionally, aged KO animals had significantly higher expression of 4-HNE when compared to juvenile KOs (2.08 ± 0.49 AU). This did not differ significantly from aged WTs though there was a trend towards it being increased. Thus, whilst nitrotyrosine levels appeared to go unchanged between experimental groups, we have found evidence for increased formation of 4-HNE products as part of the ageing process.

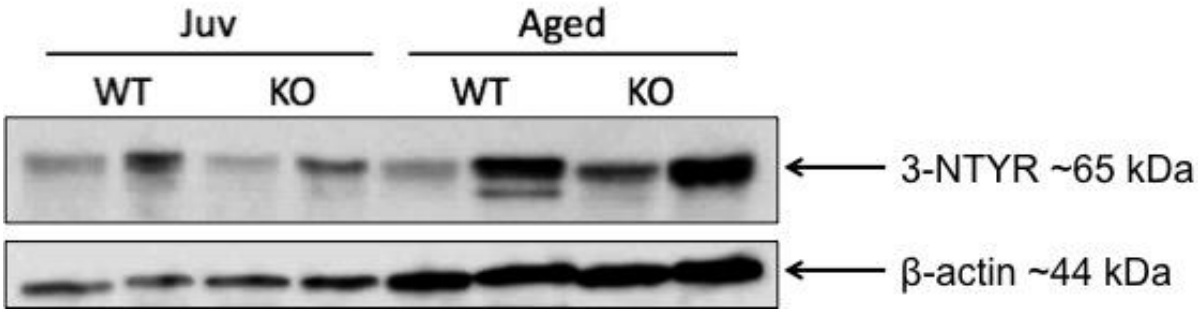

Figure 32: Representative immunoblot of aortic nitrotyrosine expression in male juvenile and aged αCGRP WT and KO mice, including β-actin loading control

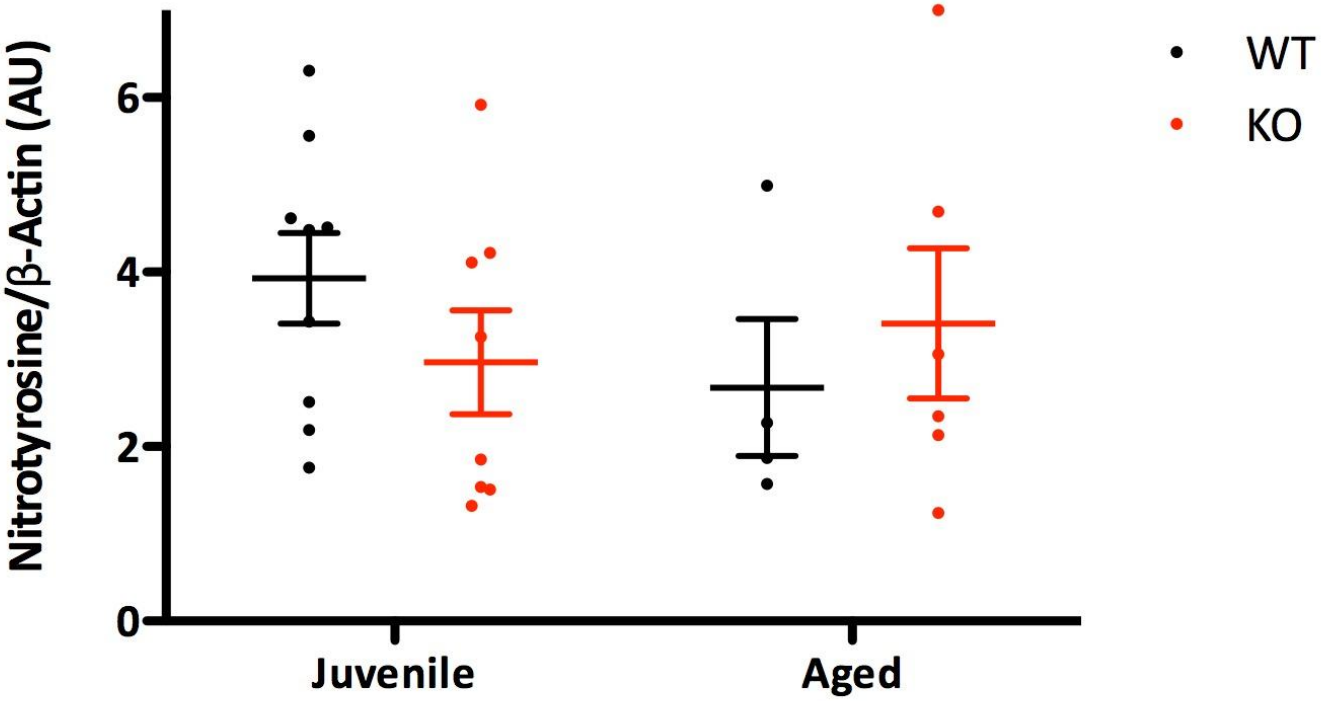

Figure 33:Immunoblot analysis of the expression of nitrated tyrosine residues in aortic protein lysates obtained from juvenile (3 months) and aged (15 months) male αCGRP WT and KO mice. Data expressed as the densitometric ratio between nitrotyrosine and β-actin, mean ± SEM, n=4-8. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. No statistically significant differences were detected between groups.

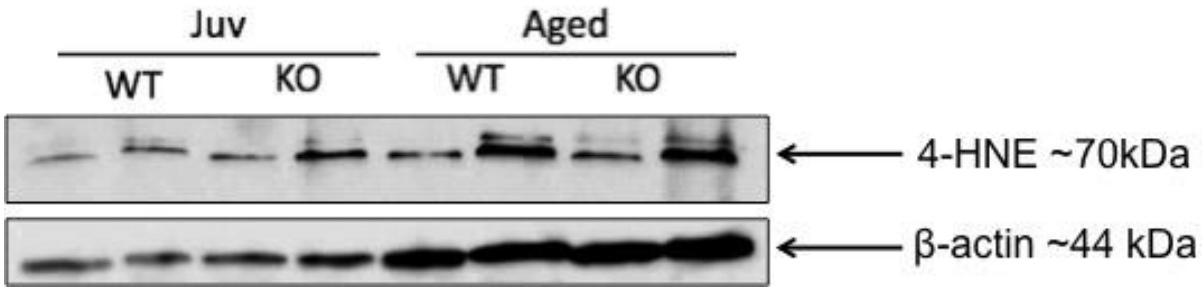

Figure 34: Representative immunoblot of aortic 4-HNE expression in male juvenile and aged αCGRP WT and KO mice, including β-actin loading control.

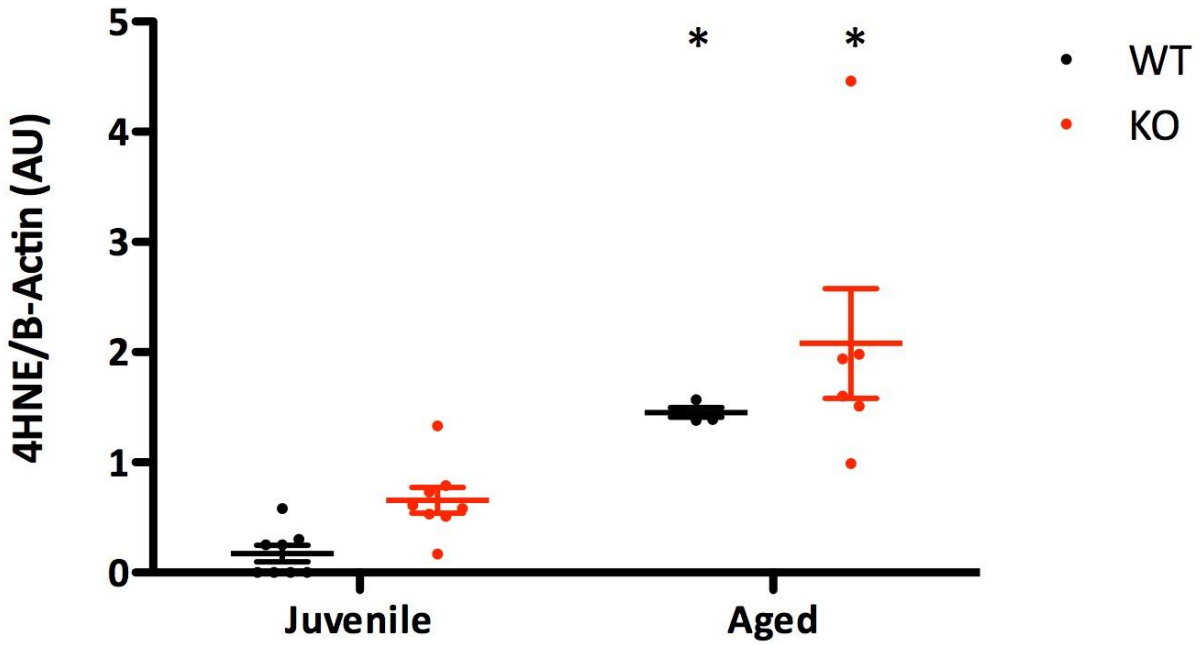

Figure 35: Immunoblot analysis of the expression of lipid peroxidation product, 4-HNE in aortic lysates obtained from juvenile (3 months) and aged (15 months) male αCGRP WT and KO mice. Data expressed as the densitometric ratio between 4HNE and β-actin, mean ± SEM, n=4-8. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. *p < 0.05 where juvenile and aged animals of the same genotype have been compared.

4.4.4 Assessment of localised vascular inflammation and leukocyte infiltration in the aortae of ageing αCGRP WT and KO mice

As a pro-oxidant environment can strongly influence the progression of vascular inflammation during ageing, we sought to gain an understanding of how this inflammation is driven and how it may be influenced by CGRP. Figure 36 illustrates data obtained from a multiplex plasma cytokine array with samples obtained from aged male αCGRP WT and KO mice. We found no significant differences in the circulating levels of cytokines IL-1, IL-6, IL-10, IL-12, TNFα and IFNγ (Figure 36A-F). However, we did observe a trend towards a potential difference in the levels of KC between these mice. Figure 36G shows that levels of circulating KC tended to be lower in aged αCGRP WT mice (799.90 ± 62.54 pg/ml) in comparison with aged KO animals (1199.00 ± 177.20 pg/ml).

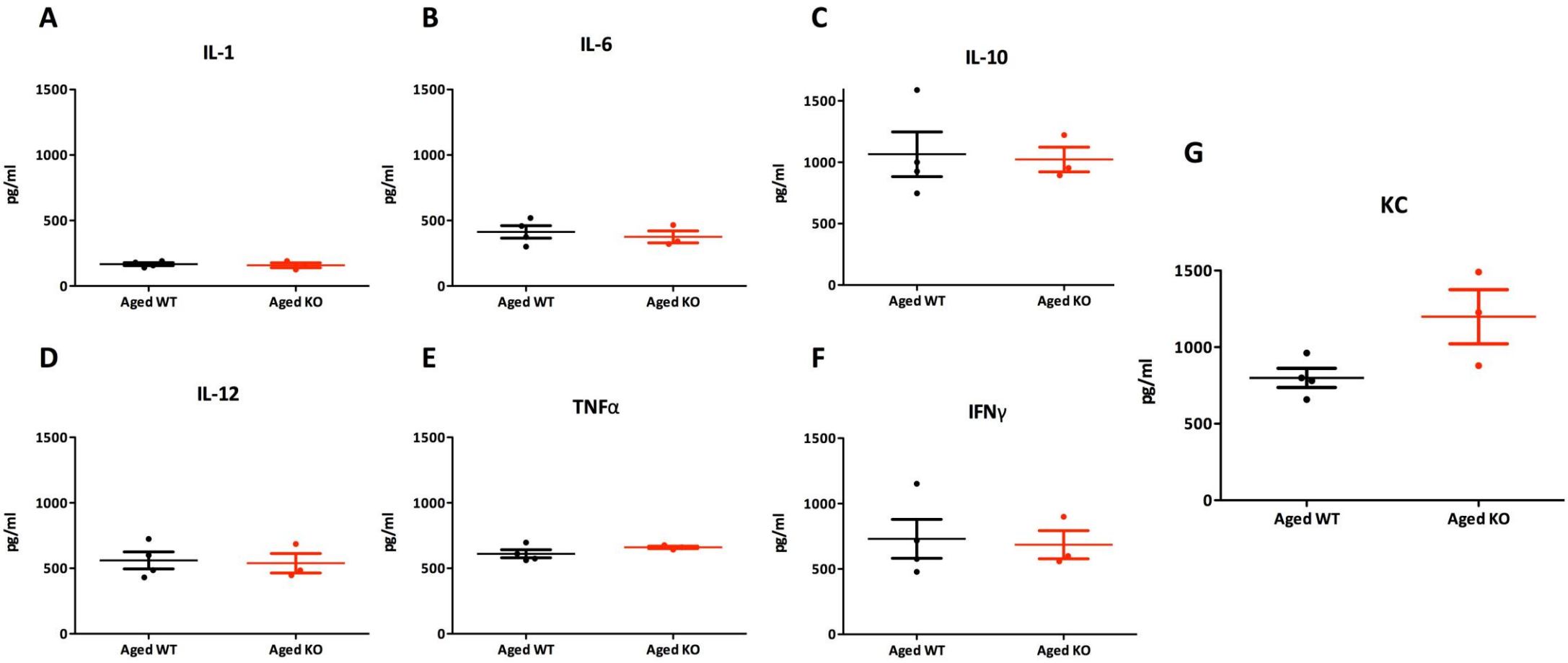

Figure 36: Analysis of circulating proinflammatory cytokine expression in plasma obtained from aged (15 months) male αCGRP WT and KO mice. Results show cytokine concentrations for A) IL-1; B) IL-6; C) IL-10; D) IL-12; E) TNF-α; F) IFN-γ; G) KC. Data expressed as pg/ml, mean ± SEM, n=3-4. Statistical significance was evaluated by paired two-tailed Student's t-test. No significant differences were found between groups.

Accompanied with ageing is the increased likelihood of cells within tissues of the vasculature to halt cell division and become senescent. The presence of senescent cells can greatly exacerbate the actions of a pro-inflammatory milieu to bring about vascular dysfunction. Figures 37A and 37B show the mRNA expression of the senescence markers p16 and p53, respectively. We found that levels of aortic p16 did not change significantly with age and was no affected by αCGRP gene deletion. However, we did observe an elevation in aortic p53 expression in WT animals as they aged from three to fifteen months (85.56 ± 9.12 copies/µl in juveniles *vs* 144.95 ± 16.35 copies/µl in aged mice). There was a trend towards KO tissues having higher p53 expression as they got older, though this difference did not reach statistical significance.

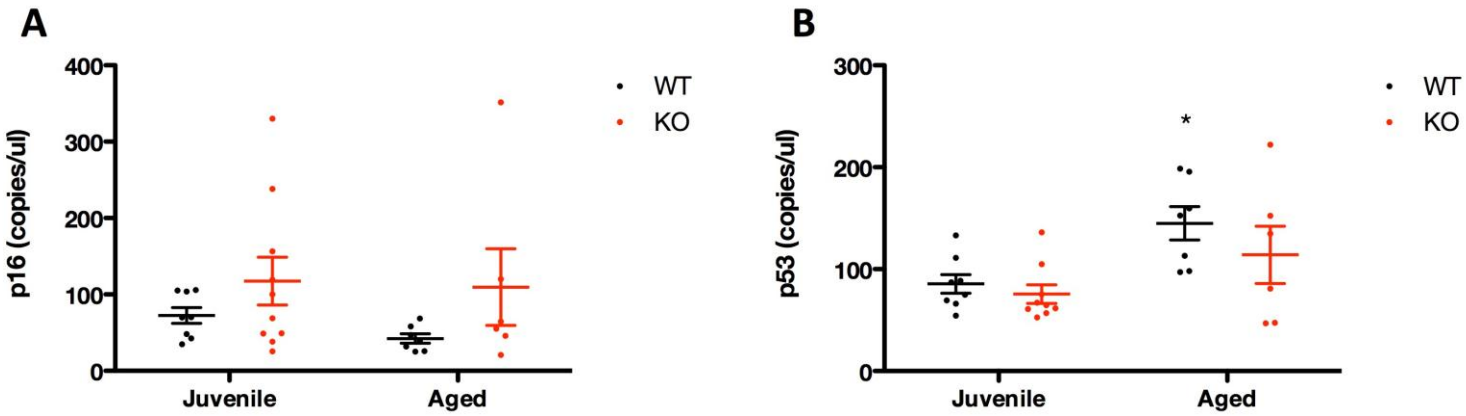

Figure 37: Gene expression analysis of cellular senescence markers associated with ageing obtained from aortae of juvenile (3 months) and aged (15 months) male αCGRP WT and KO mice. Results show A) mRNA expression of p16 in aortic lysates; B) mRNA expression of p53 in aortic lysates. Data expressed as copies mRNA per microliter, mean ± SEM, n=6-10. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. *p < 0.05 where juvenile and aged animals of the same genotype have been compared.

As a consequence of increased pro-inflammatory factors with age, there is the increased likelihood of circulating inflammatory cells being recruited into the vascular wall to further contribute to inflammation. To measure this, we looked for differences in VCAM-1 mRNA expression in the aortae obtained from juvenile and aged male αCGRP WT and KO mice. Figure 38A shows that VCAM-1 expression in juvenile WT and KO mice was comparable (23179.79 ± 3297.79 copies/µl *vs* 32231.60 ± 5308.63 copies/µl, respectively). As mice aged, levels of VCAM-1 expression were significantly elevated when compared to their juvenile counterparts (105133.70 ± 13018.94 copies/µl in aged WT *vs* 89596.00 ± 25418.29 copies/µl for aged KOs). However, there was no effect from the deletion of αCGRP in either juvenile or aged mice on VCAM-1 expression. In line with this pattern of expression, we observed raised F4/80 mRNA expression in aortic lysates as animals aged from three to fifteen months, indicative of monocyte/macrophage infiltration (Figure 38B). However, αCGRP deletion had no effect on this process. Investigations into neutrophil recruitment into the vascular wall by analysing aortic myeloperoxidase (MPO) expression yielded a similar pattern of results as mentioned before, although copy numbers were very low, indicating perhaps that neutrophil infiltration is not a key aspect of the ageing phenotype (Figure 38C).

.

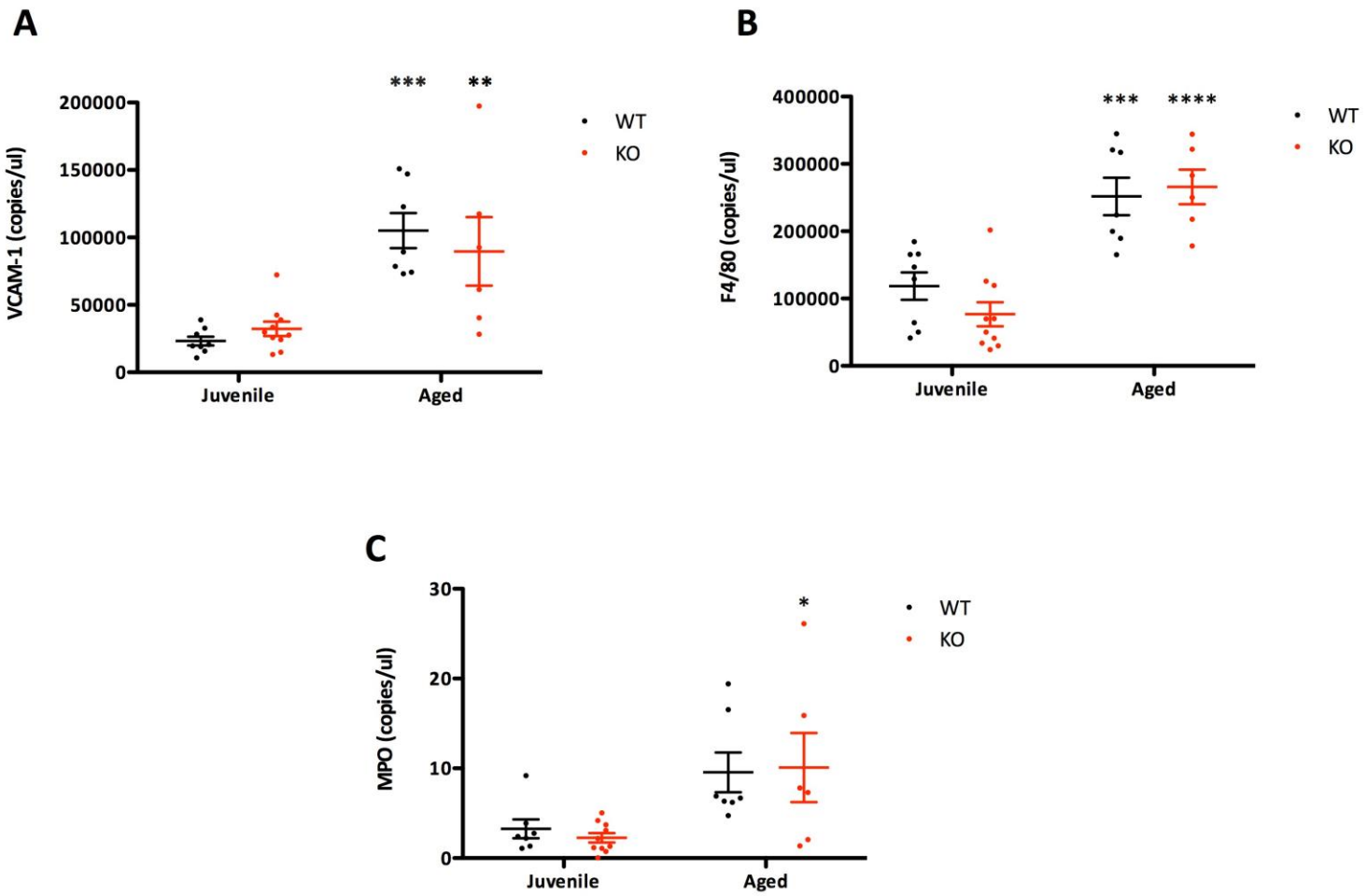

Figure 38: Gene expression analysis of vascular markers associated with leukocyte infiltration obtained from aortae of juvenile (3 months) and aged (15 months) male αCGRP WT and KO mice. Results show A) mRNA expression of VCAM-1 in aortic lysates; B) mRNA expression of F4/80 in aortic lysates; C) mRNA expression of MPO in aortic lysates. Data expressed as copies mRNA per microliter, mean ± SEM, n=6-10. Statistical significance was evaluated by two-WAY ANOVA plus Bonferroni *post hoc* test. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 where juvenile and aged animals of the same genotype have been compared

4.5 Discussion

In Chapter 3, I showed that deletion of αCGRP in male juvenile and aged mice did not lead to impairments in gross parameters of murine development. In this Chapter, I aimed to further characterise the response to ageing in αCGRP WT and KO mice with respect to *in vivo* haemodynamic parameters, associated markers of vascular remodelling and markers for leukocytic vascular inflammation. We hypothesised that as vascular reactivity to CGRP was diminished with age, as shown in Chapter 3, intracellular signalling pathways related to CGRP might be less capable of mounting a defense against elevated blood pressure and associated vascular inflammation.

4.5.1 Of mice, men and mechanisms of ageing

Mice are undoubtedly the favourite mammalian model in use to study a wide range of (patho)physiological processes. Regardless of the research field, they are used because they have many advantages in a laboratory setting: they are small, easy to care for, and breed well in captivity, such that the cost of maintaining large populations is not overwhelmingly prohibitive. Furthermore, their basic biology and genome are strikingly similar to that of humans and are largely well understood. This feature, combined with advances in genetic technologies, has facilitated considerable advances in scientific endeavour. Despite these advantages, the use of mice has been called into question with regards to the study of the biological processes of ageing, owing to the perception that murine ageing is not directly comparable to that of humans. It is obvious that the lifespan of a mouse is significantly shorter than that of man, pointing to differences in our evolutionary histories that might have helped shape these phenotypes. More subtle differences between the two organisms begin to emerge when one examines their biology in more detail. For example, mice have considerably long telomeres in comparison to humans (so long they are known as 'mega-telomeres') and high telomerase activity in many of their somatic tissues, meaning that the relative contribution of replicative senescence to the ageing phenotype in mice may be less of that than in humans (Kipling and Cooke, 1990). Furthermore, mice are capable of synthesising their own vitamin C whilst humans cannot. Vitamins are now understood to play a crucial role in contributing towards the ageing process so mice may well be 'protected' against an ageing phenotype that may be associated with vitamin deficiencies. However, the main problem with the use of mice (and, indeed, most laboratory animals) as models of human ageing is that they do not demonstrate all the typical age-related diseases seen in humans, including CVD. Thus, it has been posited that mice are unrepresentative of human ageing and therefore may not be as useful as we once thought.

The argument that mice are not a useful research tool used to study ageing and age-associated disease because they do not succumb to traditional gerontological diseases is unhelpful. The very fact that mice do not appear to develop CVD as they age, despite having a highly homologous genome and evolutionarily conserved ageing processes to humans is an important discovery. This tells us that it is likely that differences in the way our respective *environments* interact with our genomes is the real driver of the development of age-induced disease. Mice bred in standard laboratory conditions do not smoke, drink alcohol, eat a typical western diet or encounter infection. These are all significant risk factors for the development of CVD with age in humans. It is often the case that one has to introduce a significant environmental stressor in order to induce pathology in mice (for example, the feeding of a high-fat diet to induce hyperlipidaemia-associated disease, such as atherosclerosis). The advantage to using mice in ageing research is that one can often take a relatively reductionist approach by eliminating a multitude of confounding environmental factors in order to study the key mechanisms that underpin the ageing process. This approach would be impossible to take in a human cohort.

Many of the fundamental mechanisms of ageing appear to be conserved throughout the tree of life and many aspects of ageing are quite malleable throughout the species. Indeed, mice appear to be susceptible to aspects of oxidative stress-induced ageing and manipulation of the systems that govern this process can result in alterations in healthspan. That is to say, the amount of time spent in good health, rather than a simple extension of life. For example, genetic deletion of antioxidant defense enzymes can lead to an increased risk of age-related disease whilst mice that overexpress these types of enzymes can often live for longer in good health. Additionally, mice engineered to have a reduced susceptibility to cellular senescence or an improved clearance of senescent cells leads to retention of youthful characteristics (Baker et al., 2011). Taken together, a reduction of the impact of “inflammaging” processes can promote healthspan in laboratory animals, which translates to humans. Furthermore, pharmacological and dietary interventions implemented to improve lifespan *and* healthspan have been more or less successful in most eukaryotic organisms, starting in yeast and including mice(Blagosklonny et al., 2009; Ungvari et al., 2010). This indicates a level of evolutionary conservation between organisms in the malleability of ageing processes. Some of the positive results obtained from intervention studies have been recapitulated in laboratory animals such as mice and also non-human primates. Therefore it is not too much of a stretch of the imagination to consider this may be a phenomenon also present in humans.

4.5.2 Deletion of αCGRP does not adversely affect baseline haemodynamics or vascular remodeling in the ageing mouse

One of our early findings from the *in vivo* profiling of juvenile and aged αCGRP WT and KO mice was that there appeared to be no differences in baseline haemodynamics between these groups of mice. Blood pressures were normal in juvenile mice (roughly 120/80 mmHg) and these healthy pressures were maintained throughout life as animals aged to fifteen months, where the removal of αCGRP gene had no outcome. This is perhaps unsurprising given what is already known about the haemodynamic phenotype of this particular mouse. Lu *et al*. were the first to characterise blood pressure and heart rate in the juvenile αCGRP KO mouse. They showed that mean arterial pressure and heart rate were similar in resting animals and, further to this, blood pressure was unchanged between genotypes following subjection to a forced-swimming test. However, it is of note that the heart rate of the KO mice was lower than WT following exercise (Lu et al., 1999). Others using the same mouse have shown increases in both basal blood pressure and heart rate (Oh-hashi et al., 2001; Kurihara et al., 2003), however our group has recently published showing that baseline blood pressures are similar in WT and KO mice and differences only emerge when the cardiovascular system becomes stressed (Smillie et al., 2014). Other groups have made use of a CGRP KO mouse generated *via* the complete interruption of the CALC I gene, where both CGRP and calcitonin have been deleted. Results obtained from these mice have been a little more unanimous as to the role of CGRP in the regulation of haemodynamics, where deletion of both peptides tends to favour the development of spontaneous hypertension (Oh-hashi et al., 2001; Li et al., 2004). These results are often over-interpreted as supporting a role for CGRP in the regulation of basal blood pressure, though obviously this is an inappropriate conclusion to arrive at for a mouse that is essentially a "double knockout".

As the regulation of vascular tone is a process that requires such detailed fine-tuning, it is perhaps unsurprising that evolution has not favoured the development of a relatively expensive biosynthetic pathway to regulate this process at a tonic level of activity. When one considers the role of sympathetically-derived noradrenaline (NA) in regulating vascular tone, this small molecule lends itself to high-frequency activity. The biosynthetic pathway is initially expensive to build (requiring multiple enzymes), but once built, production of NA from tyrosine is energetically cheap. Furthermore, released NA is readily taken back up by the neuron to be stored for future release, meaning this is an efficient system for a process that is constantly active (Burnstock, 2009). One also only has to look at the characteristics of interneuronal chemical signalling within the central nervous system to appreciate that similar high-frequency activities most often make use of small molecule transmitters and employ more complex molecules as modulators of this activity. The *de novo*

synthesis of a peptide, its cleavage, axoplasmic transport to the nerve terminal, storage and release is energetically cumbersome on the cell, never mind the fact that CGRP is usually broken down within minutes of release by potentially more than one enzyme, with no strong evidence to support its re-uptake(Sams-Nielsen et al., 2001; Gupta et al., 2010; Sheykhzade et al., 2011). Therefore, it seems incongruous that CGRP should be involved in the tonic regulation of blood pressure and rather is involved in the fine-tuning of the vasomotor response in the face of stress and defined stimuli.

We have shown that there is no effect of CGRP gene deletion on haemodynamic parameters when compared to WT mice and I have postulated that this is the case owing to the relative expense of maintaining the CGRP signalling axis at a tonic level of activity under normal physiological conditions. Perhaps unsurprisingly, we also have shown that ageing does not induce perturbations in blood pressure in mice. As mentioned in the previous section, mice do not spontaneously develop cardiovascular complications as they get older and disease in mice more often than not needs to be induced by some kind of stressor. It is possible that the ageing process hitherto the fifteen-month mark does not represent enough of a biological stress upon the cardiovascular system to propagate an overtly abnormal phenotype, such as hypertension. A figurative interpretation of this may better emphasise this point by describing intrinsic biological ageing as the fertile substrate in which the extrinsic seed of disease can be planted and encouraged to grow.

Associated with haemodynamic changes during hypertension are structural alterations to the vasculature, in response to changes in pressure and also inflammation that accompanies the disease. For this reason, we thought it prudent to investigate these structural changes of the vessel wall as a marker of increased blood pressure that may potentially occur in the absence of detectable increases in blood pressure. We have shown that there were no differences in aortic smooth muscle cell content in CGRP WT and KO mice, either in juveniles or aged animals. Recent data published by our group has shown that hyperplasia of the aortic vascular smooth muscle can occur in response to ATII-induced hypertension. In this setting, hypertensive juvenile CGRP KO mice were shown to have a significantly higher degree of hyperplasia in comparison to WTs (Smillie et al., 2014). Administration of rutaecarpine (a reported TRPV1 agonist, discussed in the introduction to this thesis)to renovascular hypertensive rats has also been demonstrated to reverse mesenteric resistance vessel VSMC remodeling *via* a mechanism that is speculated to involve activation of TRPV1, leading to subsequent CGRP release (Qin et al., 2007). Others have also shown that CGRP can protect against adverse VSMC remodeling, particularly within the setting of pulmonary hypertension. Here, *in vivo* adenoviral transfer of the CGRP gene can limit VSMC remodeling in the

pulmonary vasculature of mice subjected to chronic hypoxia (Champion et al., 2000; Bivalacqua et al., 2002). CGRP also has an antiproliferative effect on VSMCs in response to mitogenic stimuli, such as ATII(Qin et al., 2004). This inhibitory effect of CGRP on VSMC proliferation has been ascribed to regulation of an ERK/MAPK-dependent pathway (Qin et al., 2004). Therefore, it has been shown that CGRP has the *potential* to regulate VSMC activity in certain conditions. A study performed in ageing rats demonstrated that aortic morphology was unchanged when compared to younger animals and VSMC proliferation and migration could only be induced by balloon injury (Wu et al., 2007). With this and our own results in mind, it is entirely possible that ageing to fifteen months in the mouse does not represent a significant stress to the cardiovascular system to allow for VSMC remodeling and a more substantial insult is required to reveal the relative contribution of ageing to the disease process.

Whilst we detected no changes in VSMC morphological phenotype across experimental groups, we did detect a significant increase in adventitial collagen width when WT animals aged to fifteen months. A similar trend was observed in KO animals that did not reach statistical significance. Further, there was a general trend towards juvenile KO mice having an increased aortic collagen width measurement. Curiously, this significant difference was lost when the total area of collagen was measured. Again, results from our laboratory have shown that collagen deposition is enhanced in the setting of ATII-induced hypertension and CGRP can protect against this accumulation (Smillie et al., 2014). Others have shown that CGRP is protective against hepatic fibrosis in a Concanavalin A model of chronic hepatitis (Kamiyoshi et al., 2009) and also against cardiac fibrosis during pressure-overload heart failure (Li et al., 2013b). Therefore, it would appear that CGRP has the potential to protect against aberrant fibrotic remodeling in certain disease conditions. It is known that there is a significant vascular remodeling response to ageing with respect to fibrosis, including in the large elastic arteries. This usually results in the conduit vessels (such as the aorta) becoming stiff. Stiffening of the aorta can often be a precursor to isolated systolic hypertension due to pressure waves emanating from the heart following systole not being appropriately absorbed. This results in the pressure wave being reflected back towards the heart, increasing cardiac afterload and thereby increasing systolic blood pressure(Jani and Rajkumar, 2006). The mechanisms behind which vessels can become stiff with age are numerous, although generally speaking an increase in collagen deposition and a reduction in elastin availability is a hallmark characteristic of the response (Fleenor, 2013). The discrepancy in our findings (increased collagen width but no significant increase in total area) may have transpired from a statistical underpowering of this experiment. Collagen width is measured by averaging eight replicate measurements from one section of aorta, pertaining to one animal. This measurement lends itself to a reduction in variability, which improves the chances of a

statistically significant event being identified. This indeed may be the case, as we do see a trend towards an increase in collagen area in both groups with age. Alternatively, this may be considered an artefactual finding attributable to the preparation of the sample during fixation and embedding. The vessel's shape may have become distorted, owing to stretching of regions of the vessel, which may falsely give the impression of a larger width. Despite these shortcomings, we generally identify an increase in collagen with age, which is in keeping with the published literature. We saw no evidence for CGRP in worsening this phenomenon in KO mice.

4.5.3 eNOS is not differentially expressed in juvenile and aged αCGRP WT and KO mice but CGRP gene deletion does regulate enzyme activity

A key regulator of vascular homeostasis is NO. The principle site of synthesis of NO within the vasculature is the EC, where endothelial NO synthase (eNOS, NOS3) constitutively generates NO*via* the conversion of L-arginine. A reduction in NO bioavailability (commonly referred to as 'endothelial dysfunction') is associated with a wide range of cardiovascular complications, including hypertension and atherosclerosis. Furthermore, it is generally considered that the vascular ageing response is characterised by a reduction in NO bioavailability. NO was a key molecular candidate for us to study within the context of ageing, not only because it is a significant player in the regulation of vascular tone and thus systemic blood pressure, but also owing to its potent anti-inflammatory effect which may occur in the absence of changes in pressure.

We made efforts to probe multiple components of the NO pathway in juvenile and aged αCGRP WT and KO mice. We have shown that both aortic eNOS mRNA and protein expression was unchanged as mice aged to fifteen months and there appeared to be no effect of αCGRP gene deletion at either stage of development on these processes. The literature surrounding the regulation of eNOS gene and protein expression with age is substantial, but also very unclear. Analysis of mRNA gene expression by other groups has yielded results that both support increases (Barton et al., 1997) and decreases of eNOS gene expression (Tang and Vanhoutte, 2007) in the aortae of ageing rats. Others investigating changes in eNOS mRNA in the mesenteric circulation of ageing humans found that expression did not change over time (Cernadas et al., 1998a). Studies performed in cultured human ECs from different vascular beds have also supported the finding that eNOS mRNA expression goes unchanged with age (Donato et al., 2009). Results are so variable owing to the variability in the research methods used to investigate this phenomenon. Factors including choice of organism studied, the particular strain of that organism and the timepoint at which the organism is studied, never mind the vascular bed called into investigation can all present confounding variables that lead to a wide range of conclusions. Those studying eNOS protein have also not come to a unanimous conclusion regarding its differential expression over time. Some have reported significant increases in protein expression in the aortae and mesenteric arteries of ageing rats. However, others have failed to show a difference in expression in the mesenteric vessels of the same species (Sun et al., 2004). Thus, our data concerning mRNA and protein expression of aortic eNOS in ageing mice contributes towards the burgeoning literature surrounding this phenomenon, but unfortunately does not result in the crystallization of a solid conclusion. We have recently shown that CGRP gene deletion can influence eNOS expression in an ATII model of hypertension. Hypertensive KO mice were shown to have an exacerbated loss of eNOS mRNA and protein at both 14 and 28 days of

hypertension (Smillie et al., 2014). However, it is not clear whether this is a reflection on the loss of a potent vasodilator in the face of hypertension or if CGRP actively regulates eNOS expression in these mice. Our results from ageing mice that do not suffer from hypertension would suggest alterations in total eNOS expression might be at least partly-dependent on rises in pressure.

It is evident that data concerning the expression of vascular eNOS at both the levels of mRNA and protein is unclear. However, this analysis does not reflect the full picture concerning NO bioavailability. Clearly, other regulatory processes must be changing in the absence of a clear expression phenotype, as NO bioavailability is overall generally considered to decrease as one ages. In addition to transcriptional and translational regulations, eNOS presents a significant degree of regulation *via* post-translational modifications. Specifically, eNOS is significantly regulated by the phosphorylation status of a critical serine residue (Ser1177), typically mediated by PI3K/Akt (Sessa et al., 1999). Using phospho-specific antibodies for this residue, we have shown a complex pattern of regulation in juvenile and aged αCGRP WT and KO mice. In young animals, we found that the degree of phosphorylation was significantly higher in KO animals *versus* WT. Further to this, as WT animals aged from three months to fifteen, phosphorylation of eNOS was significantly increased. This was a response that failed to be retained in the aged KO animals, where Ser1177 phosphorylation was significantly lower than juvenile KO mice and age-matched WTs. This indicates a complete flattening of the response and an inability to maintain the hyperphosphorylation of eNOS enzyme present in juvenile KOs throughout the ageing process. Others have shown that total eNOS enzyme present within the mesentery of aged rats was preserved as the animals grew older, yet the amount of phosphorylated eNOS was decreased (Sun, 2004). This may be linked to a loss in Akt activity (the enzyme responsible for ser1177 phosphorylation) (Soucy et al., 2005) with age and a decline in the amount of enzyme localised to the caveolae of the plasma membrane (Smith et al., 2006) – a process essential for eNOS function. It is interesting that in our WT mice, we witness an increase in phospho-eNOS expression where others see a loss. This may be due to differences in species studied and also the particular timepoint we have chosen to examine – there may be a compensatory upregulation in enzyme activity in 'middle-age', whereby the cardiovascular system is attempting to restore homeostasis until it is no longer able to maintain this at a later timepoint. Indeed, studies in aged humans have shown increased eNOS phosphorylation in isolated ECs (Donato et al., 2009). Despite this discrepancy, it is clear that the aged KO mice are less capable of mediating this compensation and may suffer vascular inflammation as a result.

4.5.4 The vascular redox status of αCGRP WT and KO mice is unaltered in the ageing mouse

One of the hallmark features of the ageing process is an increased propensity to develop a pro-oxidant vascular environment. For this reason, we sought to investigate how the redox status of the vasculature altered as part of the ageing process in mice and how the deletion of αCGRP impinges upon this process.

We initially characterised the vascular redox status of juvenile and aged CGRP WT and KO mice by profiling aortic mRNA expression of pro- and anti-oxidant enzymes. Whilst it is generally thought that the mitochondria represent one of the biggest producers of ROS as part of the ageing process, undoubtedly other enzymes and systems can converge upon this process. Of note in ageing is the NOX family of enzymes. Numerous evidences of either increased NOX expression and/or activity within the ageing cardiovascular system have been reported (Assar et al., 2013) and, indeed, ROS production by this family of enzymes has been thought to contribute towards reduced NO bioavailability with age and pharmacological blockade of these enzyme systems can attenuate age-associated endothelial dysfunction (McCrann et al., 2009; Zhou et al., 2009). We identified a general upregulation in aortic mRNA expression of NOX2 and NOX4 as CGRP WT and KO mice aged from three to fifteen months. However, no differences were detected between WT and KO mice at either three or fifteen months of age. Thus, CGRP deletion had little influence on the mRNA expression of these ROS-producing enzymes. To further investigate the induction of oxidative stress, we examined the expression of the anti-oxidant enzyme, HO-1. We chose to investigate this particular gene as its expression is rapidly induced following activation of the Nrf2 gene transcription pathway and is thus a useful readout of elevated ROS(Baird and Dinkova-Kostova, 2011). Nrf2 is a transcription factor that is usually retained in the cytoplasm by its repressor protein, Keap1. Keap1 is removed from this complex by electrophiles (such as ROS), allowing Nrf2 to transmigrate into the nuclear compartment to induce gene expression of antioxidant and phase 2 detoxifying enzymes, including HO-1 (Lewis et al., 2010). We found that aortic mRNA expression of HO-1 was raised in aged animals when compared to juveniles. This elevation may be interpreted in one of two ways. First, one may assume that an elevation in HO-1 would render the aged aorta to be protected against free radical-induced damage. However, one must conduct a more robust study in order to make this conclusion by examining protein expression and even activity of this enzyme to understand its action with age. Second, there is a wide range of anti-oxidant enzymes present within the vasculature to counteract damage from ROS and these would need to be more thoroughly profiled. A safer interpretation of our limited study might be that elevated HO-1 at the mRNA level is a marker of a pro-oxidant vascular milieu. However, we once again showed that CGRP gene deletion had no influence on the level of HO-1 expression, indicating that the degree of ‘stress’ present within the vasculature of WT

and KO mice may be comparable. Conversely, we have published showing elevations of HO-1 expression in a severe model of hypertension where KO mice were less able to maintain antioxidant defenses over longer periods of disease (Smillie et al., 2014), indicating that CGRP has the potential to regulate this process in some disease models.

Further evidence of vascular oxidative stress with ageing was investigated by immunoblotting for ROS-modified macromolecules within the aortae of juvenile and aged αCGRP WT and KO mice. We first investigated the expression of proteins containing nitrated tyrosine residues. Nitrotyrosine residues are indicators of oxidative stress due to the nature of their formation. These post-translational modifications are created *via* the interaction between NO and superoxide radical, forming peroxynitrite. Peroxynitrite is a highly reactive molecule that can modify cellular macromolecules, including amino acids present with protein structures (Pacher et al., 2008). This reaction is irreversible, but at a very slow rate, and thus presence of nitrated proteins can be used as a long-term marker of oxidative/nitrative stress. Indeed, other groups have shown that nitrotyrosine expression increases as part of the ageing process, in laboratory animals (van der Loo et al., 2000; Miller et al., 2007) and in humans (Donato et al., 2007). With this in mind, we were surprised to uncover that nitrotyrosine expression in juvenile WT and KO mice was similar to that found in aged WT and KO mice, where once again genetic deletion of αCGRP had little influence on this process. This may be because the formation of nitrated tyrosine residues is dependent on two independent radicals (NO and superoxide) coming together to react to form peroxynitrite. Thus, if levels of one radical are high but the other low, this will result in a poor formation of peroxynitrite and thus poor nitration of proteins. Following this result, we then sought to quantify the expression of another macromolecular marker of oxidative stress, 4-HNE. 4-HNE is formed by the peroxidation of cellular lipids by ROS (Zarkovic, 2003) and its formation is increased as part of the ageing process (Şakul et al., 2013). Our results have shown that formation of 4-HNE adducts was relatively low in the aortae of juvenile mice and was comparable between αCGRP WT and KO mice. As animals aged, there was a significantly increased formation of HNE adducts in WT mice and KO mice also experienced a significant elevation in HNE expression at fifteen months of age, indicating a greater degree of oxidative stress. Interestingly, we recently showed that both nitrotyrosine and 4-HNE expression in the aortae of CGRP WT and KO mice was unchanged in normotensive and hypertensive animals (Smillie et al., 2014). Therefore, it is possible that longer timescales or greater insults are needed to reveal build-ups of macromolecular damage related to oxidative and nitrative stresses.

4.5.5 αCGRP gene deletion does not influence the development of vascular inflammation in aged mice

As a consequence of endothelial dysfunction and increased oxidative stress within the vascular compartment as a function of age, the risk of the development of vascular inflammation is high. We investigated aspects of the vascular inflammatory response in male juvenile and aged αCGRP WT and KO mice. In a small cohort of aged WT and KO animals we performed analysis of circulating cytokines. On the whole, we showed that the cytokine profiles of WT and KO aged animals were largely similar. However, we did uncover a trend towards an increased bioavailability of KC (CXCL1). A link has been demonstrated between CGRP and CXCL1 in the past, where treatment of LPS-stimulated human dermal microvascular ECs with CGRP was sufficient to attenuate cytokine release from these cells, of which CXCL1 was one such cytokine (Huang et al., 2011). Therefore, genetic deletion of CGRP may over time lead to an increased bioavailability of KC/CXCL1, which may predispose the vasculature to the development of inflammation. CXCL1, when bound to its receptor CXCR2, acts as a potent neutrophil chemotactic agent(Ley, 2003). If the KC/CXCL1 signal is not tightly regulated, then this may result in collateral damage from inappropriate activity of cells of the innate immune system. It is interesting that we did not observe a difference in TNFα bioavailability as a complex physioimmunological antagonism is thought to exist between this cytokine and CGRP. For example, treatment of endotoxic mice with CGRP improves mortality whilst concomitantly decreasing TNFα production (Gomes et al., 2005; Kroeger et al., 2009). The lack of difference between groups, coupled with the subtle difference in plasma availability of KC/CXCL1 may simply be a reflection of the severity of this model, meaning that differences owing to the contribution of CGRP gene deletion are harder to see in a relatively subtle model of cardiovascular stress.

Inherent to the ageing process is the accumulation of senescent cells within the vascular tissues. Senescence is usually brought about by attrition of the telomeres past a certain critical point on the chromosome, due to excessive mitotic cell division. Markers of cellular senescence include the cell cycle inhibitor proteins p16 and p53 (Muñoz-Espín and Serrano, 2014). As senescent cells are capable of changes in phenotype that help propagate vascular inflammation by virtue of their 'senescence-associated secretory phenotype' or 'SASP', we investigated the expression of p16 and p53 in order to gain an understanding of how significant a contribution cellular senescence may have been towards vascular inflammation in our model (Muñoz-Espín and Serrano, 2014). We showed that p16 mRNA expression did not change as our mice aged from three to fifteen months and there was no significant effect related to the deletion of αCGRP. However, we did detect a significant increase in p53 mRNA expression in aged WT animals versus their juvenile counterparts, indicating that 15 months of age is a substantial time to induce senescence. Admittedly, we have no direct

measurement of senescent cells (detected, for instance, by senescence-associated beta-galactosidase activity) but the expression of these markers appears to be tightly correlated with other measures of senescence (Muñoz-Espín and Serrano, 2014) . Regions of senescent cell populations within the vasculature are associated with atherosclerotic lesions and, indeed, those regions of the vasculature that are at higher risk of developing lesions (i.e. arteriolar bifurcations) also appear to be populated by senescent cells at an earlier timepoint than other regions of the vascular tree (Huzen et al., 2011).

As a result of the low-grade stress that accompanies ageing as detailed above, we also sought to investigate how classical markers of vascular inflammation were affected in our model. To do this, we measured mRNA expression of the inflammatory markers VCAM-1, F4/80 and MPO in the aortae of juvenile and aged αCGRP WT and KO mice. We have shown that vascular mRNA expression of the cell adhesion molecule VCAM-1 was significantly increased as both WT and KO animals aged from three to fifteen months. However, there was no contribution from the genetic deletion of αCGRP. Increases in VCAM-1 expression with age within the aortae of mice has been shown several times before (Hemmeryckx et al., 2013). Indeed, interactions between VCAM-1 expression and CGRP have also been examined, particularly in the setting of renal injury. In hypernatraemic models of renal stress, calcitonin/αCGRP KO mice were found to have increased glomerular VCAM-1 expression in comparison to WT counterparts (Bowers, 2005). As VCAM-1 is a critical molecule in the regulation of firm arrest of mononuclear cells (specifically, monocytes) onto the activated endothelium, any alteration in its expression can have a profound effect on immune cell recruitment. In a subsequent study, elevation of cell adhesion molecule expression was found to increase the infiltration of F4/80-positive mononuclear cells into the renal cortex. F4/80-positive cell populations were higher in the KO animals, even after the hypertension induced by high sodium was normalised by hydralazine (Li et al., 2013a). We also investigated the presence of F4/80-positive cells in the aortae of our mice *via* RTqPCR. We found that the deletion of αCGRP had no influence on aortic mononuclear cell infiltration in juvenile or aged mice when compared to WTs. However, as expected, we did witness a significant increase in the amount of F4/80-positive cells infiltrating into the vasculature as the animals aged.

4.6 Conclusion

In conclusion, this chapter has shown that αCGRP deletion in juvenile (3 months) and aged (15 months) mice does not contribute to an adverse cardiovascular phenotype, at least with respect to adverse haemodynamics and associated vascular remodeling. We have also shown that the oxidative environment within the aortae of these mice is similar, with comparable levels of pro- and anti-oxidant gene expression that all appear to elevate with age. Whilst we could not detect changes in nitrated tyrosine residues as evidence of peroxynitrite activity, we did observe a significant elevation in the peroxidised lipid product, 4-HNE in aged KO animals. As a consequence of aberrant oxidative signalling, we investigated aspects of leukocyte infiltration into the vascular wall. We found VCAM-1 expression to be higher as the animals aged, an effect that αCGRP gene deletion did not influence. Further to this, aortic F4/80 copy numbers also increased in old animals, but once again monocyte/macrophage recruitment was unaffected by αCGRP.

Whilst markers of inflammation did not appear to differ in mice where αCGRP had been removed, its deletion appeared to have a significant influence on vascular NO generation. CGRP had no influence on both the transcription and translation of eNOS gene within the aorta of male juvenile and aged αCGRP WT and KO mice, though it did have an effect at the post-translational level. Juvenile KOs appeared to have a hyperphosphorylated critical serine residue (ser1177), indicative of increased eNOS activity. This degree of hyperphosphorylation was not maintained as animals reached 15 months, despite WT aged mice having elevated ser1177 phosphorylation. These subtle post-translational modifications may have consequences on how CGRP might influence signalling through the NO axis, without necessarily impinging on normal physiology to result in a hypertensive phenotype.

In summary, whilst we have obtained evidence to suggest that CGRP might influence vascular NO generation in juvenile and aged αCGRP WT and KO mice, this does not translate to alterations in the vascular hypertensive or inflammatory phenotype. WT and KO mice have largely similar inflammatory profiles as they age from three to fifteen months, although we have uncovered evidence to suggest that KO mice might suffer slightly more from a pro-oxidant vascular milieu. A schematic diagram representing key results obtained in this chapter is shown in Figure 39.

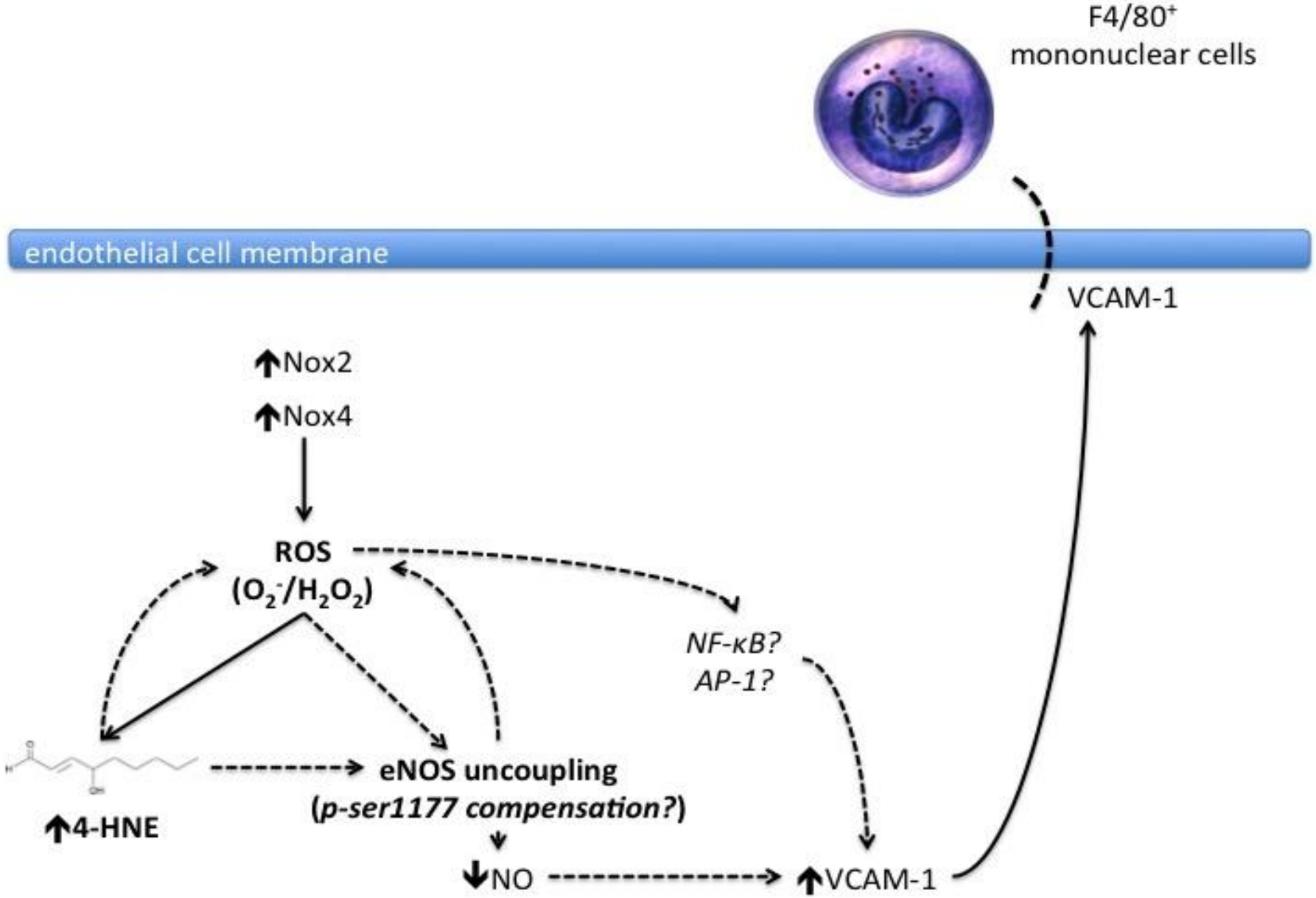

Figure 39: Schematic diagram representing key results obtained from Chapter 4 and potential related signalling pathways contributing towards the phenotype. Solid lines depict events observed in this thesis. Dotted lines depict potential signalling pathways related to what is currently known within the published literature.

5. | Chapter Five: Investigating a role for αCGRP in monocyte-endothelial cell interactions under dynamic flow conditions *in vitro*

5.1 Introduction

We have shown in Chapter 4 that ageing in mice is sufficient to induce vascular inflammation characterised by an increased propensity towards developing a pro-oxidant environment, elevated vascular gene expression of cell adhesion molecules and increased expression of vascular F4/80 mRNA, indicative of monocyte/macrophage infiltration. However, as we deemed that ageing was not a severe enough stimulus to produce CGRP-mediated protection in this model at this particular timepoint, we next aimed to investigate what anti-inflammatory activity, if any, CGRP directly possessed, in the absence of complex *in vivo* physiological factors. Thus, this Chapter will investigate how CGRP may drive or limit leukocytic inflammation *in vitro*.

5.1.1 The Leukocyte Adhesion Cascade

One of the key processes in driving the proinflammatory reaction underlying many CVDs is that of leukocyte capture and recruitment from the circulation by the vascular endothelium. The leukocyte adhesion cascade is a multi-step, complex process that is regulated by events and molecular interactions occurring on both the side of the endothelium and the leukocyte. Many subtleties and nuances are emerging to challenge and build upon the classical paradigm of the adhesion cascade. However, the traditional descriptive model is still of great value and can broadly be broken down into three main stages: tethering and rolling, firm adhesion and diapedesis. These steps and the key molecules that regulate them vary depending on which leukocytes are to be recruited but very general aspects of recruitment will now briefly be discussed, in turn.

1. **Tethering and Rolling**: Quiescent ECs do not typically recruit leukocytes, as they do not express the necessary adhesion molecules to capture circulating cells. This helps limit aberrant inflammation and only allows for inflammatory cell recruitment when it is required, for example during injury or infection. ECs can become activated in response to legitimate exogenous threat (such as release of bacterial endotoxins or secondary release of pro-inflammatory cytokines from resident leukocytes) or from stimuli related to non-infectious disease pathogenesis, including aberrant activation

of the renin-angiotensin system in hypertension or from increased ROS formation during ageing (Alom-Ruiz et al., 2008). Activation of the endothelium results in a shift in phenotype to one that is characterised by increased leukocyte-endothelial interactions, by virtue of the upregulation of cell adhesion molecules and integrins, and also liberation of chemokines to facilitate the transmigration process.

Initial tethering and rolling of the leukocyte is predominantly mediated by the selectins. P-selectin is rapidly liberated from the Weibel-Palade bodies of activated ECs and translocates to the plasma membrane, whereas E-selectin is synthesised *de novo* and appears in the membrane within a few hours. L-selectin is predominantly expressed by the leukocyte. Endothelial-expressed selectins typically interact with P-selectin glycoprotein ligand 1 on the leukocyte with a high on-off rate. These interactions are facilitated by laminar blood flow, allowing for the 'rolling' of the leukocyte along the luminal side of the endothelium (Ley et al., 2007).

2. **Leukocyte Activation and Firm Adhesion:** Rolling of the leukocyte along the activated endothelium is not sufficient to facilitate transmigration. Activated ECs express and release a number of potent chemoattractants that can reach high local concentrations due to their retention by the endothelial glycocalyx. These chemokines bind with high specificity to their cognate GPCRs located on the leukocyte, leading to their activation. CXCL8 appears to be important for neutrophil activation, whilst CXCL4 seems to induce monocyte firm adhesion (Ley et al., 2007). Activation of the leukocyte triggers an incredibly rapid inside-out signalling system, whereby cell-surface integrins become modified to increase their affinity for their counter-ligands. For example, in the case of monocyte adhesion, leukocyte Very Late Antigen-4 is positively modified to encourage interactions with VCAM-1 located on the endothelium to initiate arrest. These high affinity interactions between leukocyte and endothelium lead to the arrest and firm adhesion of the white blood cell to the vessel wall in preparation for endothelial transmigration, or diapedesis.

3. **Endothelial Transmigration:** The final step in leukocyte recruitment is that of the movement of the white blood cell from the circulation into the interstitium *via* the carefully coordinated process of endothelial transmigration. Leukocytes can traverse the vascular endothelium in one of two ways – transcellularly, where the leukocyte is internalised by the EC and transported from the luminal to abluminal membrane and subsequently released, or paracellularly, where the leukocyte manouevres itself through endothelial intercellular junctions to reach the abluminal side. Though both processes occur, paracellular migration is the most common way leukocytes transmigrate through the endothelium (Wittchen, 2009). Paracellular transmigration usually involves the structural reorganization of endothelial tight junctions. ECs may become contractile and pull away from each other to generate gaps between cells for leukocytes to move through. Furthermore, ECs may redistribute junctional molecules to and from the junctional membrane in order to facilitate paracellular transmigration. For example, junctional VE-cadherin may be removed from the cellular interface in order to reduce junctional stability(Mamdouh et al., 2008). Conversely, molecules such as platelet endothelial cell adhesion molecule-1 (PECAM-1) and junctional adhesion molecule-A (JAM-A) may be mobilised towards the junctional membrane to act as counterligands to bind to leukocyte adhesion molecules to subsequently 'guide' the leukocyte through the intercellular gap (Ley et al., 2007). Once having navigated the endothelial junction, the leukocyte is free to then move towards the source of tissue damage. This is usually achieved by the migration of the cell up a concentration gradient of chemokine.

5.1.2 The role of αCGRP in regulating leukocytic inflammation

Considering that CGRP has been associated for some time as a "pro-inflammatory neuropeptide", it is surprising that only a handful of studies have been performed investigating the direct role of this molecule in the regulation of leukocyte-EC interactions. Much of the work contributing towards the idea of CGRP being 'pro-inflammatory' stems from *in vivo* studies and, indeed, recent studies into CGRP biology may be challenging this misconception. Generally, studies of acute inflammation provide evidence to suggest that CGRP is a pro-inflammatory agent, whereby it can potentiate the activity of other inflammatory neuropeptides, such as SP (Brain and Williams, 1985; Gamse and Saria, 1985). Several *in vivo* studies have investigated the role of CGRP in the modulation of the inflammatory response to sepsis. Investigations in this field largely support a protective role for CGRP, where treatment of septic mice with the neuropeptide improved survival whilst simultaneously limiting neutrophil-mediated peritonitis, decreasing availability of pro-inflammatory cytokines and increasing availability of anti-inflammatory cytokines (Gomes et al., 2005). This protective phenotype might be produced by induction of ICER activity (Kroeger et al., 2009). It is of note that RAMP1-deficient mice are also more susceptible to tissue immune cell infiltration and increases in pro-inflammatory cytokines following both endotoxaemia and systemic polymicrobial sepsis (Tsujikawa et al., 2007; Jusek et al., 2012).

Early studies examining the capacity of CGRP to influence leukocyte adhesion found that the neuropeptide could induce adhesion of both primary neutrophils and U937 monocyte-like cells to endothelial monolayers (Sung et al., 1992; Zimmerman et al., 1992), an effect that was apparently mediated by the endothelium, rather than the leukocyte and did not require *de novo* protein synthesis. However, it should be noted that some of these experiments were performed using supraphysiological concentrations of CGRP, under static conditions and thus are not ideally representative of *in vivo* conditions. Some groups have shown that high concentrations of CGRP can activate the neutrophil directly (Richter et al., 1992), whilst others have provided evidence to the contrary (Tanabe et al., 1996). These mixed results are also reflected when considering the role of CGRP in the regulation of monocyte/macrophage activity, especially with respect to its ability to regulate antigen presentation by these cells (Grant, 2001). An excellent study published by Huang et al. in 2011 showed that CGRP was capable of attenuating LPS-induced chemokine release from human microvascular ECs *via* inhibition of NFκB-dependent gene transcription. This resulted in decreased neutrophil and mononuclear cell transmigration through a transwell apparatus *in vitro*(Huang et al., 2011). To my knowledge, minus the work performed by Sung et al. (1992) and Huang et al. (2011),

no studies have been performed investigating the direct role of CGRP in the regulation of monocyte-EC interactions, and certainly none have been performed investigating recruitment under physiological flow conditions.

I believe that CGRP has been identified as a pro-inflammatory neuropeptide in some cases *in vivo* by virtue of its profound vasodilator activity. Potent vasodilatation leads to increased tissue blood flow. This not only allows for increased numbers of erythrocytes into the vessel, but also circulating inflammatory cells and associated circulating chemotactic factors. Thus, in experimental models of inflammation where presumably there is a basal level of endothelial activation and vascular inflammation, a potent vasodilator like CGRP will act to bring in more leukocytes to contribute to inflammation. Thus, CGRP's "pro-inflammatory" nature is a secondary effect due to enhanced blood flow. What is less clear is how CGRP *directly* mediates leukocytic inflammation, in the absence of complex physiological responses. The best way this question can be addressed is through reductive, mechanistic *in vitro* experiments. To this end, we will use HUVEC to act as endothelial platforms to recruit inflammatory cells. We have chosen to make use of THP-1 monocytic cells to investigate leukocyte-EC interactions, with results in mind from our *in vivo* experiments, where we witnessed increases in infiltration of F4/80-positive cells (indicative of cells of monocyte/macrophage lineage) into the vascular wall of aged mice, but not increases in MPO-positive cells (largely indicative of neutrophils). Whilst THP-1s were once derived from a juvenile acute monocytic leukaemia patient (Tsuchiya et al., 1980) and might not necessarily be truly representative of primary monocyte activity, they are frequently used as a model system for monocytes. Unlike primary monocytes obtained from donors, THP-1s possess a homogeneous genetic background, which allows for a simpler interpretation of results obtained from their use. Furthermore, they are easier to maintain than primary cells and larger cell numbers can be used, meaning they are a more attractive cell line to use for preliminary experiments for practical reasons. Functionally, they closely resemble primary monocytes with respect to morphology and their expression of classical monocyte markers. The ability of THP-1s to differentiate into macrophages following stimulation with phorbol esters is also comparable to primary cells. Finally, their ability to interact with ECs is well-documented and therefore we deemed this an appropriate cell line to use(Qin, 2012).

5.2 Hypothesis

Treatment of TNFα-activated HUVEC (HUVEC) with CGRP will attenuate THP-1 monocyte recruitment under dynamic flow conditions *in vitro*.

5.3 Aims

1. To investigate how CGRP influences monocyte-EC interactions under flow conditions in quiescent ECs.
2. To investigate how CGRP influences monocyte-EC interactions under flow conditions in activated ECs.
3. To investigate the mechanisms by which alterations in monocyte-EC interactions under flow conditions are achieved.

5.4 Results

5.4.1 Acute stimulation of THP-1 monocytes with CGRP does not induce adhesion to HUVEC under flow, nor does it attenuate TNFα-induced adhesion

With early CGRP leukocyte-EC interaction studies from the literature in mind, we first performed experiments investigating how acute stimulation of THP-1 cells for 5 minutes with 300nM CGRP would affect leukocyte-endothelial interactions under flow, in quiescent and TNFα-activated HUVEC (see Figures 7 and 8 in Chapter Two for an overview of the experimental protocol). Figure 40 illustrates that activation of HUVEC by TNFα for six hours is absolutely required for THP-1 recruitment, as non-activated HUVEC recruited 0 cells over the course of the fifteen minute flow experiment. Further to this, we have shown that stimulation of THP-1 cells for five minutes with 300nM CGRP is not sufficient to induce adhesion in non-activated HUVEC.

Following a six-hour pre-treatment with 10 ng/mL TNFα to activate HUVEC, we witnessed a significant increase in recruitment of 79.09 ± 6.88 THP-1 cells per field of view at the fifteen minute timepoint, in comparison to non-activated controls. Further to this, acute stimulation of THP-1s with 300nM CGRP for five minutes had no effect on adhesion events (78.72 ± 12.86 cells per field of view). Thus, we conclude from these results that:

1. HUVEC activation by TNFα is required to induce THP-1 adhesion.
2. Stimulation of THP-1s for five minutes with 300nM CGRP does not facilitate adhesion to quiescent HUVEC.
3. Stimulation of THP-1s for five minutes with 300nM CGRP does not change number of adherent cells to TNFα-activated HUVEC.

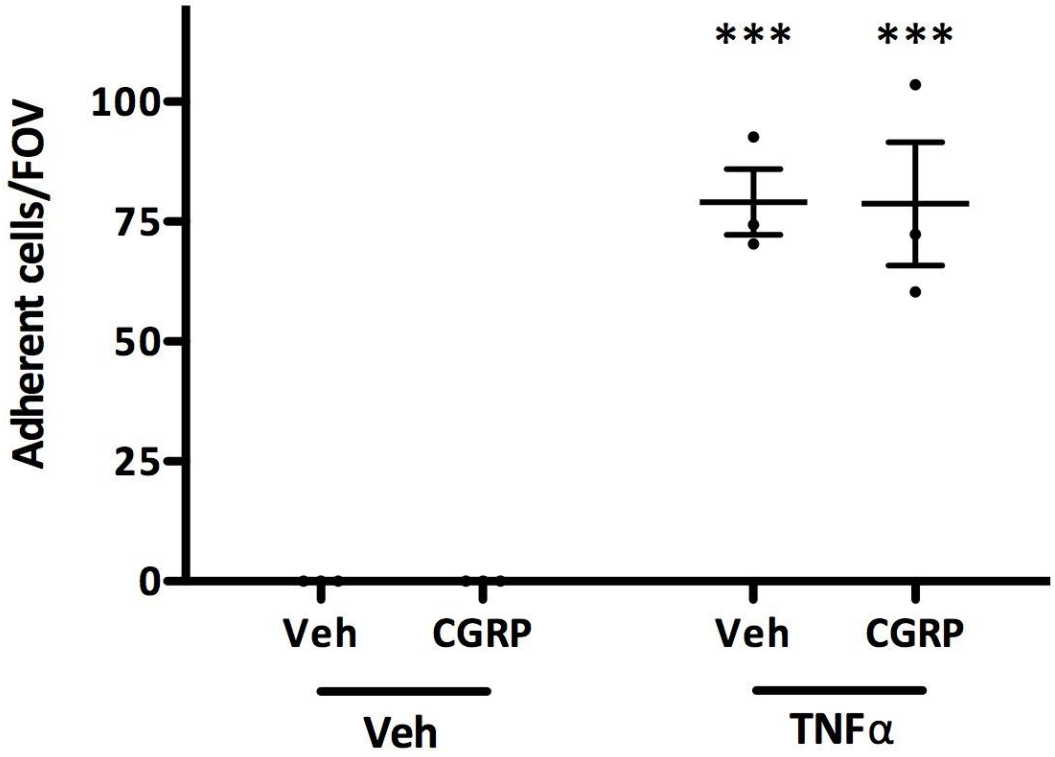

Figure 40: *In vitro* parallel plate THP-1 adhesion assay, investigating the effect of acute (5 minutes) THP-1 stimulation with 300nM CGRP on adhesion to quiescent and TNFα-activated HUVEC under flow conditions for 15 minutes. Data expressed as adherent cells per microscopic field of view, mean ± SEM, n=3. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. ***p<0.001 where quiescent and activated HUVEC have been compared.

5.4.2 Prolonged stimulation of HUVEC with CGRP attenuates TNFα-induced THP-1 adhesion in a time-dependent manner

With the finding that acute stimulation of THP-1 cells with CGRP did not modulate adhesion to HUVEC, we sought to understand how stimulation of the endothelium with CGRP influenced this process (see Figure 8 in methods for schematic detailing the protocol for this experiment). When HUVEC were activated, they were stimulated with TNFα for six hours. When cells were also treated with CGRP, this was either for the full six hours, three hours (halfway through TNFα stimulation) or one hour (at the five-hour TNFα timepoint). Vehicle treatment for each compound (TNFα or αCGRP) was stimulation of cells for the full six hours (i.e. the maximal amount of time each compound was used for). Figure 41 illustrates that, again, quiescent ECs do not recruit THP-1 cells under flow and stimulation with TNFα is required for this to happen. Further to this, co-treatment of quiescent ECs with 300nM CGRP for 1, 3 or 6 hours was not found to induce adhesion.

As expected, we discovered that 6 hours 10 ng/mL TNFα pre-treatment was required to induce THP-1 adhesion to HUVEC, where at the maximal perfusion experiment timepoint of 15 minutes, we observed 44.70 ± 8.57 adherent cells per field of view. There was a trend towards a reduction in THP-1s adherent to HUVEC treated with CGRP for 1 hour (41.76 ± 9.50 cells per field of view) and 3 hours (36.10 ± 7.66 cells per field of view), although these findings were not statistically significant. Interestingly, however, we witnessed a significant reduction in the number of adherent THP-1s adherent to HUVEC treated with CGRP for 6 hours (19.73 ± 5.38 cells per field of view). Indeed, HUVEC treated for 6 hours with CGRP had a significantly reduced adherent THP-1 population by the ten-minute perfusion timepoint (9.96 ± 3.07 cells per field of view), when compared to vehicle-treated TNFα-activate controls (28.72 ± 4.87 cells per field of view). Thus, we conclude from these results that:

1. Stimulation of quiescent HUVEC for up to 6 hours with 300nM CGRP is not sufficient to induce THP-1 adhesion.
2. Stimulation of TNFα-activated HUVEC for 6 hours with 300nM CGRP is sufficient to significantly attenuate THP-1 adhesion from the 10-minute perfusion time point onwards under physiological flow conditions.

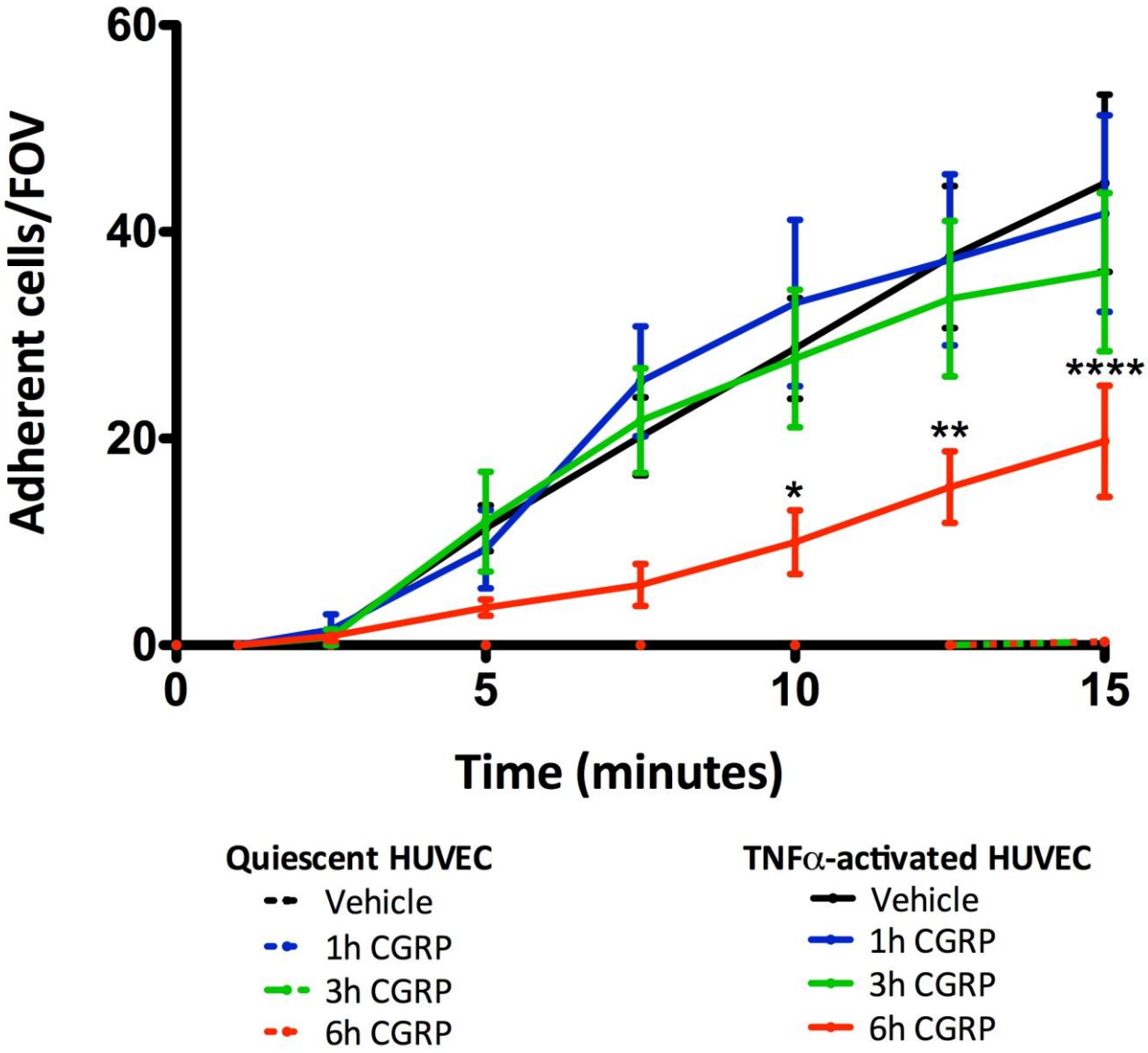

Figure 41: *In vitro* parallel plate THP-1 adhesion assay, investigating the effect of HUVEC pre-treatment with 300nM CGRP on adhesion to quiescent and TNFα-activated HUVEC under flow conditions for 15 minutes. Data expressed as adherent cells per microscopic field of view, mean ± SEM, n=3. Statistical significance was evaluated by repeated-measures two-way ANOVA plus Bonferroni *post hoc* test. ****p<0.0001; **p<0.01; *p<0.05 where activated, CGRP-treated HUVEC have been compared against vehicle control.

5.4.3 Reduced THP-1 adhesion to activated HUVEC is not dependent on changes in total cellular VCAM-1 expression

Following the observed phenotype that pre-treatment of TNFα-activated HUVEC with 300nM CGRP for six hours resulted in reduced THP-1-EC interactions, we sought to identify a mechanism that could explain this phenomenon. We first immunoblotted total HUVEC protein lysates treated in a similar manner to those used in flow assay experiments for the expression of VCAM-1, a well-known regulator of monocyte firm-adhesion to endothelium. Figures 42 and 43 show that quiescent HUVEC did not express VCAM-1 and treatment of quiescent endothelium for up to six hours with 300nM CGRP was not sufficient to induce expression of this protein.

Following stimulation of HUVEC with TNFα, we witnessed a robust induction of total cellular VCAM-1 protein expression that was found to be statistically significant versus quiescent cells. Treatment of TNFα-activated cells with 300nM CGRP for up to six hours had no effect on total VCAM-1 expression.

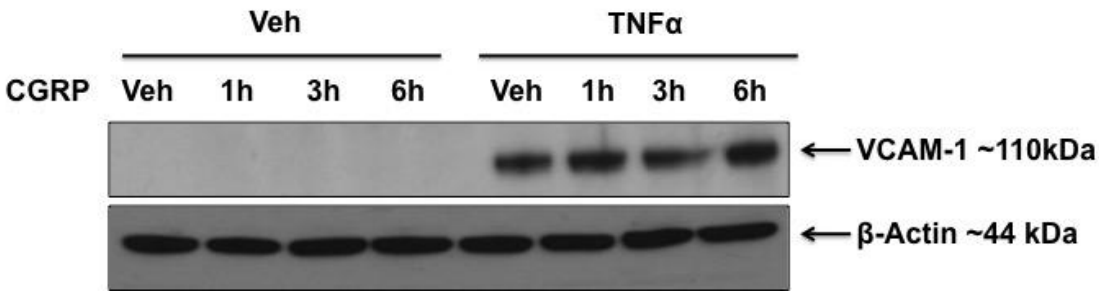

Figure 42: Representative immunoblot of total cellular HUVEC lysates, probing for VCAM-1, following stimulation with TNF and CGRP, including β-actin loading control.

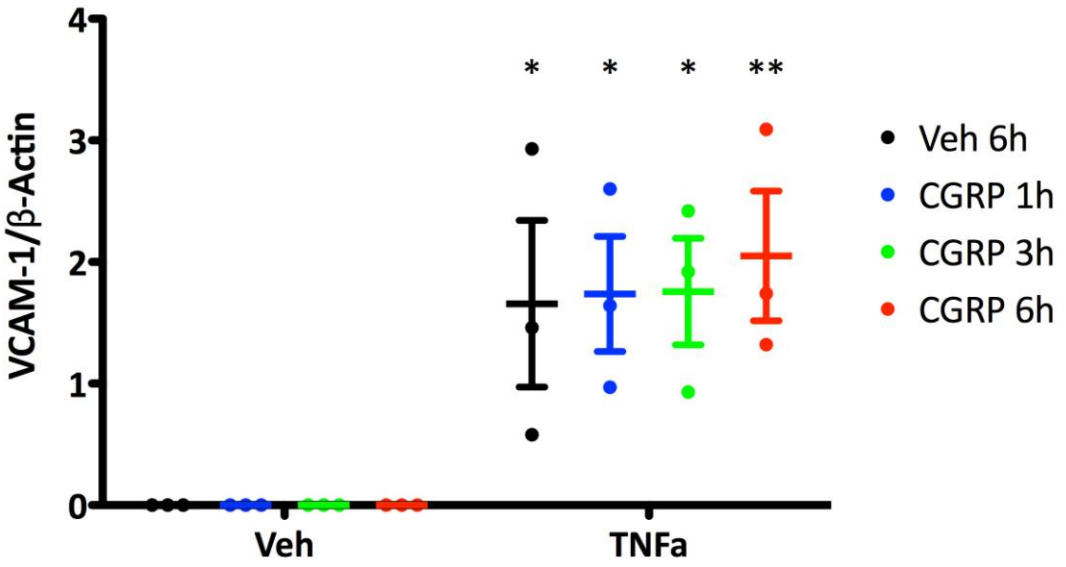

Figure 43: Densitometric analysis of immunoblotting of total cellular HUVEC lysates, probing for VCAM-1. Representative blots show VCAM-1 immunoreactivity at ~110 kDa and loading control β-actin at ~44 kDa Data expressed as arbitrary densitometric units normalised to β-actin, mean ± SEM, n=3. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. **p<0.01; *p<0.05 where TNFα-activated HUVEC were compared to their respective quiescent controls.

5.4.4 Reduced THP-1 adhesion to activated HUVEC is not dependent on changes in total cellular PECAM-1 expression

We next investigated changes in PECAM-1 expression by immunoblotting total HUVEC protein lysates treated in a similar manner to those used in flow assay experiments. PECAM-1 is important in mediating leukocyte transmigration. Figures 44 and 45 show that PECAM-1 expression is unchanged by treatment for up to six hours with 300nM CGRP in quiescent ECs. Further to this, activation of HUVEC with TNFα did not change the level of expression of PECAM-1 and neither did the co-treatment of these cells with CGRP.

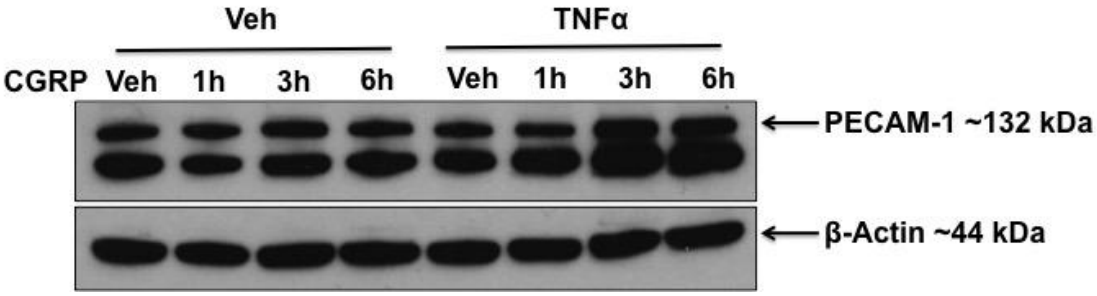

Figure 44: Representative immunoblot of total cellular HUVEC lysates, probing for PECAM-1, following stimulation with TNF and CGRP, including β-actin loading control.

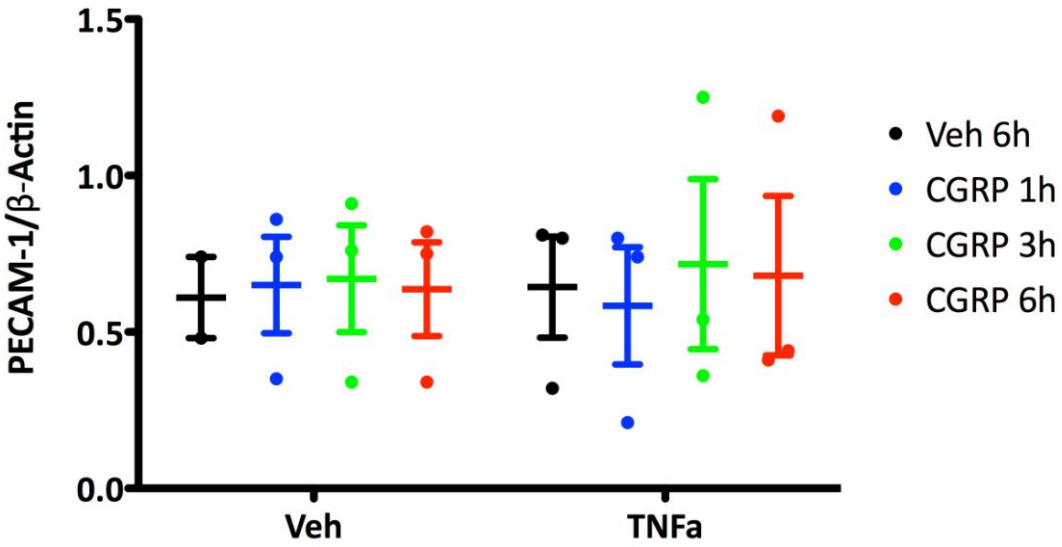

Figure 45: Densitometric analysis of immunoblotting of total cellular HUVEC lysates, probing for PECAM-1. Representative blots show PECAM-1 immunoreactivity at ~132 kDa and loading control β-actin at ~44 kDa. Data expressed as arbitrary densitometric units normalised to β-actin, mean ± SEM, n=3. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. No significant differences were detected between groups.

5.4.5 Reduced THP-1 adhesion to activated HUVEC is not dependent on changes in total cellular JAM-A expression

We next investigated changes in JAM-A expression by immunoblotting total HUVEC protein lysates treated in a similar manner to those used in flow assay experiments. JAM-A is important in the regulation of endothelial tight junctions and has a role in facilitating leukocyte transmigration through this axis. Figures 46 and 47 show that JAM-A expression is unchanged by treatment for up to six hours with 300nM CGRP in quiescent ECs. Further to this, activation of HUVEC with TNFα did not change the level of expression of JAM-A and neither did the co-treatment of these cells with CGRP.

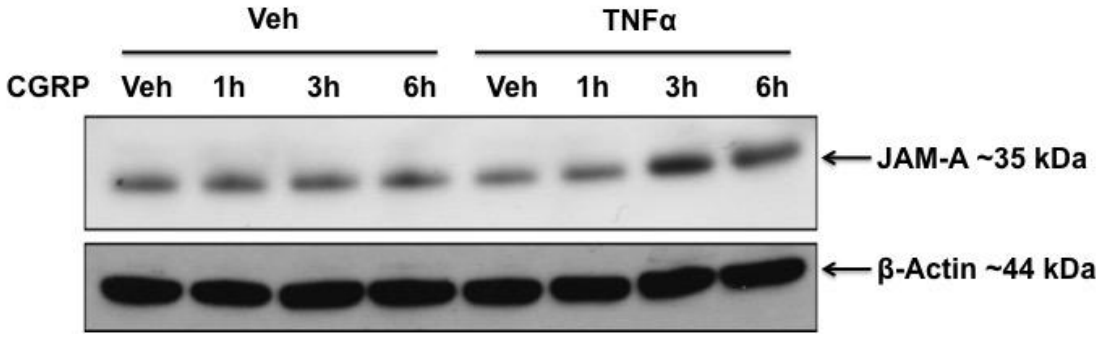

Figure 46: Representative immunoblot of total cellular HUVEC lysates, probing for JAM-A, following stimulation with TNF and CGRP, including β-actin loading control.

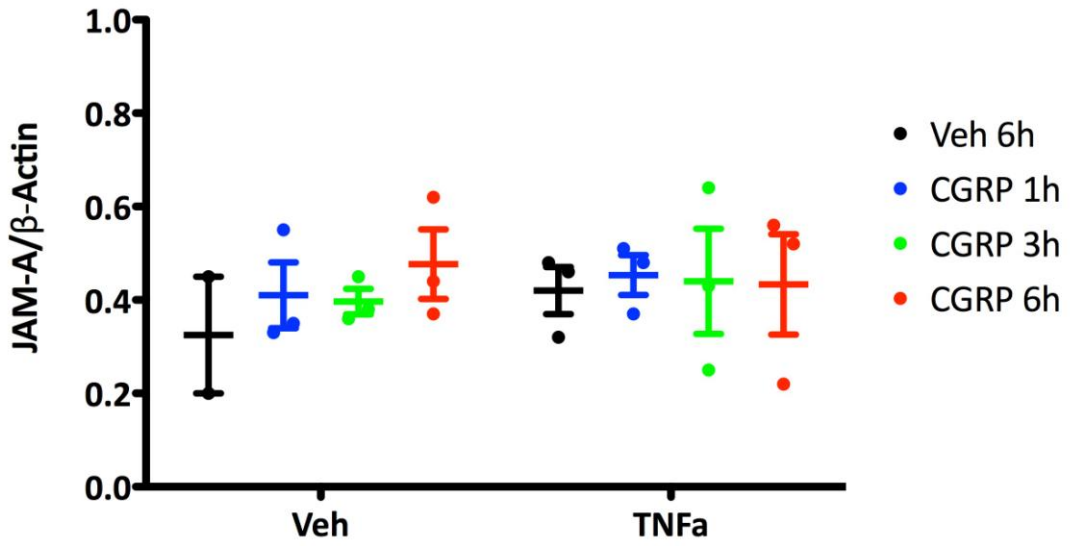

Figure 47: Densitometric analysis of immunoblotting of total cellular HUVEC lysates, probing for JAM-A. Representative blots show JAM-A immunoreactivity at ~132 kDa and loading control β-actin at ~44 kDa. Data expressed as arbitrary densitometric units normalised to β-actin, mean ± SEM, n=3. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. No significant differences were detected between groups.

5.4.6 Reduced THP-1 adhesion to activated HUVEC is not dependent on changes in total cellular JAM-C expression

We next investigated changes in JAM-C expression by immunoblotting total HUVEC protein lysates treated in a similar manner to those used in flow assay experiments. JAM-C is important in the regulation of endothelial tight junctions and has a role in facilitating leukocyte transmigration through this axis. Figures 48 and 49 shows that JAM-C expression is unchanged by treatment for up to six hours with 300nM CGRP in quiescent ECs. Further to this, activation of HUVEC with TNFα did not change the level of expression of JAM-C and neither did the co-treatment of these cells with CGRP.

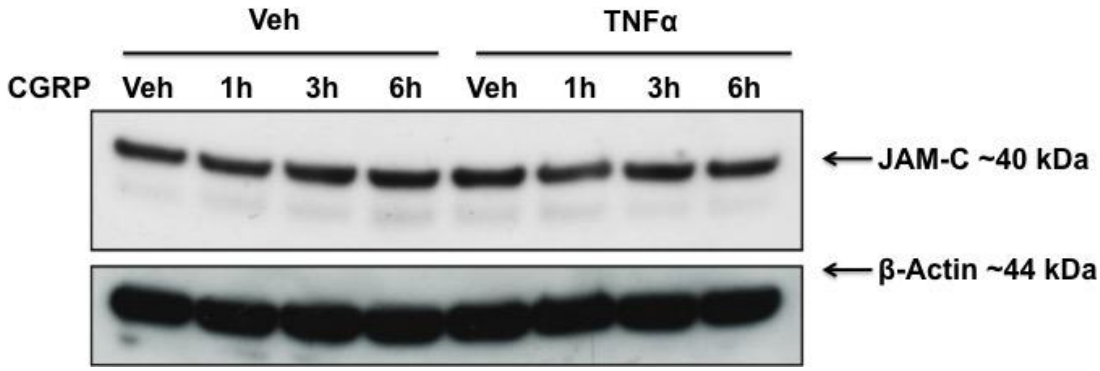

Figure 48: Representative immunoblot of total cellular HUVEC lysates, probing for JAM-C, following stimulation with TNF and CGRP, including β-actin loading control.

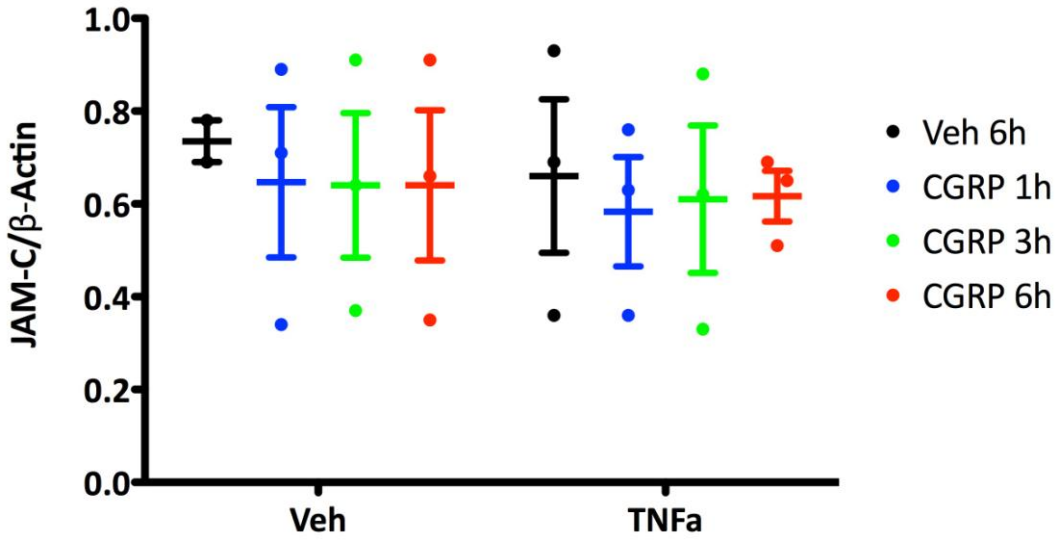

Figure 49: Densitometric analysis of immunoblotting of total cellular HUVEC lysates, probing for JAM-C. Representative blots show JAM-A immunoreactivity at ~132 kDa and loading control β-actin at ~44 kDa Data expressed as arbitrary densitometric units normalised to β-actin, mean ± SEM, n=3. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. No significant differences were detected between groups.

5.4.7 Microarray analysis reveals that CGRP does not alter TNFα-induced changes in global endothelial gene expression

Following the investigation of changes in whole cell protein expression of various cell adhesion molecules and identifying no clear candidates, we next sought to examine how CGRP affected the TNFα response in HUVEC at the level of the transcriptome. Here, we found that stimulation of HUVEC with 10 ng/mL TNFα for six hours was sufficient to induce a robust pro-inflammatory gene transcription network. Setting a false discovery rate (FDR) of q = 0.05 to decrease the chance of identifying genes that changed their expression artefactually, as a result of multiple-comparison testing, we identified 1351 genes whose expression differed following TNFα exposure versus cells treated with vehicle. Surprisingly, however, we found that treatment of HUVEC for six hours with 300 nM CGRP had no effect on HUVEC gene expression at baseline. When q = 0.05 for this data set, 0 genes had changed their level of expression. Furthermore, co-stimulation of TNFα-activated HUVEC with CGRP resulted in no change in the gene expression pattern *versus* TNFα treatment alone.

For simplicity and clarity, we have shown in Table 10 the top 25 genes that downregulated their level of expression in HUVEC, following TNFα treatment, including their p-values and fold change in expression. Table 11 shows similar data for the top 25 genes that upregulated their level of expression under similar treatment circumstances. Tables 12 and 13 show that 300 nM CGRP for 6 hours did not result in a change in the transcriptome induced by TNFα. Using GeneGo MetaCore software, we then grouped these genes into functional groupings, according to the signalling pathways that they belong to. As there is considerable overlap in these pathways owing to no single gene having one single function, we have shown the top 10 enrichment pathways that the genes we have identified clustered into (Figure 50). Pathways with high levels of significance included those related to NFκB and cytokine signalling, particularly that related to IL-18 (gene enrichment map illustrated in Figure 51).

Gene Symbol	Gene Title	p-value	Fold change
MAP2K6	mitogen-activated protein kinase kinase 6	0.000622073	0.205617
DUSP4	dual specificity phosphatase 4	7.07E-06	0.299751
DACH1	dachshund homolog 1 (Drosophila)	3.79E-06	0.306102
OR1L8	olfactory receptor, family 1, subfamily L, member 8	0.00127583	0.318431
HTR1B	5-hydroxytryptamine (serotonin) receptor 1B, G protein-coupled	0.000111942	0.318502
PCDH7	protocadherin 7	8.81E-06	0.326367
PCDH9	protocadherin 9	8.03E-06	0.327213
TMC7	transmembrane channel-like 7	5.57E-05	0.356045
SOX18	SRY (sex determining region Y)-box 18	8.31E-06	0.3565
HTR1D	5-hydroxytryptamine (serotonin) receptor 1D, G protein-coupled	9.04E-05	0.360248
FAT4	FAT tumor suppressor homolog 4 (Drosophila)	1.31E-06	0.36051
GPRIN3	GPRIN family member 3	0.001122046	0.363434
GPR126	G protein-coupled receptor 126	6.44E-06	0.376928
GJA5	gap junction protein, alpha 5, 40kDa	0.000221768	0.382617
CLEC12B	C-type lectin domain family 12, member B	0.000379586	0.386513
LONRF3	LON peptidase N-terminal domain and ring finger 3	0.000678512	0.389914
CCNE2	cyclin E2	0.000338144	0.398083
PCDH17	protocadherin 17	9.94E-05	0.407927
CALCRL	calcitonin receptor-like	3.45E-05	0.409431
CDH6	cadherin 6, type 2, K-cadherin (fetal kidney)	8.77E-05	0.415264
PLAC8	placenta-specific 8	0.000148325	0.415722
KRT80	keratin 80	0.000103213	0.41708
MANSC4	MANSC domain containing 4	8.70E-05	0.418563
DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4	0.000118824	0.420874
ALX1	ALX homeobox 1	2.67E-05	0.421508

Table 10: Table illustrates top 25 genes decreasing their expression in HUVEC following six hour TNFα stimulation *versus* vehicle, thresholded by a false discovery rate of q = 0.05.

Gene Symbol	Gene Title	p-value	Fold change
IFIH1	interferon induced with helicase C domain 1	4.77E-06	7.37101
C2CD4B	C2 calcium-dependent domain containing 4B	1.08E-06	7.66832
IL1A	interleukin 1, alpha	4.92E-07	7.78733
ICOSLG	inducible T-cell co-stimulator ligand	6.46E-06	7.83087
TNFAIP2	tumor necrosis factor, alpha-induced protein 2	2.67E-05	8.04515
CSF2	colony stimulating factor 2 (granulocyte-macrophage)	1.42E-06	8.67158
CXCL11	chemokine (C-X-C motif) ligand 11	6.39E-07	8.6982
CCL20	chemokine (C-C motif) ligand 20	3.12E-05	8.82469
RND1	Rho family GTPase 1	2.04E-06	9.77696
BCL2A1	BCL2-related protein A1	1.38E-06	9.85974
GABBR1 /// UBD	gamma-aminobutyric acid (GABA) B receptor, 1 /// ubiquitin D	0.000189399	11.3131
IL18R1	interleukin 18 receptor 1	5.16E-07	12.3721
TNFAIP3	tumor necrosis factor, alpha-induced protein 3	2.04E-07	12.8676
EBI3	Epstein-Barr virus induced 3	6.97E-06	13.0558
ICAM1	intercellular adhesion molecule 1	0.000113497	14.2779
SELE	selectin E	1.74E-05	17.4448
TNFRSF9	tumor necrosis factor receptor superfamily, member 9	2.91E-05	17.7804
IL8	interleukin 8	3.53E-07	19.4468
TRAF1	TNF receptor-associated factor 1	1.47E-06	19.567
CD69	CD69 molecule	9.83E-06	22.5417
BIRC3	baculoviral IAP repeat containing 3	9.26E-07	27.2492
CX3CL1	chemokine (C-X3-C motif) ligand 1	3.55E-05	35.0929
UBD	ubiquitin D	4.49E-05	45.2061
VCAM1	vascular cell adhesion molecule 1	7.37E-07	54.972
CXCL10	chemokine (C-X-C motif) ligand 10	6.65E-07	67.5932

Table 11: Table illustrates top 25 genes increasing their expression in HUVEC following six hour TNFα stimulation *versus* vehicle, thresholded by a false discovery rate of q = 0.05.

Gene Symbol	Gene Title	p-value	Fold change
MAP2K6	mitogen-activated protein kinase kinase 6	0.000622073	0.205617
DUSP4	dual specificity phosphatase 4	7.07E-06	0.299751
DACH1	dachshund homolog 1 (Drosophila)	3.79E-06	0.306102
OR1L8	olfactory receptor, family 1, subfamily L, member 8	0.00127583	0.318431
HTR1B	5-hydroxytryptamine (serotonin) receptor 1B, G protein-coupled	0.000111942	0.318502
PCDH7	protocadherin 7	8.81E-06	0.326367
PCDH9	protocadherin 9	8.03E-06	0.327213
TMC7	transmembrane channel-like 7	5.57E-05	0.356045
SOX18	SRY (sex determining region Y)-box 18	8.31E-06	0.3565
HTR1D	5-hydroxytryptamine (serotonin) receptor 1D, G protein-coupled	9.04E-05	0.360248
FAT4	FAT tumor suppressor homolog 4 (Drosophila)	1.31E-06	0.36051
GPRIN3	GPRIN family member 3	0.001122046	0.363434
GPR126	G protein-coupled receptor 126	6.44E-06	0.376928
GJA5	gap junction protein, alpha 5, 40kDa	0.000221768	0.382617
CLEC12B	C-type lectin domain family 12, member B	0.000379586	0.386513
LONRF3	LON peptidase N-terminal domain and ring finger 3	0.000678512	0.389914
CCNE2	cyclin E2	0.000338144	0.398083
PCDH17	protocadherin 17	9.94E-05	0.407927
CALCRL	calcitonin receptor-like	3.45E-05	0.409431
CDH6	cadherin 6, type 2, K-cadherin (fetal kidney)	8.77E-05	0.415264
PLAC8	placenta-specific 8	0.000148325	0.415722
KRT80	keratin 80	0.000103213	0.41708
MANSC4	MANSC domain containing 4	8.70E-05	0.418563
DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4	0.000118824	0.420874
ALX1	ALX homeobox 1	2.67E-05	0.421508

Table 12: Table illustrates top 25 genes decreasing their expression in HUVEC following six hour TNFα stimulation and co-stimulation with CGRP for six hours, thresholded by a false discovery rate of q = 0.05.

Gene Symbol	Gene Title	p-value	Fold change
IFIH1	interferon induced with helicase C domain 1	4.77E-06	7.37101
C2CD4B	C2 calcium-dependent domain containing 4B	1.08E-06	7.66832
IL1A	interleukin 1, alpha	4.92E-07	7.78733
ICOSLG	inducible T-cell co-stimulator ligand	6.46E-06	7.83087
TNFAIP2	tumor necrosis factor, alpha-induced protein 2	2.67E-05	8.04515
CSF2	colony stimulating factor 2 (granulocyte-macrophage)	1.42E-06	8.67158
CXCL11	chemokine (C-X-C motif) ligand 11	6.39E-07	8.6982
CCL20	chemokine (C-C motif) ligand 20	3.12E-05	8.82469
RND1	Rho family GTPase 1	2.04E-06	9.77696
BCL2A1	BCL2-related protein A1	1.38E-06	9.85974
GABBR1 /// UBD	gamma-aminobutyric acid (GABA) B receptor, 1 /// ubiquitin D	0.000189399	11.3131
IL18R1	interleukin 18 receptor 1	5.16E-07	12.3721
TNFAIP3	tumor necrosis factor, alpha-induced protein 3	2.04E-07	12.8676
EBI3	Epstein-Barr virus induced 3	6.97E-06	13.0558
ICAM1	intercellular adhesion molecule 1	0.000113497	14.2779
SELE	selectin E	1.74E-05	17.4448
TNFRSF9	tumor necrosis factor receptor superfamily, member 9	2.91E-05	17.7804
IL8	interleukin 8	3.53E-07	19.4468
TRAF1	TNF receptor-associated factor 1	1.47E-06	19.567
CD69	CD69 molecule	9.83E-06	22.5417
BIRC3	baculoviral IAP repeat containing 3	9.26E-07	27.2492
CX3CL1	chemokine (C-X3-C motif) ligand 1	3.55E-05	35.0929
UBD	ubiquitin D	4.49E-05	45.2061
VCAM1	vascular cell adhesion molecule 1	7.37E-07	54.972
CXCL10	chemokine (C-X-C motif) ligand 10	6.65E-07	67.5932

Table 13: Table illustrates top 25 genes increasing their expression in HUVEC following six hour TNFα stimulation and co-stimulation with CGRP for six hours, thresholded by a false discovery rate of q = 0.05.

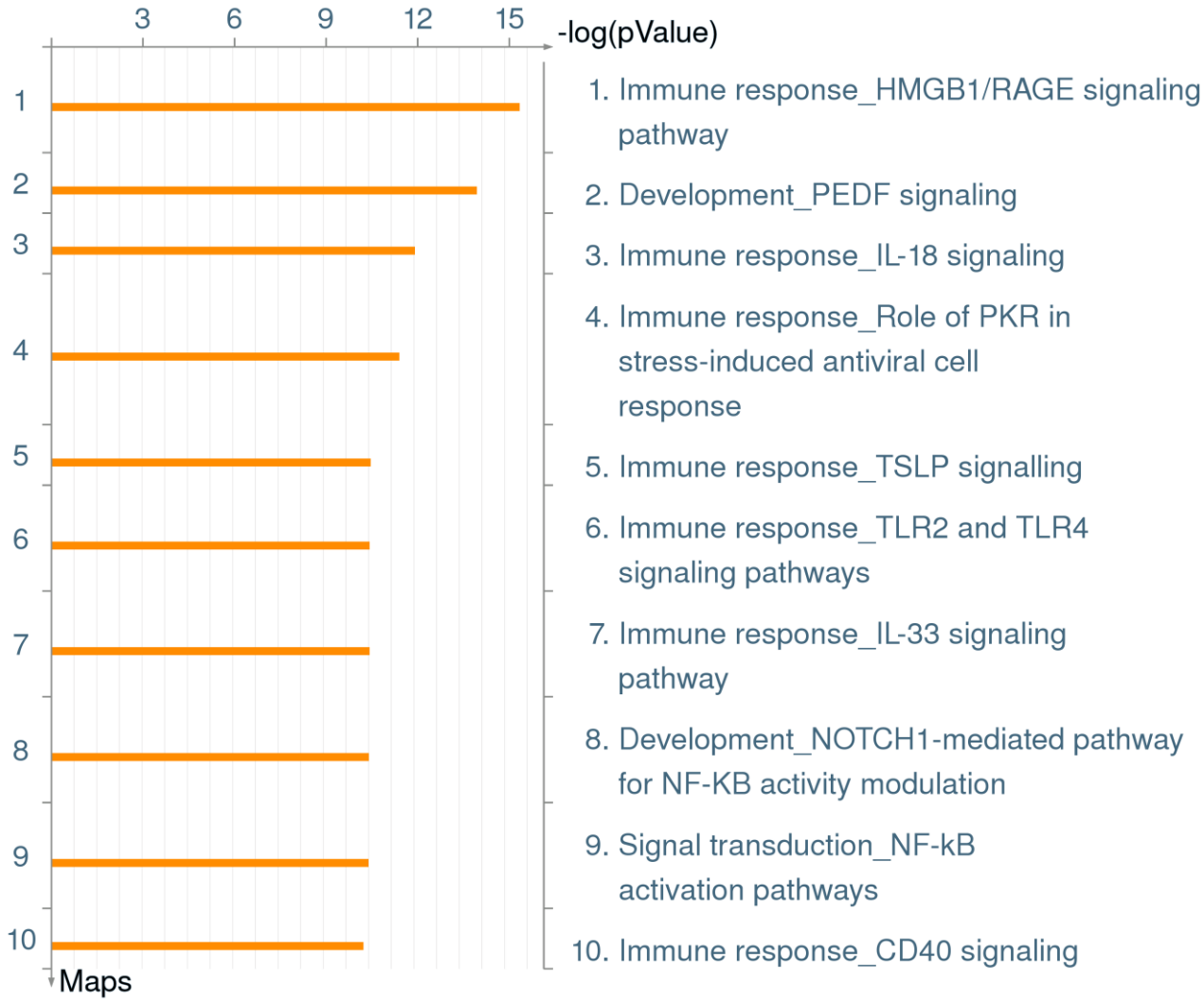

Figure 50: Enrichment of 1351 genes altering their expression in HUVEC treated for six hours with 10 ng/mL TNFα. Data ranked according to likelihood that genes observed appear in pre-defined gene pathways, as measured by –log(p-value).

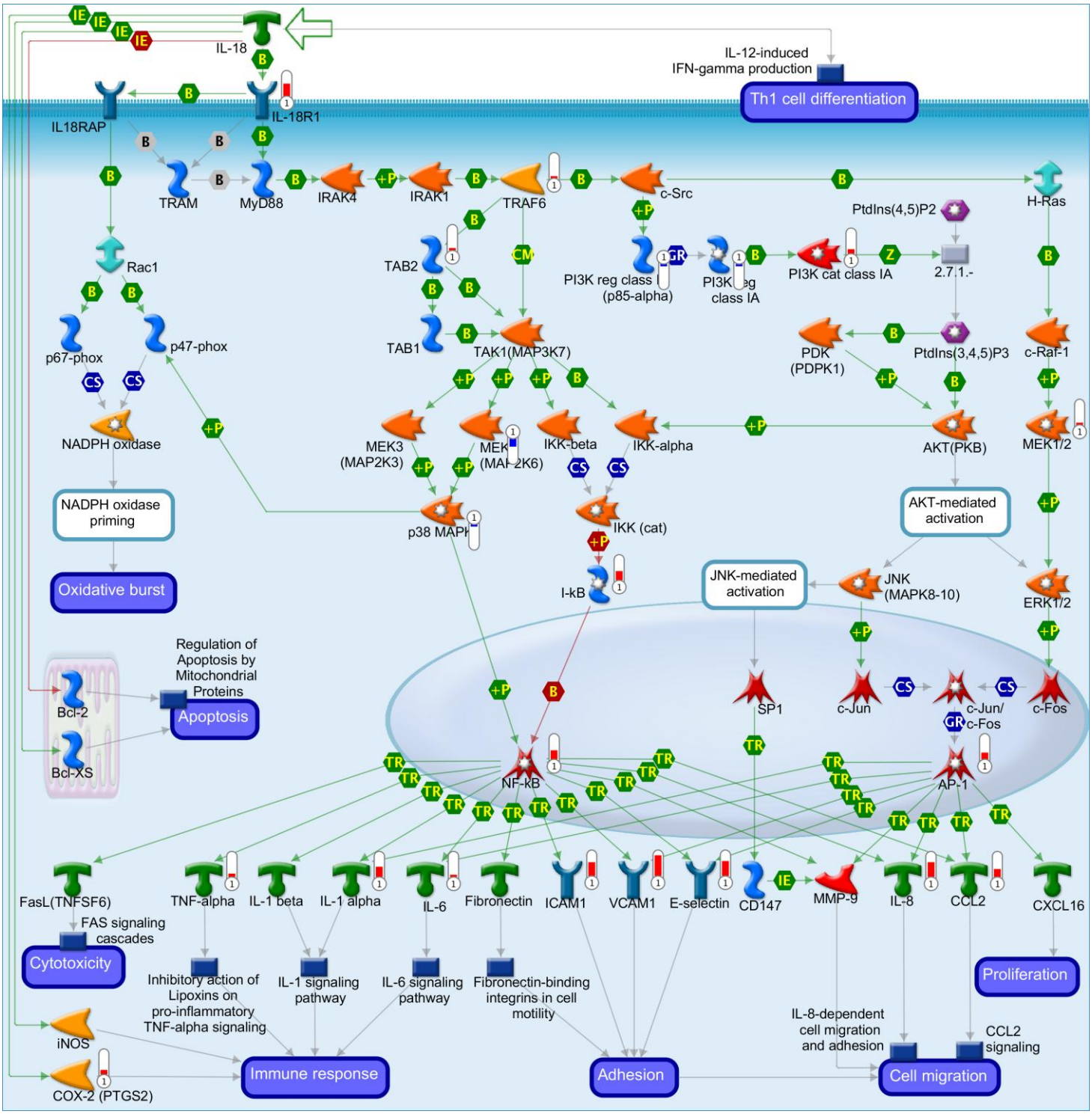

Figure 51: Gene enrichment map pertaining to IL-18 signalling.network. 21/60 genes identified in microarray screen appeared in this pre-defined network, yielding a p value= 1.422e-^{12} with a FDR of q = 3.64e-^{10}. B: binding; CM: covalent modifications; CS: complex subunit; GR: group relation; IE: influence on expression; P: phosphorylation; TR: transcriptional regulation Z: catalysis. Genes with a red thermometer are positively regulated with TNFα, where fuller thermometers indicate a strong level of expression. Genes with a blue thermometer are negatively regulated with TNFα, where fuller thermometers indicate a lower level of expression.

5.5 Discussion

In this chapter, I have shown that CGRP has the potential to act as a direct anti-inflammatory agent *in vitro*, by virtue of its ability to attenuate THP-1 monocyte-EC interactions, under physiological flow conditions. In Chapter Four we have shown that in the mouse, ageing to fifteen months is sufficient to induce infiltration of F4/80-positive mononuclear cells into the aortic wall. Deletion of αCGRP did not affect this process and I deemed this due to the mouse model of natural ageing not being harsh enough of a stimulus to provoke CGRP-dependent vascular protection. Despite this, there is a small but mixed literature concerning the role of CGRP in the regulation of leukocyte trafficking, none of which has investigated leukocyte recruitment under flow conditions. Thus, we thought this a sensible avenue of research to pursue. We used THP-1 cells as a human monocyte surrogate for practical reasons. This monocytic cell line is well-characterised and highly-representative of primary monocytes (Qin, 2012) and their use circumvents the problems associated with obtaining high cell yields of high viability from human donors and further reduces variability in data from donor to donor. Monocytic cells were used in place of other innate immune cells (such as neutrophils), as we obtained a clear signal from the *in vivo* ageing study in Chapter 4 that F4/80-positive (i.e. monocyte/macrophage) cells infilitrated into the vascular wall with advancing age. Conversely, we saw no evidence of neutrophil accumulation with ageing and thus deemed this the best approach to take.

5.5.1 HUVEC activation with TNFα is absolutely required to promote leukocyte-endothelial cell interactions *via* the induction of pro-inflammatory gene and protein expression

In this chapter we have reproduced the data of other groups by showing that perfusion of inflammatory cells (such as THP-1s) over quiescent, non-activated HUVEC does not lead to the recruitment of these cell types to the endothelium (Gamble et al., 1985; Furie and McHugh, 1989). In order to achieve recruitment, we had to stimulate the endothelium with 10 ng/mL TNFα for six hours to allow for the induction of pro-inflammatory gene expression and the upregulation of cell adhesion molecules at the plasma membrane. Cell adhesion molecules that are typically involved in leukocyte recruitment by the EC include the P- and E-selectins, VCAM-1, ICAM-1, PECAM-1 (Golias et al., 2007) and, to some extent, JAM isoforms (Ostermann, 2005; Schmitt et al., 2014). This paradigm of the leukocyte adhesion cascade is supported by the results we obtained investigating the HUVEC transcriptome following TNFα treatment and through some of our protein expression studies *via* whole-cell immunoblotting. We found that VCAM-1 gene expression was upregulated almost 55-fold over quiescent HUVEC. Further, we showed that HUVEC do not constitutively express this cell adhesion molecule under non-stimulated conditions but they robustly and significantly induce VCAM-1 protein expression following TNFα stimulation. Furthermore, we witnessed an elevation of E-selectin gene expression, at around 17-fold over control cells and an elevation of ICAM-1 gene expression, at around 14-fold over control. Immunoblotting showed that quiescent HUVEC expressed similar amounts of PECAM-1, JAM-A, JAM-C that were unchanged by TNFα stimulation. Through microarray analysis, we also uncovered evidence for a high degree of increased transcript for various cytokines and chemokines, which may facilitate firm adhesion of the monocyte *via* activation of the cell to induce integrin engagement. We noted strong upregulation of genes encoding CXCL10, CX3CL1, IL-8, CCL20, CXCL11, CSF2, and IL-1α. Thus, we have demonstrated our ability to activate canonical TNFα-induced pathways in order to recruit leukocytes to endothelium in our assay.

5.5.2 Acute stimulation of THP-1 cells with 300 nM CGRP does not affect leukocyte adhesion to quiescent or activated HUVEC

We have shown that acute stimulation of THP-1 cells with 100 nM CGRP for five minutes prior to perfusion over activated endothelium was not sufficient to alter leukocyte-EC interactions. This finding is in contrast with an early finding within the literature, which suggests CGRP can directly activate inflammatory cells such as neutrophils, albeit at high concentrations (Richter et al., 1992). Other studies have shown that CGRP is capable of inhibiting pro-inflammatory activity in pre-activated macrophages (Torii et al., 1997). Thus, it is conceivable that had we followed a line of investigation whereby we had pre-activated monocytes with TNFα and then stimulated with CGRP, we may have identified an interesting phenotype. Following this finding, however, we decided to abandon this line of investigation, mainly owing to the biological relevance of the question. It is unlikely that circulating CGRP directly stimulates immune cells as the concentrations found within the plasma are so low, around the picomolar range (Russell et al., 2014). It is more likely that the most relevant source of CGRP is the DRG nerve terminal that innervates the vascular wall. These nerves are well placed to release CGRP and in places infiltrate through the tunicae media to make contact with the ECs of the intimal layers of the vessel. Thus, we decided to instead focus on the effect of CGRP on the EC, rather than the leukocyte.

5.5.3 Stimulation ofHUVEC with 300 nM CGRP does not promote leukocyte adhesion in quiescent endothelium, but attenuates leukocyte-endothelial interactions in TNFα-activated endothelial cells

Focusing on the effect of CGRP on the endothelium, we uncovered some interesting results. Firstly, we showed that treatment of quiescent ECs for up to six hours with 300 nM CGRP was not sufficient to encourage leukocyte-endothelial interactions under flow conditions. This indicates that CGRP likely does not induce cell adhesion molecule expression over this timeframe. This finding is in contrast with early studies investigating the effect of CGRP on endothelial biology, where treatment of endothelium with CGRP enhanced leukocyte adhesion under static conditions, using supraphysiological concentrations of peptide (Sung et al., 1992; Zimmerman et al., 1992). Further to this, cell adhesion molecule protein expression (VCAM-1, PECAM-1, JAM-A, JAM-C) was not significantly altered by CGRP stimulation for up to six hours and the transcriptome as measured by microarray was also unaffected. Thus, we concluded CGRP had a minimal effect on these aspects of endothelial biology at this concentration and within this timeframe.

Despite this lack of activity in quiescent ECs, we identified an interesting phenotype related to CGRP in TNFα-activated endothelium. We showed that co-stimulation of TNFα-treated HUVEC with 300 nM CGRP produced a time-dependent attenuation in leukocyte adhesion under flow conditions, where a significant attenuation of around 50% could be observed when cells were treated for six hours. A trend towards a reduction in leukocyte adhesion could also be observed at the three-hour time point. We concluded from this that CGRP impinges upon TNFα activity to limit leukocyte adhesion to endothelium. This effect complements a study performed by Huang et al. who showed that CGRP could inhibit the production of chemokines from LPS-activated microvascular endothelium, resulting in a reduction in leukocyte trafficking (Huang et al., 2011). Whilst harder to interrogate in terms of mechanisms, some have shown *in vivo* that CGRP is capable of attenuating leukocyte accumulation under pro-inflammatory conditions, in models of vascular preconditioning (Kamada et al., 2006; Hartmann et al., 2011) and sepsis (Gomes et al., 2005).

We were surprised at the finding that CGRP did not influence HUVEC gene transcription either in isolation or when combined with TNFα treatment. This is especially so because maximal attenuation of leukocyte recruitment occurred at six hours of treatment with CGRP, which would be characteristic of a process like gene transcription that takes many hours to complete, as opposed to a transient signalling event. TNFα treatment of HUVEC induced a

robust gene transcription profile, some of which was associated with typical NFκB-dependent pathways. Given that Huang et al. have previously shown that 10 nM CGRP is capable of inhibiting gene transcription in human dermal microvascular ECs through a LPS-NFκB network *via* the stabilisation of IκBα within the cytosol (Huang et al., 2011), we were surprised that we could not recapitulate this phenotype with the use of CGRP in attenuating TNFα transcription responses. Furthermore, more generally, increases in intracellular cAMP levels have been shown to inhibit NFκB-dependent gene transcription in ECs *via* the activation of PKA (Ollivier et al., 1996). Steps were taken to ensure that the CGRP used in this particular assay was bioactive – aliquots were used from the same batch as flow assay experiments and CGRP was assayed for bioactivity using a mesenteric artery preparation in the myograph, where it was found to produce vasodilatation at 300 nM.

We attempted to identify specific mechanisms by which CGRP might reduce monocyte adhesion to activated endothelium. Initial immunoblotting for key EC adhesion molecules did not point to a particular molecule regulating this response. Further microarray analysis of endothelial gene expression revealed that whilst TNFα induced a robust and well-characterised pattern of gene expression, CGRP did not change this response. This indicates that however CGRP is producing the observed phenotype, it is not by directly affecting TNFα-dependent gene transcription. A possibility is that CGRP is affecting intracellular protein trafficking – changes in total cellular adhesion molecule expression might not be apparent but the temporospatial distribution of adhesion molecules within the cell may be different. For instance, the removal of adhesion molecules (such as VCAM-1, for instance) from the plasma membrane and into intracellular compartments may result in a reduced adhesion phenotype. Indeed it is known, for instance, that molecules of the endothelial tight junction (including PECAM-1) can be removed from the membrane and "cycled" through the lateral border recycling compartment (Mamdouh et al., 2008), depending on the requirements of the cell to maintain its junction at a particular point in time. One way to interrogate this trafficking process and to build upon our cell adhesion molecule immunoblotting experiments would be to investigate expression of cell adhesion molecules *within* the EC membrane, perhaps by biotinylating such proteins and subsequently identifying them by mass spectrometry, for instance. Alternatively, in line with studies performed by Huang et al., it is possible that endothelial CGRP signalling is limiting chemokine presentation by the cell (Huang et al., 2011). Certainly, we showed that TNFα could induce gene expression for a range of cytokines/chemokines in HUVEC. Failure to secrete these factors would presumably result in lowered THP-1 activation to mediate firm

adhesion to the endothelium and thus a mechanism that is independent on EC adhesion molecule expression and dynamics might exist.

Another surprising result was related to our findings in microarray experiments, showing that genes encoding proteins that comprise the CGRP receptor were less active following TNFα treatment *versus*control-treated cells (CALCLR expression reduced by 0.40). This has been shown once before by Nagoshi et al. who found that treatment of coronary artery ECs with 10 ng/mL TNFα (the same concentration used in our experiments and one which is representative of that found within the inflamed vascular wall) was sufficient to bring about a ~60% reduction in CLR expression and a ~30% reduction in RAMP1 expression, which coincided with a loss of receptor function, as measured by cAMP assay (Nagoshi et al., 2004). This could be a potential mechanism by which CGRP attenuates leukocyte-endothelial interactions, though this mode of action would point to a level of constitutive receptor activation by endogenously produced ligands, which is not an phenomenon that is well-characterised of the CGRP receptor.

Whilst we have discovered an interesting phenotype regarding the ability of CGRP to attenuate monocyte-EC interaction under flow conditions *in vitro*, there are a few limitations to this arm of the study and improvements with future experiments can be made. Firstly, it would be prudent to establish a concentration-response curve to determine the active range of concentration of CGRP required toproduce this phenotype. Admittedly, the concentration we have used in this experiment could be argued as being on the supraphysiological scale, especially with respect to reported circulating concentrations of peptide, thought to be within the picomolar range. However, we designed the experiment to obtain the best chance of observing a phenotype by using a relatively high concentration. Further to this, circulating levels of peptide probably do not represent a reservoir of biologically significant CGRP and may better be considered as neuronal spillover, much like circulating noradrenaline. At the site of release, however, local concentrations of CGRP are likely to be high and whilst it is not known, it is conceivable that concentrations of peptide that reach the endothelium *in vivo* are within the range that we are using. Secondly, mechanistic analyses would take advantage of selective CLR/RAMP1 receptor antagonists (such as BIBN4096BS) to probe whether the phenomenon observed is a CGRP receptor-dependent event or if the concentration of CGRP we have chosen is high enough to activate the AM_1 receptor for AM, comprised of CLR/RAMP2.

5.5.4 Conclusion

Despite the lack of mechanism, there is some biological significance in our finding that CGRP can time-dependently inhibit leukocyte adhesion. We have shown in Chapter 4 that ageing is characterised by an enhanced infiltration of inflammatory cells into the vascular walland this can provide a pro-inflammatory platform for disease. A disease that is highly prevalent in the aged is atherosclerosis, where activation of the endothelium and trafficking of circulating monocytes is a hallmark early pathogenic component. Unfortunately, studies of atherosclerosis and CGRP are incredibly sparse, possibly owing to the difficulty of generating a double ApoE/CGRP KO mouse by conventional cross-breeding methods. Both ApoE and CGRP genes are located in close proximity to one another in the mouse genome and thus will usually cross over together during meiosis. Studies taking advantage of other mouse models of atherosclerosis (for example, in LDL receptor KO mice fed atherogenic diets) might reveal a protective role for CGRP in trafficking of circulating monocytes into the early atherosclerotic lesion. We have so far witnessed a lack of evidence supporting a protective role for CGRP within the ageing vasculature but we have gathered evidence for CGRP being protective *in vitro*. With the additional knowledge that previous studies from our lab concerning more severe CVD models reveal a degree of vascular protection afforded by CGRP (Smillie et al., 2014), Chapter 6 will attempt to recapitulate *in vivo* protection by challenging the ageing vasculature with ATII-induced hypertension.

6. | Chapter Six: Investigating a role for αCGRP in a mouse model of geriatric hypertension

6.1 Introduction

In Chapter 4, I showed that aged mice lacking the gene encoding αCGRP do not suffer from pathological increases in baseline blood pressure and have similar vascular inflammatory profiles, in comparison to WT animals. We ascribed this to the lack of severity of the ageing model and it not being a significant enough stimulus to provoke CGRP-mediated cardiovascular protection. Conversely, in Chapter 5, results suggested that CGRP could act in an anti-inflammatory manner, by virtue of attenuating leukocyte-EC interactions. With both of these findings in mind, we sought to develop a new model in ageing WT and KO αCGRP mice that would be severe enough to generate vascular dysfunction, namely, an ageing ATII model of hypertension.

There is a dense literature involving the use of the ATII model of hypertension in the mouse and, indeed, we have published recently on the role of αCGRP in this particular model (Smillie et al., 2014) (Figure 52 illustrates hypertensive data taken from this publication). However, there are significantly less studies related to the use of this model in ageing mice. Three recent publications from the Ungvari group have characterised the model and have apparently demonstrated its utility. Results from their laboratory have shown that infusion of 1.4 mg/kg/day ATII over 28 days is sufficient to induce increases in blood pressure of about 40-60 mmHg, in male C57/BL6 mice aged 24 months (Csiszar et al., 2013; Toth et al., 2013a; 2013b). Hypertension in aged mice was associated with exacerbated cerebromicrovascular injury and upregulation of proinflammatory cytokines. However, given that these three publications have emerged from the same laboratory, what remains to be seen is whether this is a reproducible model of hypertension in aged animals. In this chapter, we will apply this model to male aged αCGRP WT and KO mice, in order to provoke a pro-inflammatory vascular environment to ideally precipitate a degree of vascular protection stemming from CGRP.

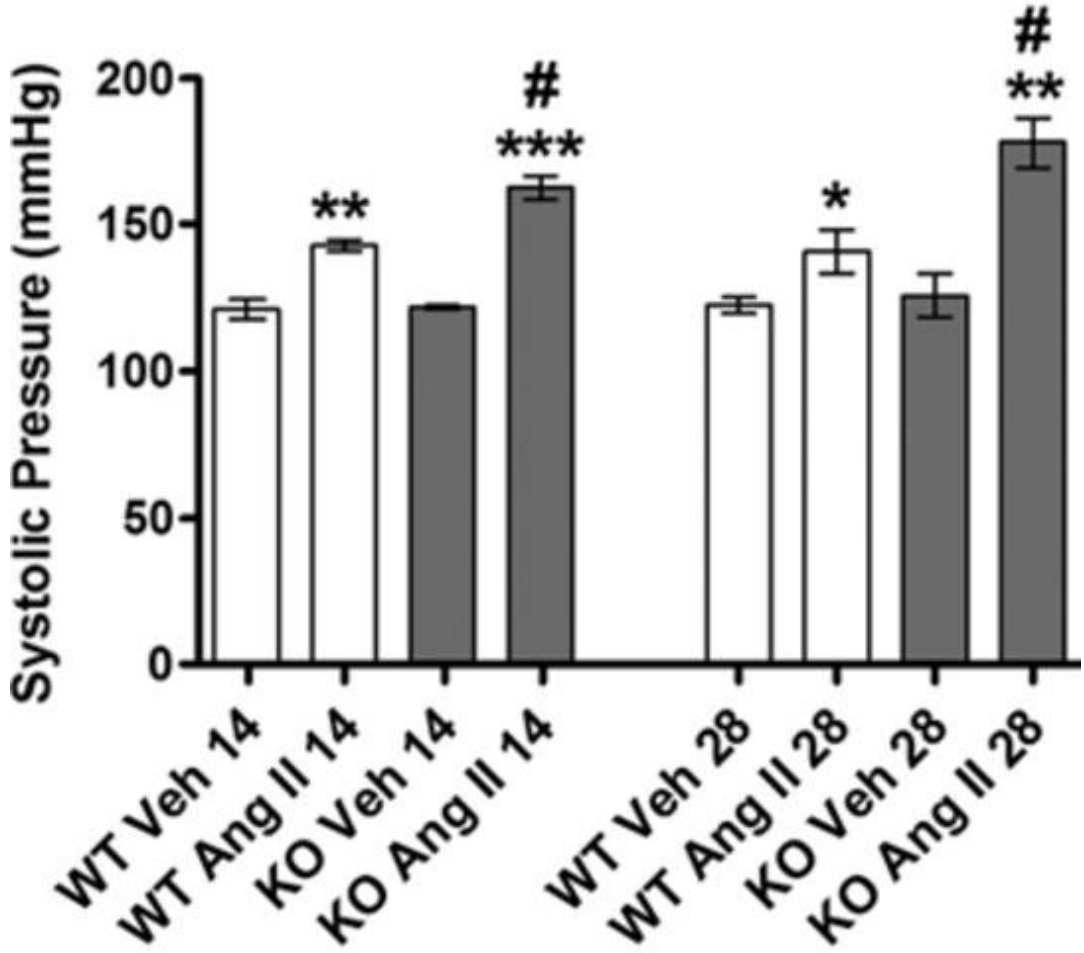

Figure 52: Figure adapted from Smillie *et al*. (2014) showing elevated systolic blood pressure in ATII-treated juvenile mice, with exacerbated hypertension in CGRP KO mice (Smillie et al., 2014).

6.2 Hypothesis

Infusion of 1.1 mg/kg/day ATII into aged (15 months) αCGRP WT and KO mice will produce hypertension and associated vascular inflammation that is exacerbated in mice lacking αCGRP.

6.3 Aims

1. To establish and characterise a new model of ATII-induced hypertension in aged αCGRP WT and KO mice.
2. To investigate a potential protective role for CGRP against hypertension-induced vascular inflammation in the ageing mouse.

6.4 Results

6.4.1 Infusion of 1.1 mg/kg/day angiotensin II in aged αCGRP WT and KO mice does not clearly produce the development of hypertension

To establish hypertension in aged αCGRP WT and KO mice, we chose to use a dose of 1.1 mg/kg/day ATII, infused *via* osmotic minipump over a course of 14 days. This dosing regime has been previously well-characterised within our group and is known to induce hypertension in juvenile mice (Bodkin et al., 2014; Smillie et al., 2014). With this and the aforementioned studies published by Ungvari in mind, we expected to observe hypertension in our mice. Figure 53 shows longitudinal blood pressure responses over the course of the experiment that, surprisingly, did not produce a clear result. Disappointingly, we found that WT animals treated with vehicle trended towards a non-significant increase in systolic blood pressure (Figures 53, 54) from baseline to day 14 (baseline 95.89 ± 3.55 mmHg *vs* 110.93 ± 7.31 mmHg). Furthermore, we discovered that WT mice infused with ATII displayed no clear trend towards the expected rise in systolic blood pressure (baseline 95.74 ± 8.10 mmHg *vs* 96.12 ± 11.04 mmHg). Vehicle-treated KO mice displayed a trend towards a decrease in blood pressure at day 14 from baseline (102.68 ± 1.80 mmHg *vs* 90.05 ± 0.92 mmHg) and WT vehicle-treated mice showed a small trend towards increased blood pressure (102.90 ± 2.66 mmHg *vs* 109.37 ± 8.37 mmHg). Diastolic pressure (Figure 54B) and MAP (Figure 54C) showed similar trends, with no significant changes detected in any of the treatment groups. It is worth noting that one WT ATII-treated mouse and one KO ATII-treated mouse went on to develop substantial renal cysts, which led to large decreases in blood pressure from baseline, potentially confounding some of the results from this experiment.

As this was such a surprising result, we thought it prudent to scrutinise the data further in order to identify evidence of a pharmacological effect of ATII. Figure 55 illustrates changes in systolic (Figure 55A), diastolic (Figure 55B) and MAP (Figure 55C) blood pressure following Veh/ATII treatment in αCGRP WT and KO mice, with trend lines matching each animal. Here, we show evidence of a mix of responders and non-responders, which might be obscuring the final result we observed. Whilst it is clear that an unexpected result was observed in vehicle-treated WT animals, we see evidence for at least two WT animals within the ATII group responding to the infusion in the expected manner. For instance, one mouse begins with a baseline systolic blood pressure of 105.83 mmHg, which then rises to 147.00 mmHg following treatment (Figure 55A), a rise in systolic blood pressure of 41.17 mmHg. Another animal was found to have a lower baseline than the rest in the group, but had an increase in

blood pressure of 29.08 mmHg following treatment, which may be considered to be hypertensive relative to baseline. Strikingly, in the ATII-treated KO group of animals, we observed four out of six animals responding to treatment, as opposed to only two out of six animals in the WT ATII group. Whilst the variable responses observed in the control group animals confound the data somewhat, looking at responses obtained from individual animals, we see some evidence that this model has been successful, to an extent, in some cases.

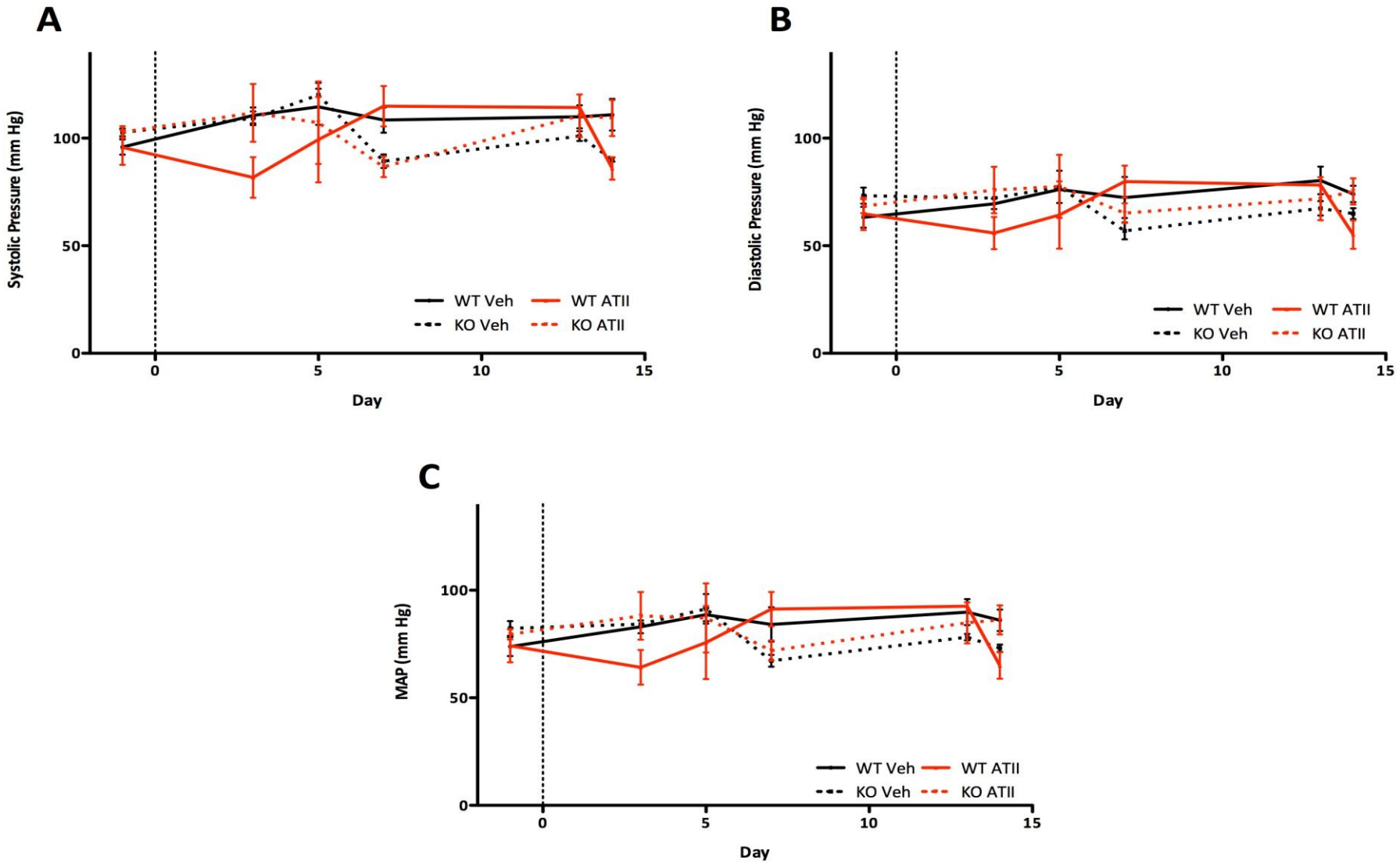

Figure 53: Longitudinal blood pressure responses of aged (15 months) αCGRP male WT and KO mice, following infusion of either vehicle (Veh, saline) or angiotensin II (ATII) over 14 days. Results show A) systolic blood pressures of male aged αCGRP WT and KO mice following treatment; B) diastolic blood pressures of male aged αCGRP WT and KO mice following treatment; C) mean arterial blood pressures of male aged αCGRP WT and KO mice following treatment. Data expressed as mm Hg, mean ± SEM, n=4 for vehicle-treated animals, n=6 for ATII-treated animals.

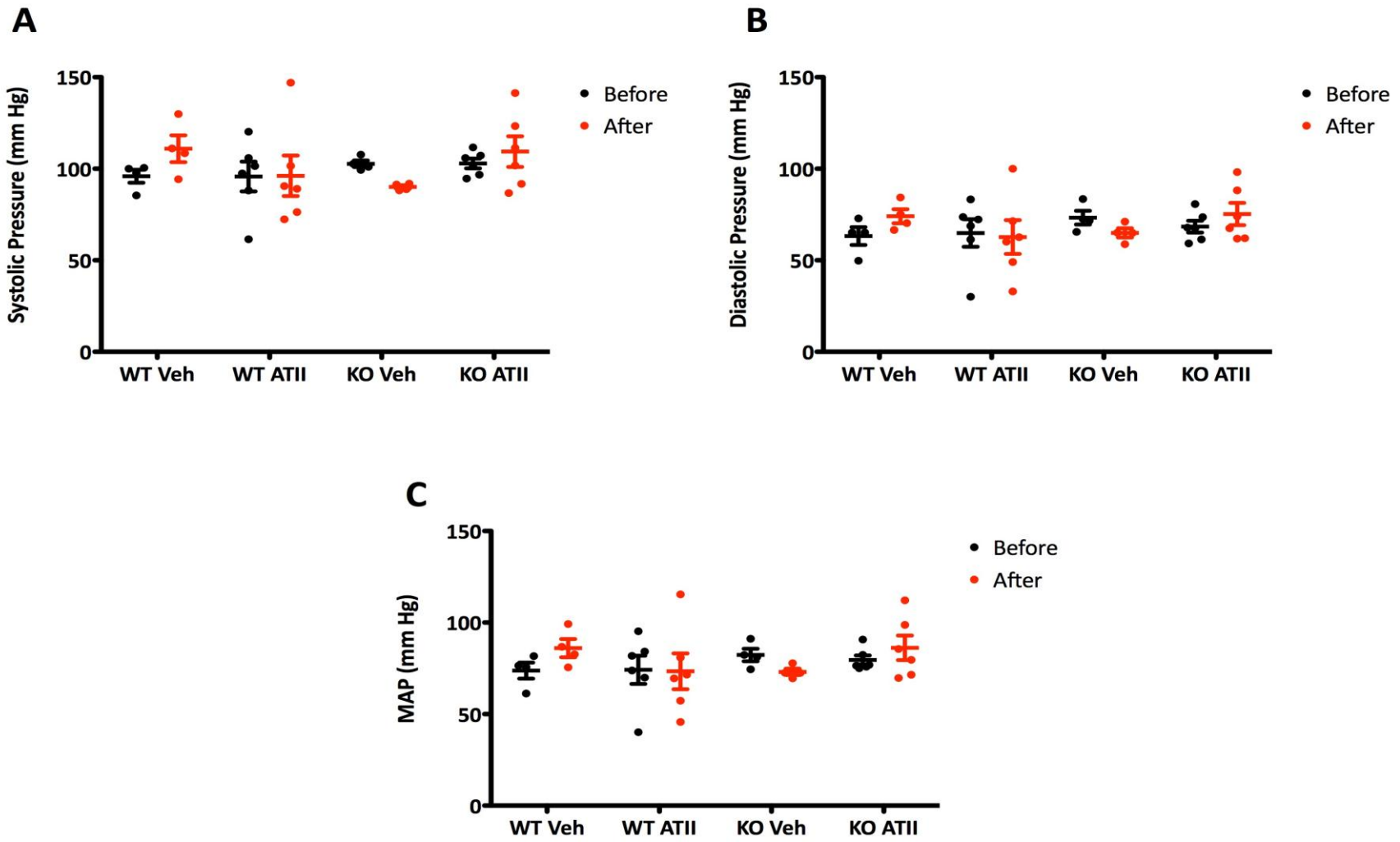

Figure 54: Blood pressures of aged (15 months) αCGRP male WT and KO mice, following infusion of either vehicle (Veh, saline) or angiotensin II (ATII). Results show baseline blood pressure ('Before) and blood pressure at day 14 of hypertension ('After'). Results show A) systolic blood pressures of male aged αCGRP WT and KO mice following treatment; B) diastolic blood pressures of male aged αCGRP WT and KO mice following treatment; C) mean arterial blood pressures of male aged αCGRP WT and KO mice following treatment. Data expressed as mm Hg, mean ± SEM, n=4 for vehicle-treated animals, n=6 for ATII-treated animals. Statistical significance was evaluated by repeated measures two-way ANOVA plus Bonferroni *post hoc* test. No significant differences were detected between experimental groups.

Owing to differences in baseline blood pressures between animals, we further sought to crystallise the blood pressure response from this experiment. To do so, Figure 56 illustrates blood pressure responses at day 14 of the experiment as a percentage change from baseline for each individual experimental subject. Figure 56A shows systolic blood pressure changes, Figure 56B diastolic and Figure 56C MAP. Indeed, when we analysed responses from individual animals, we uncovered evidence of groups of responders *versus* non-responders. Figure 56A shows that 2/6 WT ATII-treated animals responded in a hypertensive manner relative to baseline. Interestingly, 4/6 KO ATII-treated animals responded in a hypertensive manner relative to baseline.

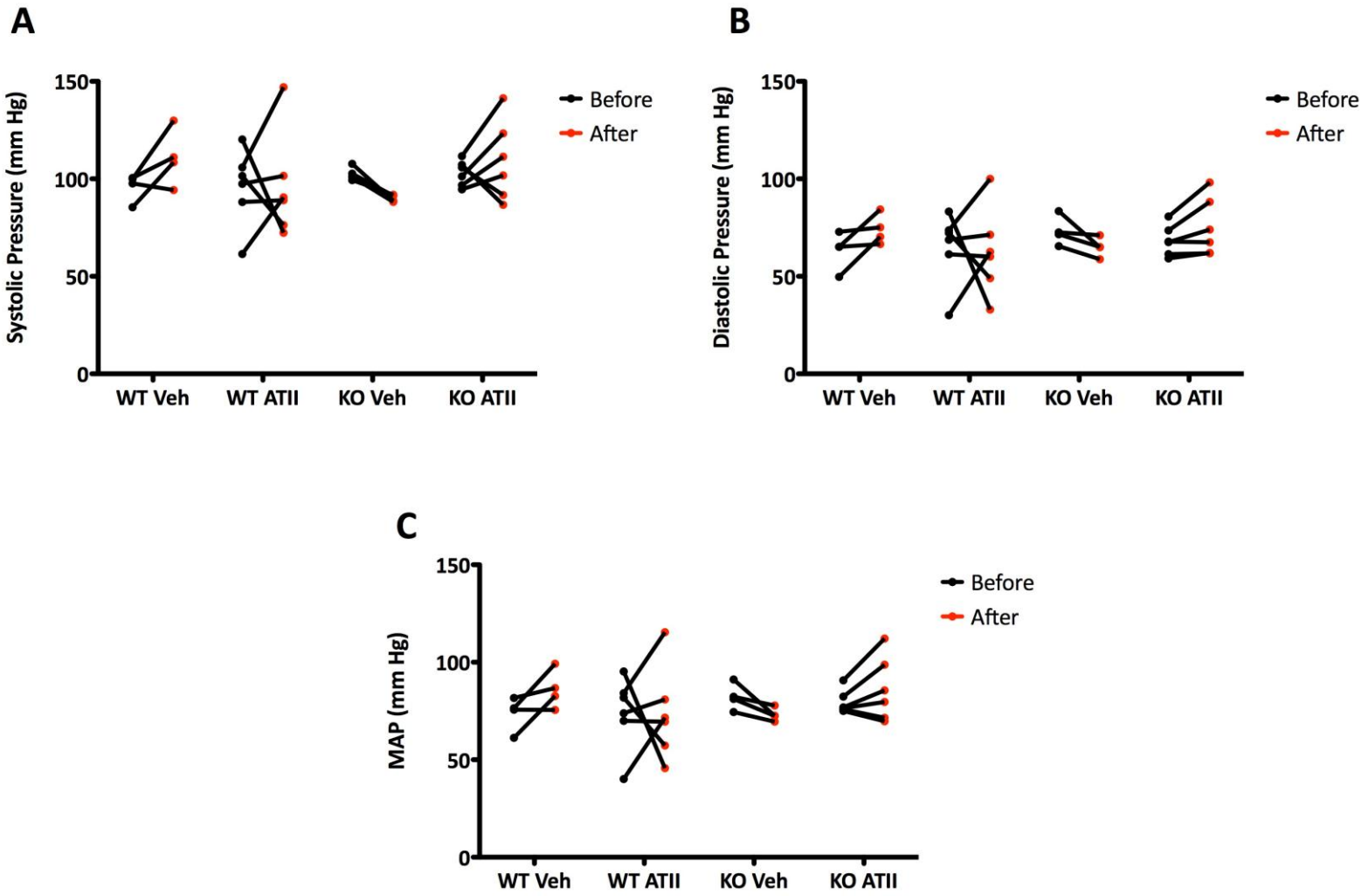

Figure 55: Blood pressures of aged (15 months) αCGRP male WT and KO mice, following infusion of either vehicle (Veh, saline) or angiotensin II (ATII). Results show baseline blood pressure ('Before') and blood pressure at day 14 of hypertension ('After'), matched for each animal. Results show A) systolic blood pressures of male aged αCGRP WT and KO mice following treatment; B) diastolic blood pressures of male aged αCGRP WT and KO mice following treatment; C) mean arterial blood pressures of male aged αCGRP WT and KO mice following treatment. Data expressed as mmHg, mean ± SEM, n=4 for vehicle-treated animals, n=6 for ATII-treated animals. Statistical significance was evaluated by repeated measures two-way ANOVA plus Bonferroni *post hoc* test. No significant differences were detected between experimental groups.

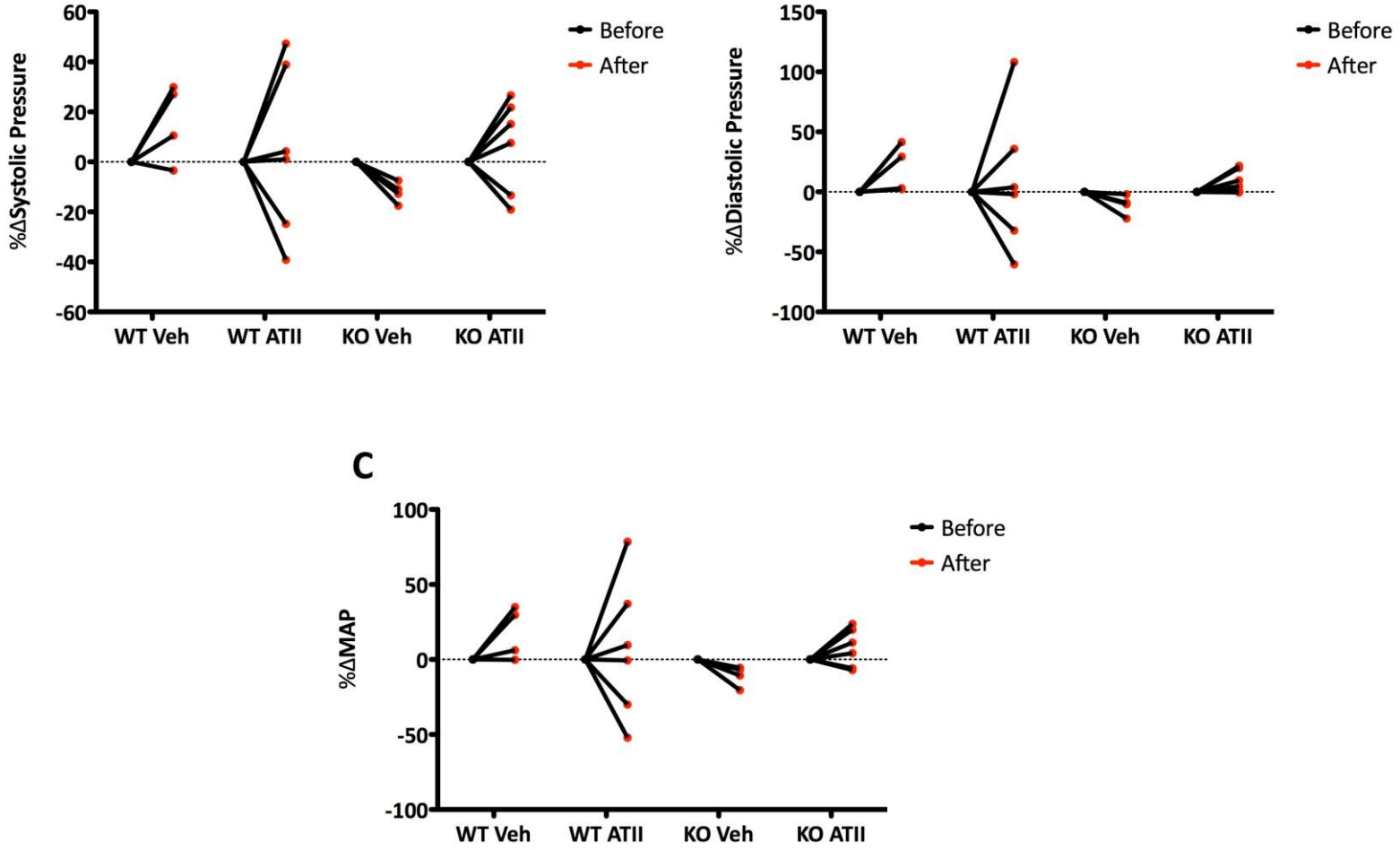

Figure 56: Blood pressures of aged (15 months) αCGRP male WT and KO mice, following infusion of either vehicle (Veh, saline) or angiotensin II (ATII). Results show % change from baseline blood pressure ('Before') and blood pressure at day 14 of hypertension ('After'), matched for each animal. Results show A) systolic blood pressures of male aged αCGRP WT and KO mice following treatment; B) diastolic blood pressures of male aged αCGRP WT and KO mice following treatment; C) mean arterial blood pressures of male aged αCGRP WT and KO mice following treatment. Data expressed as %change from baseline mm Hg, mean ± SEM, n=4 for vehicle-treated animals, n=6 for ATII-treated animals. Statistical significance was evaluated by repeated measures two-way ANOVA plus Bonferroni *post hoc* test. No significant differences were detected between experimental groups.

6.4.2 Infusion of 1.1 mg/kg/day angiotensin II in aged αCGRP WT and KO mice does not promote clear cardiac hypertrophy but does induce polydipsia

With the knowledge that ATII failed to produce a clear and consistent hypertensive response in this experiment, we sought to measure other markers of hypertension, which may have changed in the absence of an overt pressor phenotype. Figure 57 illustrates changes in water consumption in αCGRP WT and KO mice following vehicle or ATII infusion. Figure 57A shows group mean data for changes in water consumption measurements in WT and KO mice receiving either vehicle or ATII. Interestingly, here we found that vehicle-treated animals did not differ significantly in their water consumption at day 14 in comparison to baseline. However, both WT and KO animals that received 1.1 mg/kg/day ATII significantly increased their water consumption by day 14 of infusion (139.76 ± 36.34 µl/hour *vs* 281.42 ± 81.07 µl/hour for WT; 112.26 ± 14.48 µl/hour *vs* 277.93 ± 75.36 µl/hour for KO). Thus, whilst significant changes in blood pressure may not have occurred, we have some evidence to suggest that ATII is having some pharmacological effect in these mice, with respect to water consumption. Figures 58A-D illustrate correlations between percentage changes in systolic blood pressure and water intake, in order to identify a relationship between the two parameters. We did not find a significant relationship between blood pressure and water consumption.

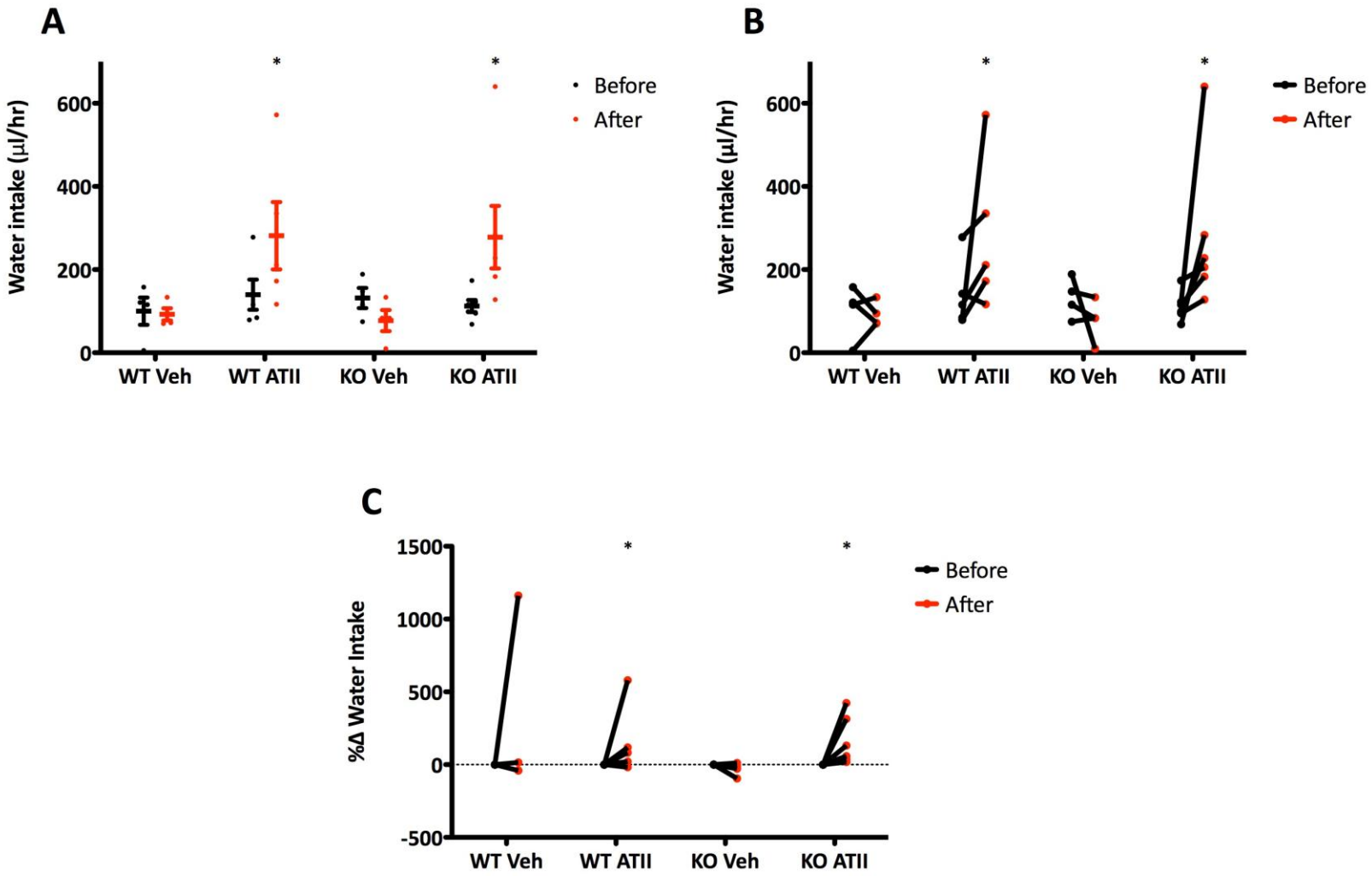

Figure 57: Water intakes of aged (15 months) αCGRP male WT and KO mice, following infusion of either vehicle (Veh, saline) or angiotensin II (ATII). Results show A) water intakes of male aged αCGRP WT and KO mice following treatment; B) water intakes of male aged αCGRP WT and KO mice following treatment, matched for each animal; C) % change from baseline water intake of male aged αCGRP WT and KO mice following treatment. Data expressed as A), B) µl/hour, mean ± SEM, n=4 for vehicle-treated animals, n=6 for ATII-treated animals; C) %change from baseline water intake. Statistical significance was evaluated by repeated measures two-way ANOVA plus Bonferroni *post hoc* test. *p<0.05 where genotype-matched treatment groups have been compared.

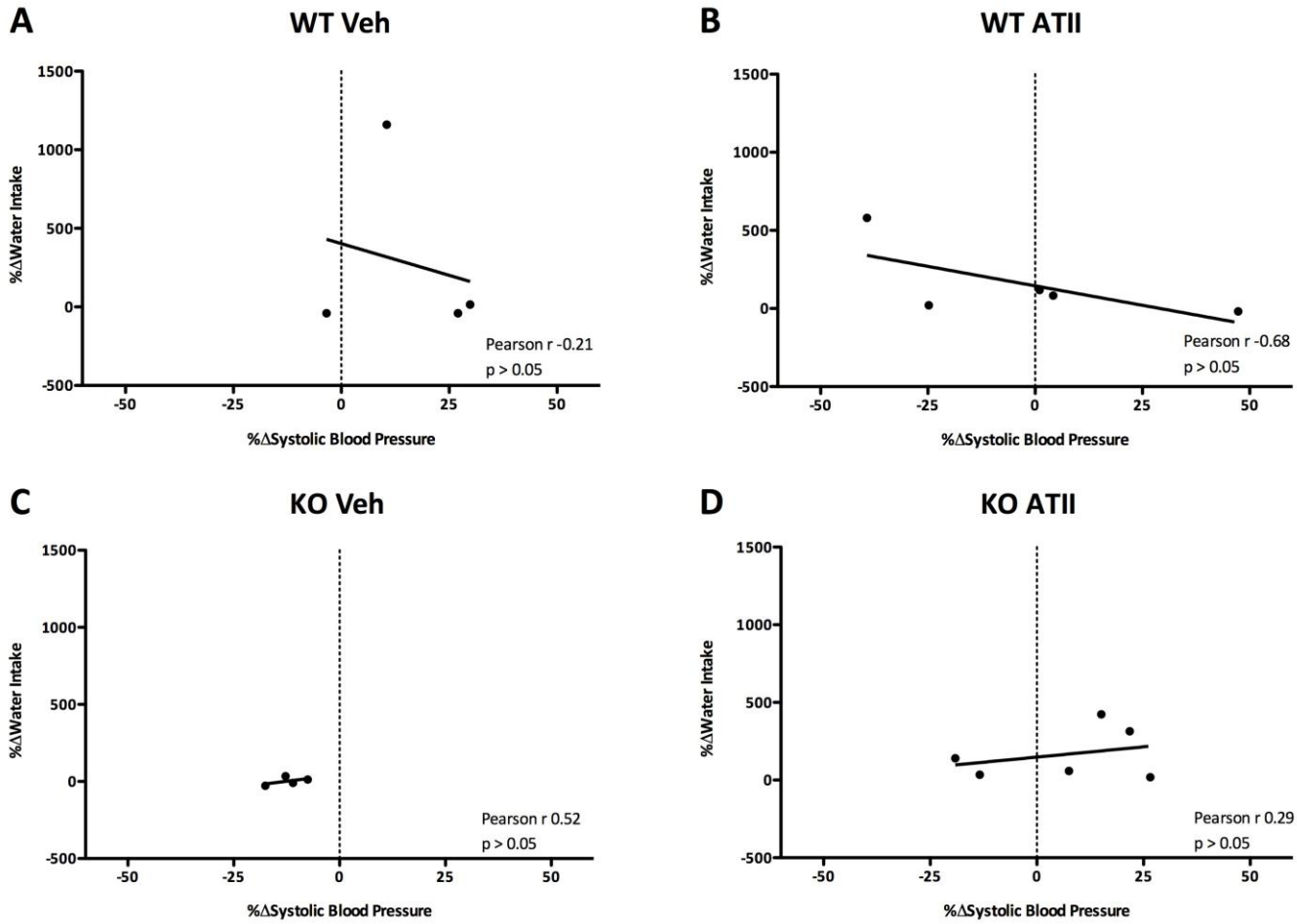

Figure 58: Correlating changes in water intake over baseline *vs* percentage changes in systolic blood pressure over baseline in aged (15 months) αCGRP male WT and KO mice, following infusion of either vehicle (Veh, saline) or angiotensin II (ATII). Results show correlations for A) WT Veh-treated animals; B) WT ATII-treated animals; C) KO Veh-treated animals; D) KO ATII-treated animals. Statistical significance was evaluated by Pearson r correlation coefficient, where $p < 0.05$ was considered to be statistically significant.

In conjunction with water consumption, we also investigated other parameters known to change following the induction of experimental hypertension. Figure 59 shows how animal heart weight was affected by ATII, both in terms of raw mass (Figure 59A) and heart mass relative to animal body size (Figure 59B). We did not observe any significant change in either total heart mass or heart mass relative to body size. However, we did witness an interesting trend towards an increase in heart mass in KO mice following ATII, though this did not reach statistical significance. Furthermore, data represented in Figures 60A-D show that there was not a significant correlation between changes in systolic blood pressure and heart mass.

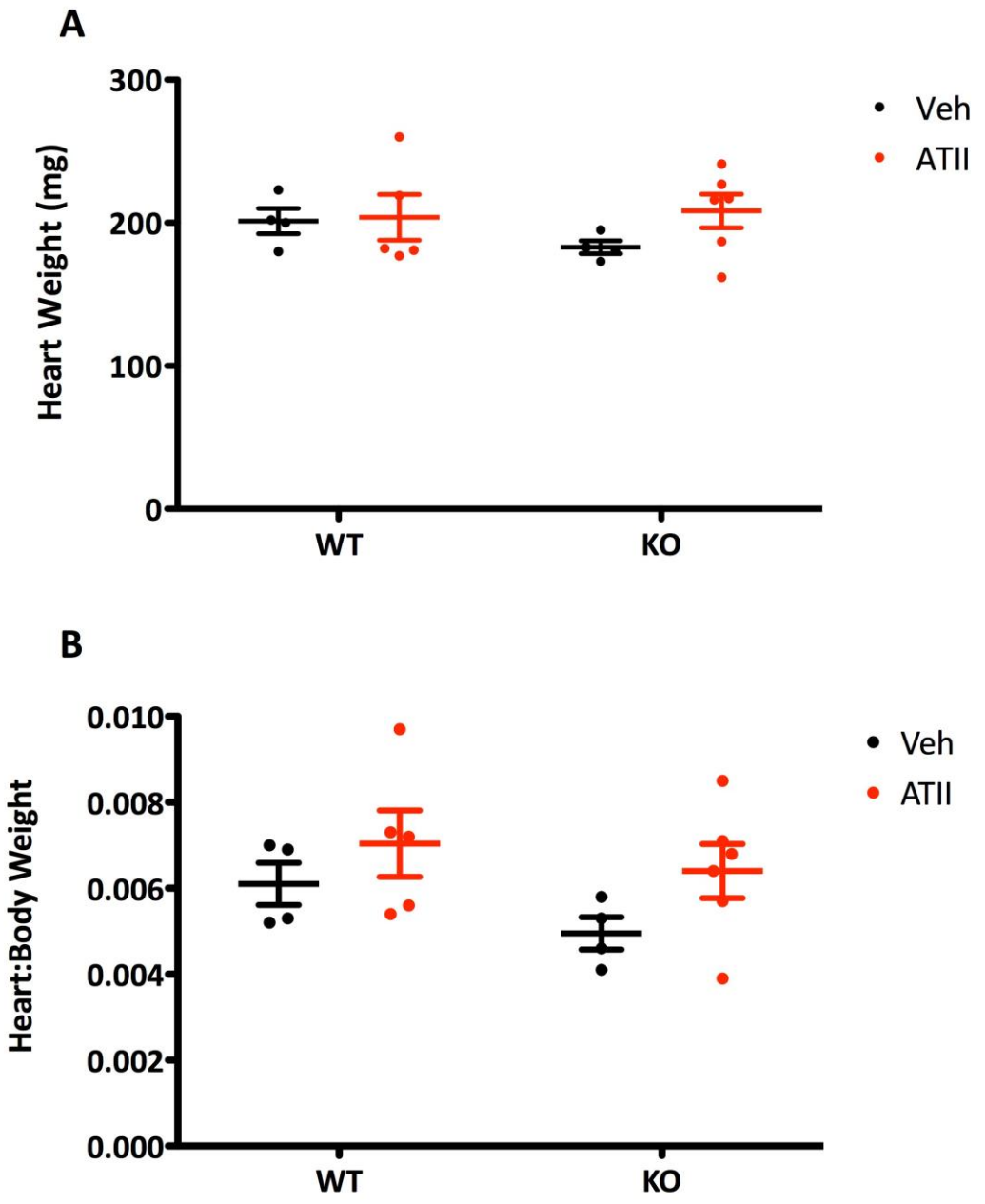

Figure 59: Changes in heart weight of aged (15 months) αCGRP male WT and KO mice, following infusion of either vehicle (Veh, saline) or angiotensin II (ATII). Results show A) changes in heart mass of male aged αCGRP WT and KO mice following treatment; B) changes in heart mass, relative to body weight of male aged αCGRP WT and KO mice following treatment. Data expressed as A) mg, mean ± SEM, n=4 for vehicle-treated animals, n=6 for ATII-treated animals; B) mg/mg body weight, mean ± SEM, n=4 for vehicle-treated animals, n=6 for ATII-treated animals. Statistical significance was evaluated by repeated measures two-way ANOVA plus Bonferroni *post hoc* test. No significant differences were detected between experimental groups.

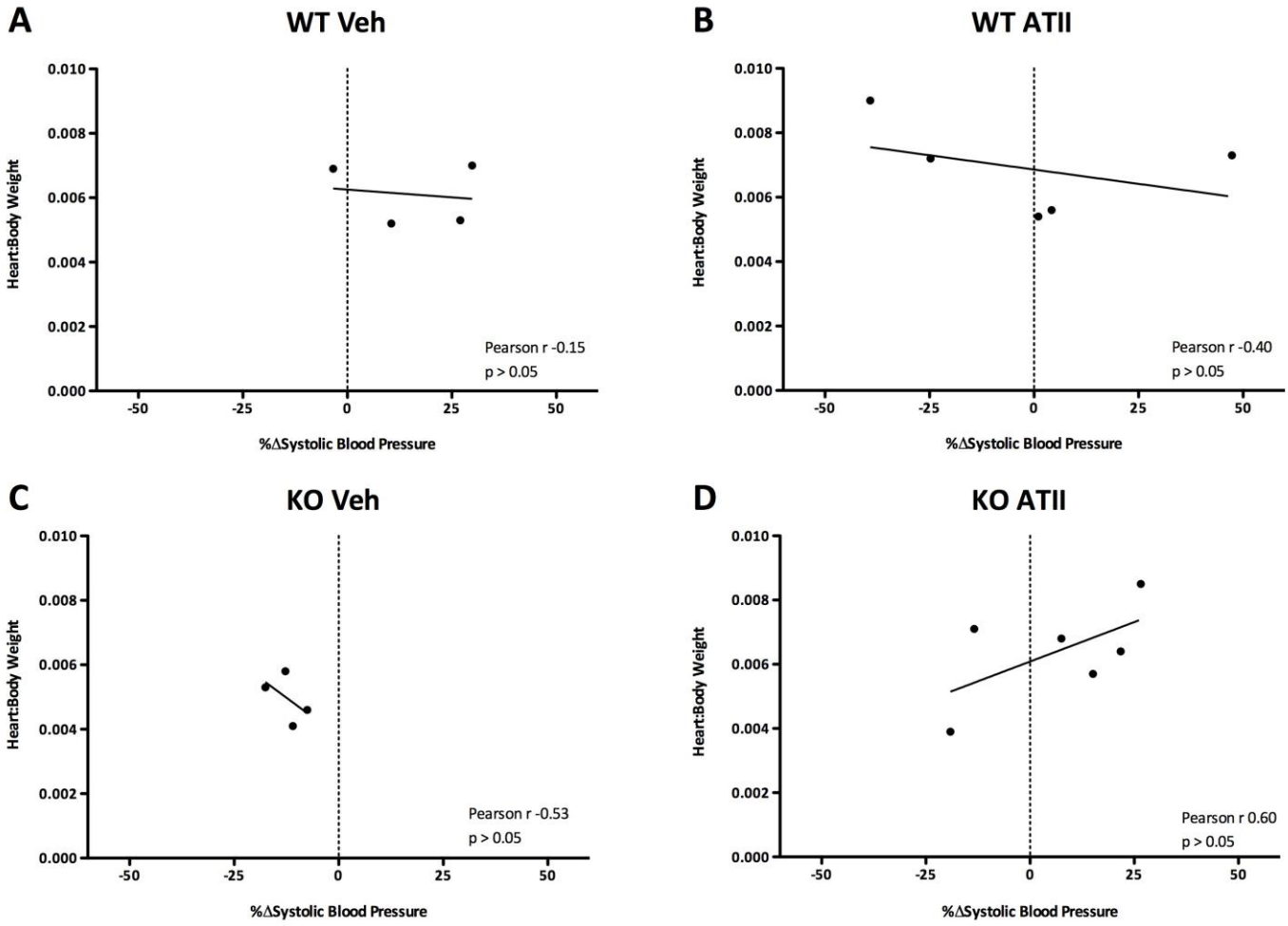

Figure 60: Correlating changes in heart weight over baseline *vs* percentage changes in systolic blood pressure over baseline in aged (15 months) αCGRP male WT and KO mice, following infusion of either vehicle (Veh, saline) or angiotensin II (ATII). Results show correlations for A) WT Veh-treated animals; B) WT ATII-treated animals; C) KO Veh-treated animals; D) KO ATII-treated animals. Statistical significance was evaluated by Pearson r correlation coefficient, where $p < 0.05$ was considered to be statistically significant.

6.4.3 Infusion of 1.1 mg/kg/day angiotensin II in aged αCGRP WT and KO mice does not induce changes in gene expression of markers of vascular dysfunction

Whilst we did not witness significant increases in blood pressure in aged αCGRP WT and KO animals receiving ATII treatment, we did see some evidence for pharmacological activity of ATII when we measured water consumption. These data combined made us consider that whilst we set out to establish a more 'severe' model of cardiovascular stress by the infusion of a pressor dose of ATII, we may have perhaps generated a 'subpressor' ATII model instead. This type of model would not increase blood pressure in the mouse, but may still have the capacity to induce vascular dysfunction and associated inflammation.

To investigate this, we probed total mRNA lysates from the aortae of aged αCGRP WT and KO mice treated with either vehicle or ATII for 14 days. We initially examined eNOS gene expression as an early marker of endothelial dysfunction and found no significant changes in the level of its expression in WT and KO mice following either vehicle or ATII administration (Figure 61A). We then investigated HO-1 gene expression as an early marker of oxidative stress and again found no significant changes in its expression (Figure 61B). Interestingly, however, we found that WT mice had a clear trend towards elevated HO-1 expression following ATII infusion, in comparison with their vehicle-treated counterparts (4015.25 ± 483.04 copies/µl for vehicle-treated *vs* 12039.00 ± 3893.33 copies/µl for ATII-treated). This did not reach statistical significance, but points to an interesting potential action of ATII in promoting oxidative stress in the absence of changes in pressure. This increase was reflected in KO animals, but to a lesser degree. Despite potential increases in HO-1 as a response to oxidative stress, we observed no significant changes or clear trends in the expression of pro-oxidant enzymes NOX2 (Figure 61C) and NOX4 (Figure 61D).

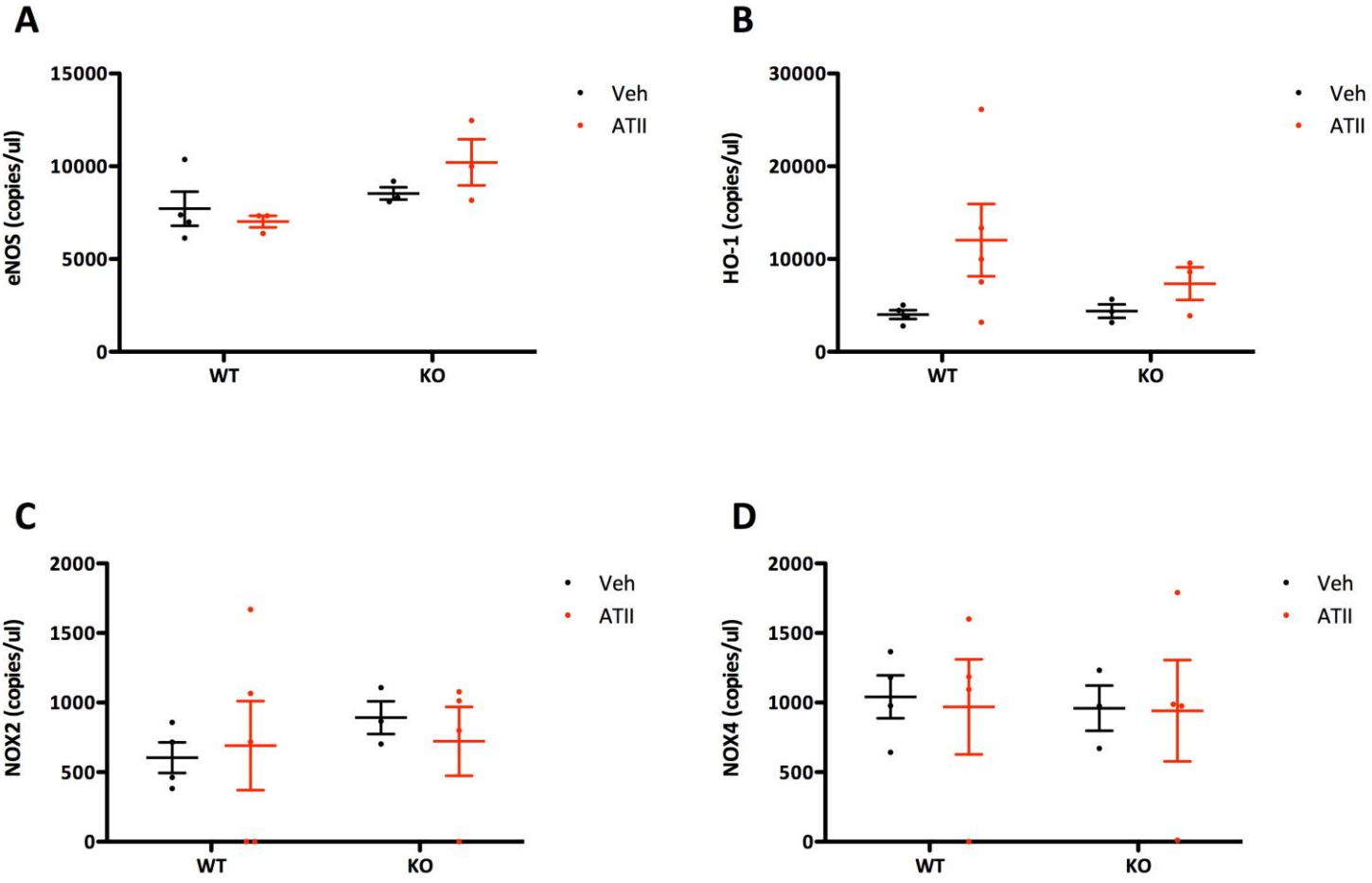

Figure 61: Gene expression analysis of vascular dysfunction markers of aortic lysates obtained from aged (15 months) male αCGRP WT and KO mice, treated with either vehicle (Veh) or ATII. Results show A) mRNA expression of eNOS in aortic lysates; B) mRNA expression of HO-1 in aortic lysates; C) mRNA expression of NOX2; D) mRNA expression of NOX4 in aortic lysates. Data expressed as copies mRNA per microliter, mean ± SEM, n=3-5. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. No significant differences were detected between groups.

6.4.4 Infusion of 1.1 mg/kg/day angiotensin II in aged αCGRP WT and KO mice does not induce changes in gene expression markers of vascular inflammation

Further to examination of gene expression of markers related to vascular dysfunction, we next studied the expression of aortic genes from aged αCGRP WT and KO animals that are more classically related to the development of leukocytic vasculitis. Figure 62A illustrates changes in aortic VCAM-1 expression in aged WT and KO mice in response to either vehicle or ATII. Echoing changes in HO-1 expression, we found a clear but non-significant increase in VCAM-1 gene expression in WT mice following ATII infusion (492.50 ± 15.74 copies/µl for vehicle-treated *vs* 1088.44 ± 325.20 copies/µl for ATII-treated). This trend was reflected in KO animals receiving ATII treatment, although it was less clear. We also investigated changes in the gene expression of another marker of vascular inflammation, MCP-1, to complement our VCAM-1 findings (Figure 62B). Here we did not observe as clear a signal, with expression appearing relatively stable across all four groups and no statistically significant changes emerging.

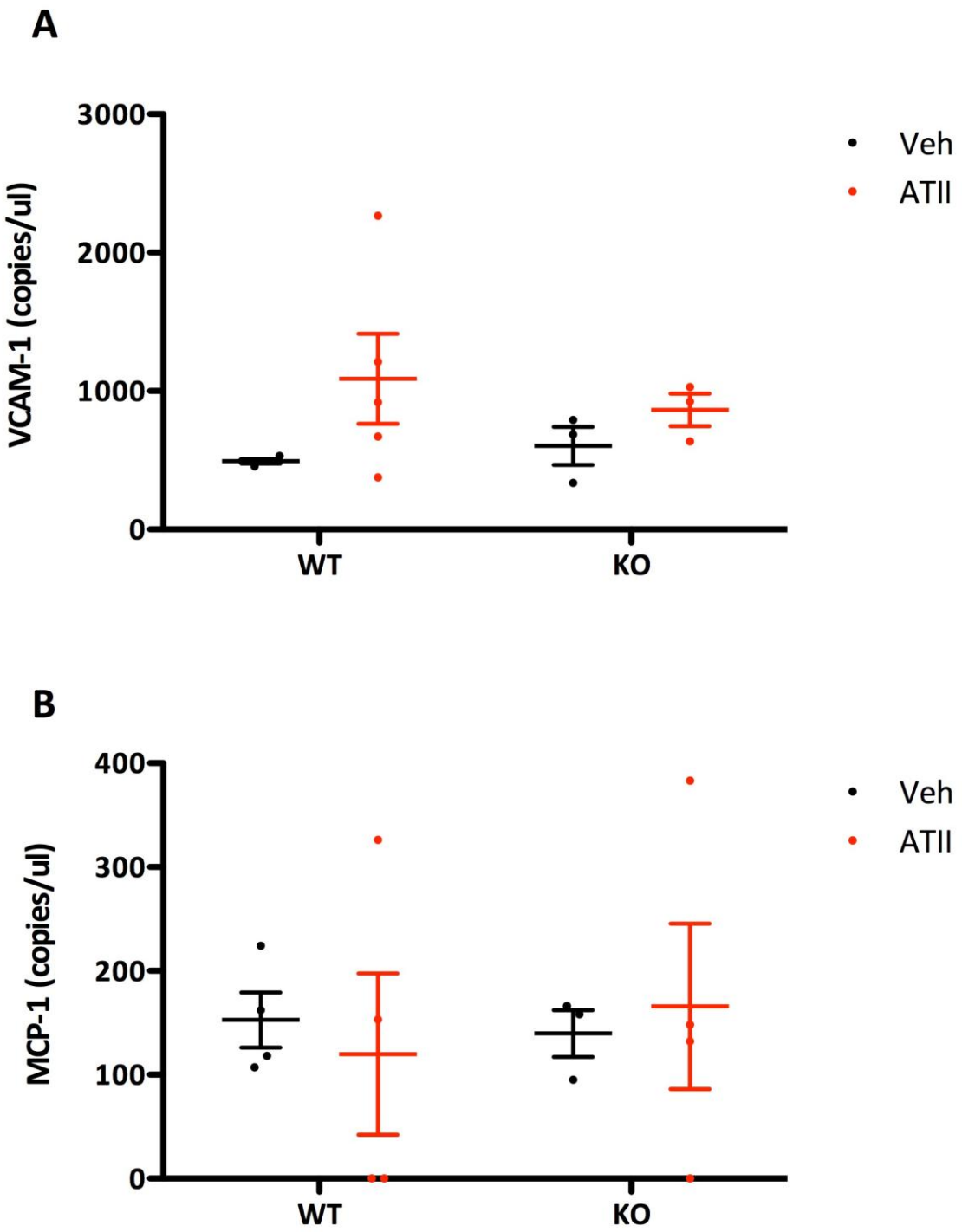

Figure 62: Gene expression analysis of vascular inflammatory markers of aortic lysates obtained from aged (15 months) male αCGRP WT and KO mice, treated with either vehicle (Veh) or ATII. Results show A) mRNA expression of VCAM-1 in aortic lysates; B) mRNA expression of MCP-1 in aortic lysates. Data expressed as copies mRNA per microliter, mean ± SEM, n=3-5. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. No significant differences were detected between groups.

6.4.5 Infusion of 1.1 mg/kg/day angiotensin II in aged αCGRP WT and KO mice induces changes in vascular CGRP receptor gene expression

We were also interested in investigating how aortic CGRP receptor gene expression might change following the induction of hypertension, in aged αCGRP WT and KO mice (Figure 63). Here, we have shown that the gene expression of CLR mRNA within the aorta does not appear to significantly change in WT and KO mice following infusion of ATII. However, we did note a non-significant trend towards an upregulation of CLR gene expression in WT mice that received ATII for 14 days (667.75 ± 35.93 copies/µl for vehicle-treated *vs* 904.25 ± 186.11 copies/µl for ATII-treated). Expression of CLR in KO animals receiving ATII appeared comparable to controls (Figure 63A)

Interestingly, when we interrogated aortic RAMP1 gene expression (Figure 63B), we found the opposite. RAMP1 appeared to be stably expressed in WT animals, with comparable levels of expression between vehicle- and ATII-treated groups. Vehicle-treated KO mice also had a very similar level of mRNA expression (335.66 ± 60.84 copies/µl). However, levels of RAMP1 mRNA significantly doubled in the ATII-treated KO group (770.25 ± 89.15 copies/µl) when compared with vehicle-treated. Levels were also significantly higher than those in the ATII-treated WT group. This is a striking result in comparison to RAMP2 mRNA expression, which appeared to be quite stable across genotype and treatment groups (Figure 63C).

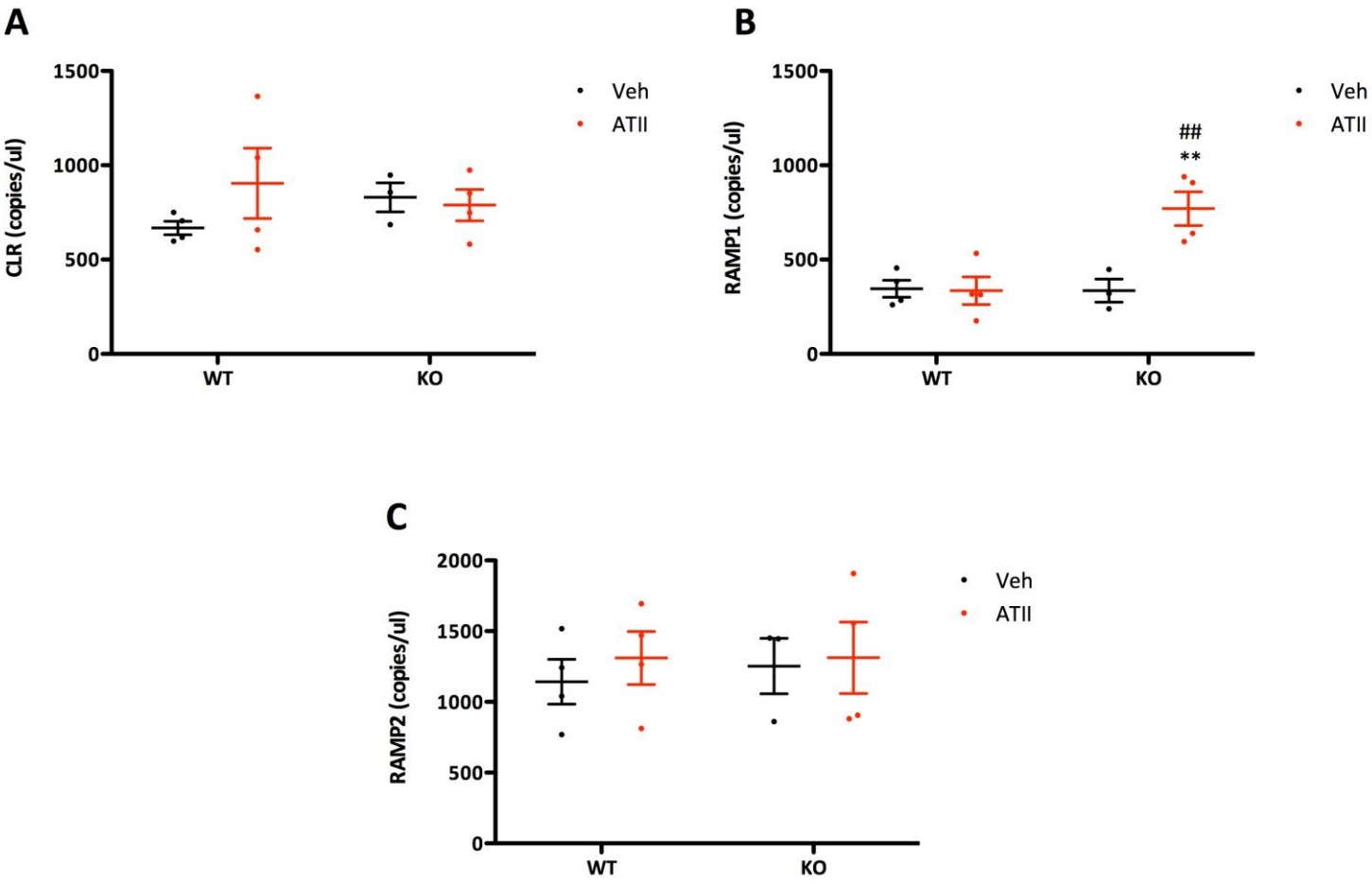

Figure 63: Gene expression analysis of vascular inflammatory markers of aortic lysates obtained from aged (15 months) male αCGRP WT and KO mice, treated with either vehicle (Veh) or ATII. Results show A) mRNA expression of CLR in aortic lysates; B) mRNA expression of RAMP1 in aortic lysates; C) mRNA expression of RAMP2 in aortic lysates. Data expressed as copies mRNA per microliter, mean ± SEM, n=3-5. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. **p < 0.01 where comparisons were made between animals of differing genotype receiving the same treatment; ##p < 0.01 where comparisons were made between animals of the same genotype receiving different treatment.

6.4.6 Infusion of 1.1 mg/kg/day angiotensin II in aged αCGRP WT and KO mice does not alter the expression of CGRP isoforms within the dorsal root ganglia

Finally, we measured the gene expression of CGRP isoforms within the DRG of aged αCGRP WT and KO mice, following infusion of either vehicle or ATII (Figure 64). We found that αCGRP gene expression did not significantly differ between WT mice receiving either vehicle or ATII. KO mice did not express any transcript for αCGRP (Figure 64A). Expression of βCGRP also did not significantly change, although there was a small non-significant trend towards an elevation of expression in WT mice following ATII treatment (94.33 ± 47.16 copies/µl *vs* 163.25 ± 30.31 copies/µl, Figure 64B). KO mice had similar levels of the alternative CGRP isoform.

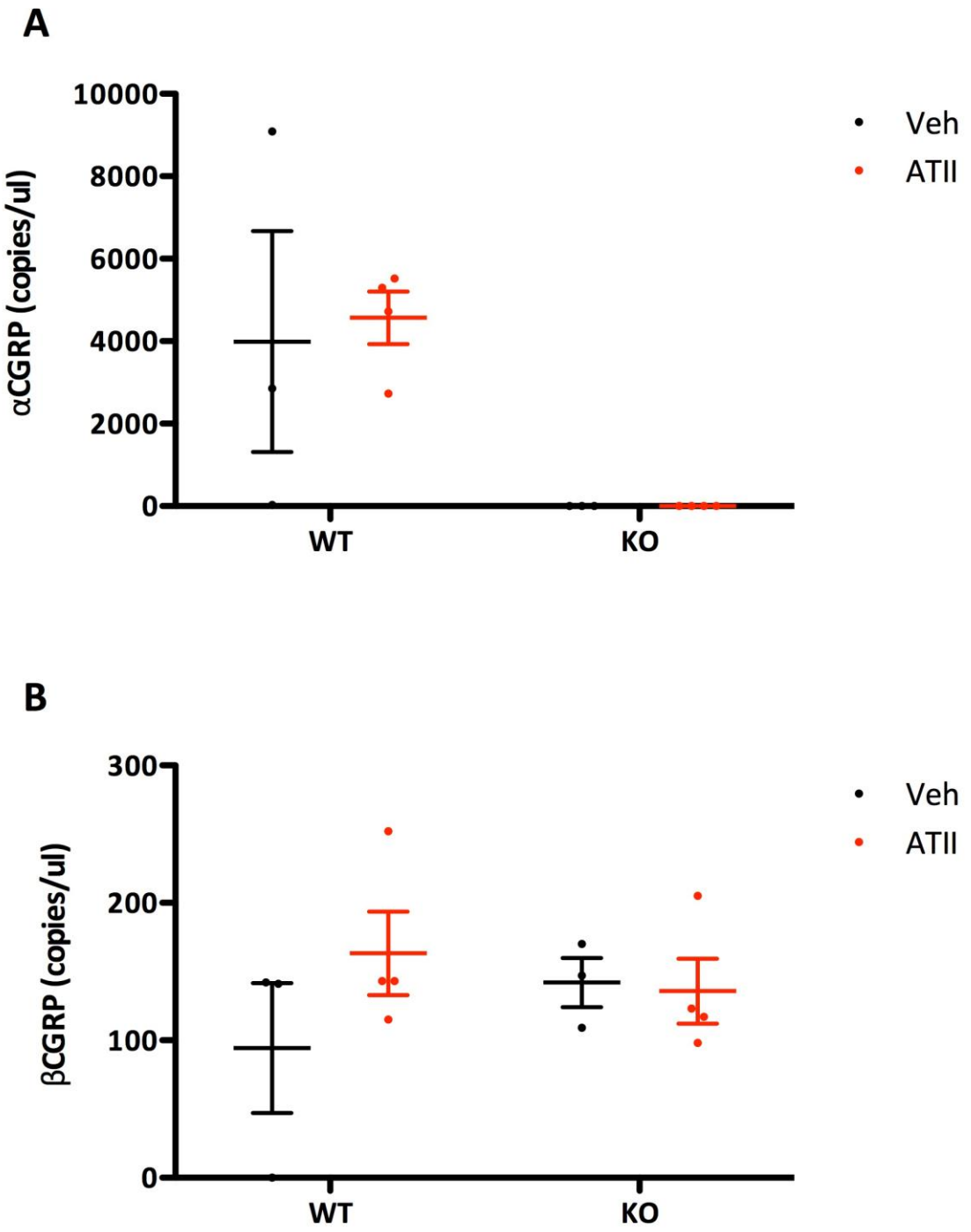

Figure 64: Gene expression analysis of CGRP isoforms of DRG lysates obtained from aged (15 months) male αCGRP WT and KO mice, treated with either vehicle (Veh) or ATII. Results show A) mRNA expression of αCGRP in DRG lysates; B) mRNA expression of βCGRP in DRG lysates. Data expressed as copies mRNA per microliter, mean ± SEM, n=3-4. Statistical significance was evaluated by two-way ANOVA plus Bonferroni *post hoc* test. No significant differences were detected between groups.

6.4.7 Infusion of 1.1 mg/kg/day angiotensin II in juvenile αCGRP WT and KO mice leads to increased blood pressure and cardiac hypertrophy

Due to our failure to generate hypertension following ATII infusion in aged male αCGRP WT and KO mice, we sought to revalidate the model in a small sample of juvenile male mice in order to exclude a lack of effect originating from technical reasons. We implanted juvenile αCGRP WT and KO mice with osmotic minipumps loaded to administer a dose of 1.1 mg/kg/day ATII or vehicle. Figure 65A shows that this was sufficient to induce hypertension in mice, increasing systolic blood pressure by approximately 30 mmHg. Vehicle-treated mice displayed no trend towards a change in blood pressure.

As further validation of the model, we also investigated the degree of cardiac hypertrophy in these mice, as a readout of ATII activity compounded by increased blood pressure (Figure 65B). We found that ATII tended to increase heart mass in WT and KO animals receiving ATII treatment, but not in those receiving vehicle. Thus, we concluded from these data that the model was still fit-for-purpose.

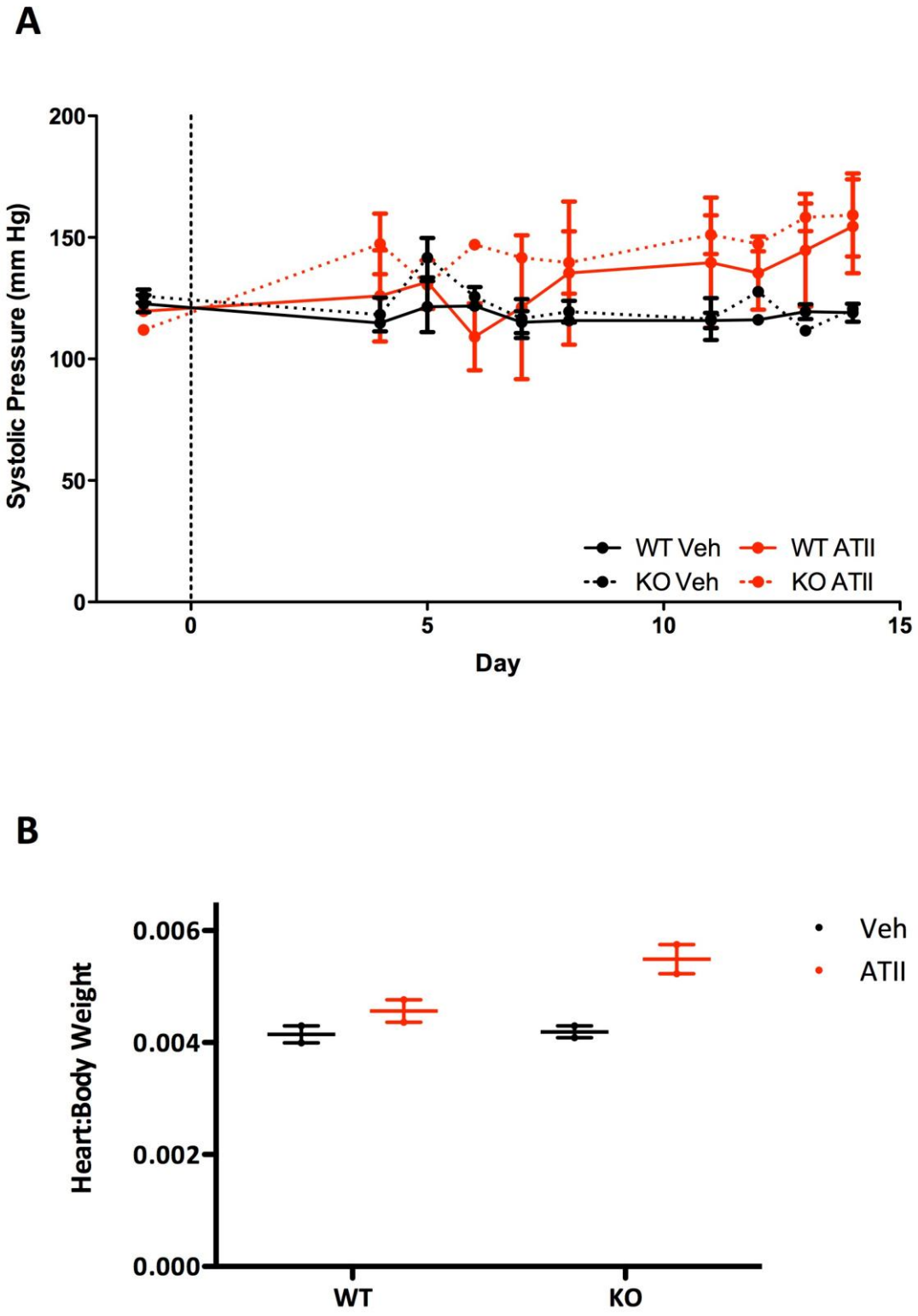

Figure 65: Validation of the ATII model of hypertension in juvenile (3 months) male αCGRP WT and KO mice, treated with either vehicle (Veh) or ATII over 14 days. Results show A) systolic blood pressures of male juvenile αCGRP WT and KO mice following treatment; B) heart to body weight ratios of male juvenile αCGRP WT and KO mice following treatment. Data expressed as A) mm Hg mean ± SEM; B) heart weight to body weight ratio mean ± SEM. n=2.

6.5 Discussion

In Chapter 4, we showed that ageing in mice did not promote an overt maladaptive cardiovascular phenotype, with respect to regulation of blood pressure. Ageing to fifteen months was sufficient to generate a pro-inflammatory vascular milieu, characterised by enhanced cell adhesion molecule expression and increased infiltration of leukocytes into the vascular wall. However, we did not find evidence for a role for CGRP in regulating this process – WT and KO mice appeared similar in most inflammatory parameters at both three and fifteen months of age. We associated this lack of protection with the mild nature of the ageing model, given that more overt stresses to the cardiovascular system can produce evidence for CGRP-mediated vascular protection (Russell et al., 2014). Despite this, in Chapter 5 we uncovered evidence to support an intrinsic anti-inflammatory role for CGRP *in vitro*, where it acted to limit leukocyte-EC interactions in a time-dependent manner *via* its action on the endothelium. With both of these findings in mind, we sought to generate a more severe model of cardiovascular stress in the ageing mouse, in order to precipitate CGRP-mediated protection *in vivo*.

6.5.1 Infusion of 1.1 mg/kg/day ATII in aged αCGRP WT and KO mice does not lead to the generation of systemic hypertension

In order to create such a model of vascular inflammation in ageing mice, we combined two existing models we have established in our group – that of ATII-induced hypertension and ageing itself. We deemed this to be an interesting experimental question that has relevance to the human condition – most humans tend to develop hypertension in middle age. We have already published a study showing that CGRP can act as a vascular protective agent in an ATII model of hypertension (Smillie et al., 2014) and others have shown a similar result in other models (Bowers, 2005; Sabharwal et al., 2010). Thus, we expected that implantation of ATII-loaded osmotic minipumps would generate hypertension that would be compounded by the subclinical inflammation of ageing to create an exacerbated phenotype. We found that infusion of 1.1 mg/kg/day ATII over 14 days did not lead to a significant increase in systemic blood pressure in aged αCGRP WT and KO mice. This was surprising to us, as others have validated this model and shown it to be viable (Csiszar et al., 2013; Toth et al., 2013a; 2013b). Additionally, upregulation of the AT1R has been observed both within the rodent heart (Heymes et al., 1998) and primate vasculature during ageing (Wang et al., 2003), suggesting that susceptibility to ATII-induced hypertension should be greater with age. It is of note that two mice did present with polycystic kidneys (one WT ATII-treated and one KO ATII-treated), which presumably led to substantial drops in blood pressure. These cysts were only observed in ATII-treated animals and anecdotal evidence from our group has shown ATII to induce renal cysts in juvenile animals, in the past. Data from these mice were not excluded, as there appears to be a link between ATII and cyst development, meaning this phenotype is likely a facet of the model rather than biological anomaly (Loghman-Adham, 2004; Kaliappan et al., 2012). We further attempted to understand why aged mice failed to develop hypertension by reproducing some of our published results performed in a small sample of juvenile mice (Bodkin et al., 2014; Smillie et al., 2014) and found that, indeed, they did go on to develop hypertension following ATII infusion. By doing this, we minimised the likelihood that the absence of phenotype observed in aged mice was related to a failure of the experiment. Thus, we were interested in this apparent failure to respond and sought to scrutinize the model further, in order to understand what kind of effect, if any, was happening elsewhere in the mouse, with particular reference to the vasculature.

6.5.2 Evidence of pharmacological activity of ATII in aged αCGRP WT and KO mice by measurement of cardiac hypertrophy and water consumption

It is well established that ATII can act in a dipsogenic manner to increase thirst and drinking behavior (McKinley and Johnson, 2004), acting to eventually increase blood volume and raise blood pressure as a result. Thus, we deemed that the monitoring of fluid consumption in aged αCGRP WT and KO mice receiving ATII infusion would be an alternative measure of ATII having a pharmacological effect in these animals. Indeed, we found that saline-treated animals all consumed similar volumes of water, regardless of genotype, but animals receiving ATII infusion significantly increased their water intake over vehicle-treated animals. This indicated that whilst animals were not becoming hypertensive, ATII was still producing a physiological response. In addition to this dipsogenic response, we also found evidence to suggest that animals receiving ATII treatment tended to have an increased heart mass, although this failed to reach statistical significance. Analysis of responses from each individual animal revealed that we had populations of responders versus non-responders. Indeed, it also appeared that CGRP KO mice had a greater population of responders to ATII with respect to increases in blood pressure (4/6 mice) versus WT mice (2/6 responders). Therefore, we investigated whether the animals that responded by increasing blood pressure also responded in a positive manner with respect to other markers of ATII activity, namely, water intake and cardiac hypertrophy. In summary, we found that there were no clear correlations between increases in systolic blood pressure and water intake/increased heart mass, indicating that the observed effects were truly independent of blood pressure and mediated by the specific activity of ATII.

From these data combined, we concluded that ATII was inducing a pressure-independent cardiovascular response. Subpressor doses of ATII have been shown to influence vascular inflammation and dysfunction in the absence of overt changes in blood pressure (Reckelhoff et al., 2000; Bataller et al., 2003) and thus we thought it possible that we would also observe a degree of inflammation in our mice, where the deletion of αCGRP might result in a worsened phenotype.

6.5.3 Infusion of 1.1 mg/kg/day ATII into aged αCGRP WT and KO mice does not lead to the generation of vascular inflammation

Having observed clear changes in the vascular inflammatory profile of ageing aortae in Chapter 4, we investigated changes in pro-inflammatory gene expression within the same vessel in this model. We first investigated changes in eNOS mRNA expression, which may have provided an indication of endothelial dysfunction in mice. We did not see any significant differences or, indeed, clear trends in changes in mRNA expression of this enzyme across experimental groups. This was surprising, as ATII is a long-established inducer of early endothelial dysfunction (Schulz et al., 2011). Indeed, some have shown that endothelial dysfunction induced by ATII in mice can be driven by inflammatory monocytes, which induce expression of iNOS, generating nitro-oxidative stress sufficient to uncouple endothelial NOS (Kossmann et al., 2014). Of course, results shown in Chapter 4 showed that eNOS mRNA expression remained stable with progression in age from three to fifteen months, but the level of eNOS activation did differ. Thus, it would be prudent to look at eNOS protein expression and activity (based on phosphorylation status) within this model in order to draw better conclusions with respect to endothelial dysfunction. In conjunction with our eNOS mRNA finding, we witnessed no distinct, statistically significant pro-inflammatory signal following ATII treatment in either αCGRP WT or KO mice. However, we did witness some interesting trends that may point to some effect. For instance, WT mice receiving ATII trended towards having an elevated HO-1 mRNA expression, which may be indicative of a pro-oxidant environment preceding classical vascular inflammation. A similar result in this group was identified with respect to a non-significant elevation in aortic VCAM-1 expression. However, we found no increase in pro-oxidant NOX enzyme expression as we did in Chapter 4, which would be characteristic of ATII infusion (Nguyen Dinh Cat et al., 2013). However, it is worth noting that this pilot study had a relatively low statistical power and increasing sample sizes in future experiments may reveal clearer effects.

6.5.4 Infusion of 1.1 mg/kg/day ATII into aged αCGRP WT and KO mice does not drastically alter aspects of the CGRP signalling axis but may affect vascular RAMP1 expression

We also interrogated aspects of the CGRP signalling system, by measurement of isoform expression within the DRG. There is known to be a link between ATII signalling and perivascular nerve function, extending to alterations in CGRP-mediated vascular responses. We have previously shown that chronic ATII administration to mice over either a 14- or 28-day period can increase bioavailable CGRP, possibly in an attempt to restore cardiovascular homeostasis in the face of a strong vasopressor stimulus (Smillie et al., 2014). Others have shown that ATII can inhibit CGRP release from perivascular nerves and subsequently reduce its bioactivity (Kawasaki et al., 1998; 2000). Further to this, it is thought that ATII can directly remodel the sensory neuron to promote sensory nerve dysfunction and even denervation in the SHR; effects that are blocked by AT1R blockade and ameliorated by AT2R activation (Kawasaki, 2002; Kawasaki et al., 2003; Hobara et al., 2005). We found no strong evidence in this study for dysregulation of expression of CGRP isoforms at the mRNA level within DRG lysates. However, we did notice a trend towards an increase in βCGRP expression in the DRG of WT animals receiving ATII treatment, which may be indicative of an early response to cardiovascular stress. It is interesting that we see this trend with respect to βCGRP expression and not αCGRP. However, we have shown changes in βCGRP expression following the induction of hypertension (Smillie et al., 2014) and thus this isoform may represent an underappreciated area of CGRP research, particularly when the αCGRP KO mouse is being used.

We further investigated CGRP signalling mechanisms by analysing the expression of CGRP receptor genes within the aortae of αCGRP WT and KO mice receiving ATII. We saw no change in the expression of CLR or RAMP2, indicating that AM receptor signalling expression remained largely unaffected within this model. However, we have shown a significant increase in RAMP1 mRNA expression *only* in KO mice receiving ATII treatment. RAMP1 levels were significantly raised in comparison to ATII-treated WTs and vehicle-treated KOs. This is an intriguing result, which may strengthen our long-term hypothesis of CGRP activity. This upregulation of RAMP1 may point to an increased sensitivity and susceptibility to a hypertensive (or, indeed, sub-hypertensive) stimulus in KO mice versus WT. Whilst we have shown that juvenile CGRP KO mice have a worsened cardiovascular phenotype in response to high-dose ATII infusion (Figure [X])(Smillie et al., 2014), in this chapter we provide evidence to suggest that a subpressor dose of ATII is still significant enough of a stimulus to provoke changes in the CGRP KO mouse. One way to think of how CGRP is acting might be to employ the analogy of an early braking system. In WT animals, where the 'brake' is intact, it is harder to generate pathology as a result of ATII infusion as CGRP is actively working against this development. However, when the brake is removed in the KO animal, the manifestation

of pathology is easier to see. Thus, RAMP1 may be increasing its expression in ATII-treated KO mice in order to amplify signal transduction through the CGRP receptor in order to counteract early perturbations in cardiovascular function. Changes in RAMP expression following the induction of hypertension have not so far been observed, although experimental manipulation of these proteins has yielded a level of cardiovascular protection, as previously discussed(Sabharwal et al., 2010; Zhang and Russo, 2010). Others have shown changes in RCP expression within the vasculature in order to mediate improved CGRP-dependent vascular responses in a model of subtotal nephrectomy salt-induced hypertension (Supowit et al., 2011).

6.5.5 Conclusion

In summary, in this chapter we attempted to establish a model of vascular inflammation with the knowledge that ageing alone does induce inflammation but the role for CGRP within this setting is negligible (Chapter 4), coupled with evidence from Chapter 5 which suggests CGRP does indeed possess intrinsic anti-inflammatory properties. Here we have shown that infusion of 1.1 mg/kg/day ATII into aged αCGRP WT and KO mice did not lead to overt hypertension, as we have previously published in juvenile animals (Smillie et al., 2014) and shown in a small pilot study within this chapter. The reason for this is unclear but we have provided evidence for small groups of responders versus non-responders when analysing data from individual animals. Furthermore, some data from this chapter may suggest that whilst overt hypertension was not generated, aged CGRP KO mice may still be more sensitive to ATII infusion, as evidenced by a greater proportion of hypertensive responders within this subgroup. Furthermore, evidence of an increased propensity of these mice to increase aortic expression of RAMP1 mRNA may point to an early damage-limitation signal in response to ATII infusion.

7. | Chapter Seven: General Discussion

7.1 Overview

Ageing in humans is a complex phenotype that is governed by many biological processes that contribute to various extents in driving the phenomenon and, certainly, not all processes will contribute in each case. Furthermore, many environmental and lifestyle factors can interact with these endogenous biological processes to exacerbate the phenotype. Despite this apparent complexity, there is a great need to investigate the basic biology of ageing, owing to the increasing ageing population demographic in most areas of the Western world, including the UK. As ageing represents the single biggest independent risk factor for the development of CVD, it is evident that we are facing an emerging ageing epidemic that is likely to represent a substantial burden to the economy and public health services(Franklin, 1999). Thus, it is important that we turn to experimental models of ageing, both *in vivo* and *in vitro*, in order to better understand the aetiology of ageing such that we can develop novel therapeutics to improve healthspan. As discussed, ageing is often comorbid with other complications (such as hypertension) and thus it is also prudent to study the interactions between the ageing body and associated diseases.

My central hypothesis was that deletion of αCGRP in mice would result in an exacerbated vascular inflammatory phenotype induced by ageing to fifteen months, driven in part by leukocytic processes. We proposed that this inflammation would impinge upon haemodynamics to generate hypertension. Using the evidence presented in this thesis, I am now able to address the previously stated aims and comment on how this evidence fits with my hypothesis.

7.2 Major and novel findings of this thesis

The data from this thesis has built upon existing knowledge suggesting a protective role for CGRP within the cardiovascular system. However, until this thesis, no studies had been performed investigating how CGRP affects the ageing process, especially with respect to vascular inflammatory processes. Thus, with this we have contributed entirely novel findings to this field. Further to this, we have expanded knowledge on how CGRP acts to attenuate leukocytic vasculitis at the level of the leukocyte adhesion cascade. The salient findings of this thesis are as follows:

7.2.1 Ageing to fifteen months in mice does not induce an adverse cardiovascular phenotype *in vivo* and genetic deletion of αCGRP has no effect on haemodynamic or murine growth parameters.

Our initial hypothesis was that ageing to fifteen months in mice would be sufficient to precipitate a maladaptive cardiovascular phenotype, specifically leading to a mild, pre-hypertensive state. Data obtained during the on-going Framingham Heart Studyshows quite clearly that systolic blood pressure rises linearly with age, between the years of 30 to 84 and over. Changes in diastolic blood pressureoccurring with age is more complex. Data seem to suggest that diastolic pressure increases until around the age of 50, but then decreases as age increases. This generates a steep rise in pulse pressure with ageing, which is potentially dangerous (Franklin, 1999). In our studies, we used αCGRP WT and KO mice bred onto a C57BL/6J background at fifteen months of age. C57BL/6J mice have been demonstrated to live for 815 ± 16 days in laboratory conditions (Jackson Laboratories, 2008). Thus, from this, we deemed our mice to be “middle-aged” – a time at which humans would typically be expected to manifest some form of CVD, such as hypertension.

We did not observe evidence of overt cardiovascular abnormalities in these mice, with respect to haemodynamic parameters and deletion of αCGRP gene had no impact on these outcomes. Certainly, these findings call into question the validity of using mice as models of human ageing if they are unable to spontaneously develop cardiovascular (or other) disease. However, I have discussed in Chapter 4 that mice generally only succumb to diseases related to cancer in their old age and the absence of other comorbidities is presumably related to the interaction between the mouse genome and its environment. It is known in humans that improvements in lifestyle (such as cessation of smoking, taking exercise and dietary improvements) can significantly improve cardiovascular outcome by lowering risk for hypertension, atherothrombotic disease and ultimately heart attack and stroke(Santos-Parker et al., 2014; Siervo et al., 2014). It is therefore likely that mice do not fall foul to CVD in old age presumably because they do not smoke, drink orhave poor diet.

Indeed, CVD can be precipitated in mice through dietary interventions, such as feeding of a high-fat diet (Marshall et al., 2013), hypercholesterolaemic diet (Xiangdong et al., 2011) or providing a hypernatraemic drinking water (Van Vliet and Montani, 2008). Thus, the interaction of the organism's genome with its environment is key in producing disease and this feature is shared between both mice and humans (and, indeed, other mammals commonly-used in the laboratory). Indeed, I conjecture that were it ethical to maintain humans under standard laboratory conditions, the prevalence of CVD would be low.

Despite finding little evidence to suggest overt signs of CVD were present in ageing mice, we were optimistic regarding the inherent stress of the ageing process in provoking cardiovascular defects in the αCGRP KO mouse. We have previously shown that in a short- and long-term ATII model of hypertension, deletion of αCGRP leads to an exacerbated hypertensive phenotype and augmented cardiovascular stress associated with the disease, despite WT and KO mice presenting normally under basal conditions (Smillie et al., 2014). This would indicate that αCGRP forms part of a stimulus-activated network that works against acute stresses to restore homeostasis. Others using αCGRP KO mice (i.e. those with αCGRP deleted and calcitonin expression left intact) in other severe models of CVD have also shown similar results (for example, in wire-induced vascular injury and hindlimb ischaemia models)(Mishima et al., 2011; Yang et al., 2013). I have argued that the lack of phenotype in KO mice in the face of age-induced stress is probably related to the subclinical and chronic nature of age-associated pathologies. These pathologies are slow in onset, slow to progress and do not represent a serious threat to the organism at any particular point in time – there is no great upset of homeostasis to provoke biological systems acting to 'regress towards the mean', quite unlike the ATII model. Given the relative biological expense of synthesising, maintaining and releasing a peptide to counteract a subtle 'disease' such as ageing, it is perhaps not surprising that deletion of CGRP precipitates no clear phenotype – its action is simply not required in the face of an apparent 'low threat'. A further confounding factor we uncovered in this study was developmental genetic compensation in our αCGRP KO mice. We showed that circulating plasma levels of the peptide remained consistent in both genotypes throughout the ageing process. Furthermore, we also showed that βCGRP transcript was increased in juvenile KO mice in comparison with WT animals and transcript of both isoforms appeared to be elevated with age. Thus, it is entirely possible that any potential phenotypes generated by ageing are masked by this compensation. Both isoforms of CGRP have high sequence homology, similar binding affinities for CGRP receptor and almost identical biological activity (Russell et al., 2014). Therefore, βCGRP might be compensating for a loss of αCGRP activity and an increase in transcript with age, if biologically active, could potentiate any level of cardiovascular protection stemming from these peptides. It would be interesting to see how the

ageing phenotype is altered in a double αCGRP/βCGRP KO mouse, though to my knowledge this mouse is currently unavailable. Further, the generation of a double KO by traditional cross-breeding methods would presumably be difficult, owing to the close proximity of both CGRP genes on chromosome 7 in the mouse. With these theories and issues in mind and also thinking of future work, I would argue that the role of αCGRP in the ageing process *in absence of other comorbidities* is likely negligible and the opportunity of extending ageing studies to a later timepoint in mice likely represents a risky research avenue that is perhaps best left untouched.

7.2.2 Ageing in mice induces changes in vascular reactivity to known pharmacological mediators of vasoconstriction and vasodilatation

Despite a lack of an obvious maladaptive cardiovascular phenotype in ageing αCGRP WT and KO mice, we did uncover interesting evidence supporting an altered vascular reactivity phenotype in these animals. We have shown that aged mice display a differential contractile response in mesenteric arteries, depending on the agonist used. Mice aged fifteen months displayed a significant reduction in sensitivity to the α-adrenoceptor agonist, PE, whilst the thromboxane receptor agonist U46619 displayed increased potency in this experimental group. Interestingly, we also witnessed diminished vasodilator bioactivity to exogenous CGRP, which may point to disturbances in CGRP receptor signalling. This phenotype has been shown once before in ageing rats (Chan and Fiscus, 2003), although no clear mechanism for this response was identified in this study. Despite these changes in vascular reactivity in this isolated preparation, we witnessed no change in *in vivo* haemodynamic parameters. Whilst initially this may seem surprising, these results are perhaps not so remarkable. We have studied only three physiological regulators of vascular tone and therefore are only examining a snapshot of what might be happening at this stage of the ageing process. Doubtless, other compensatory mechanisms will exist to allow the ageing organism to re-establish homeostasis. Changes in isolated pharmacological preparations are certainly academically interesting but might not be reflective of a relevant *in vivo* endpoint, such as changes in blood pressure.

7.2.3 αCGRP gene deletion may worsen early endothelial dysfunction in this model by increasing pro-oxidant damage (characterised by increased 4-HNE expression) and reducing eNOS activity by altering its phosphorylation status, leading to vascular inflammation.

Despite a lack of a maladaptive cardiovascular phenotype in αCGRP WT and KO mice produced by ageing, we did collate some interesting preliminary evidence to support a degree of endothelial dysfunction in our mice. Endothelial dysfunction is typically defined as a reduction in NO bioavailability. This phenomenon very often presents in ageing humans and certainly in those who have other cardiovascular comorbidities, such as hypertension or atherosclerosis (Virdis et al., 2010). Multiple biological mechanisms can converge to produce endothelial dysfunction and many of these are associated with pro-oxidant processes. In Chapter 4, we show evidence for elevated pro-oxidant enzyme expression and the accumulation of oxidised macromolecules (specifically, the accumulation of peroxided lipid moieties). This is characteristic of the ageing process and might act as a surrogate marker of endothelial dysfunction. We also showed evidence for an increased level of eNOS activity in aged WT mice, as measured by ser1177 phosphorylation. This may be interpreted as the endothelium trying to boost NO signalling in face of oxidative challenges. It was interesting to observe that whilst WT mice had hyperphosphorylated eNOS, aged KO mice were not able to maintain this level of hyperphosphorylation (which was high in juvenility). Endothelial dysfunction, as characterised by impaired endothelial-dependent vasodilatation exists both in aged laboratory animals and in humans free from other known comorbidities thought to contribute towards this phenotype, suggesting a primary role for ageing alone in driving this response (Küng and Lüscher, 1995; Taddei et al., 1995; Gerhard et al., 1996; Lesniewski et al., 2009). The primary mechanism by which endothelial dysfunction manifests is through the induction of oxidative stress. However, other mechanisms may contribute, such as the enhanced production of vasoconstrictor autacoids with age. This is quite common with respect to the shift in the balance of vasodilator prostanoids in juvenility to more vasoconstrictor eicosanoids with age (Tang and Vanhoutte, 2008; Seals et al., 2011). Indeed, as previously discussed, in Chapter 3 we witnessed an enhanced sensitivity of isolated mesenteric arteries from aged mice to the thromboxane receptor agonist, U46619, which may be an additional marker of endothelial dysfunction. We observed an induction in pro-oxidant enzyme gene expression and also an induction in anti-oxidant enzyme gene expression, potentially induced as a response to elevated oxidative stress. This was accompanied by elevated peroxidised lipid bioavailability in aged mouse aorta, which has been shown to be a hallmark of vascular ageing by other laboratories (Sin et al., 2014). Surprisingly, we did not find evidence for increases in nitrosylated tyrosine residues, which is consistently seen with ageing (Hemmeryckx et al., 2013). However, this may be a reflection of a reduction in NO generation, meaning less NO is available to

react with superoxide to form peroxynitrite, the molecule responsible for the nitration of macromolecules. In order to gain a more concrete understanding of what is happening at the level of the endothelium in juvenile and aged αCGRP WT and KO mice, it would be essential to investigate endothelial-dependent NO production directly, as our markers may only be considered surrogates at this stage. Experiments may take advantage of isolating aortic ECs from juvenile and aged αCGRP WT and KO mice and measuring NO liberation from these cells following exposure to a pharmacological agonist, such as acetylcholine.

In conjunction with evidence for endothelial dysfunction occurring with ageing, that perhaps manifested more clearly in αCGRP KO mice, we also observed the appearance of vascular inflammatory markers within the aortae of aged mice. This, to my mind, validates the use of mice to study the fundamental processes of ageing – both mice and humans appear to share features of vascular inflammation as part of this biological process. We witnessed significant elevations in the mRNA expression of pro-inflammatory vascular markers, including VCAM-1 and F4/80, a marker of macrophage infiltration. Increases in expression of these types of molecules associated with leukocyte trafficking have been shown in the mouse aorta before (Hemmeryckx et al., 2013). Indeed, elevations in cell adhesion molecule expression and infiltration of inflammatory cells into the vascular wall are strongly associated with the ageing process, especially in patients who have early atherosclerotic lesion development subsequent to early endothelial dysfunction (Woollard and Geissmann, 2010).

7.2.4 Co-treatment of TNFα-activated endothelial cells with CGRP results in the attenuation of leukocyte adhesion through an unknown mechanism that appears to be independent of regulation of gene transcription.

In Chapter Five we showed some evidence to support an anti-inflammatory role for CGRP, owing to its ability to reduce THP-1 monocyte recruitment to inflamed endothelium under physiological flow conditions *in vitro*, by virtue of its activity on the EC itself. Furthermore, treatment of endothelium for up to six hours with CGRP in the absence of TNFα did not induce leukocyte recruitment. These findings combined suggest that CGRP possesses a neutral property at the level of the endothelium.Our finding is consistent with a recent study by Huang et al. who showed that CGRP could inhibit mononuclear cell transmigration by inhibiting EC NF-κB activity (Huang et al., 2011). The phenotype we observed appeared to be independent in changes of whole cell expression of the cell adhesion molecules VCAM-1, PECAM-1, JAM-A and JAM-C. Further to this, microarray experiments showed that CGRP did not alter the pro-inflammatory endothelial transcriptome induced by TNFα. This was surprising to us, given that CGRP is known to induce transcriptional activity in some cell types (Walker et al., 2010a).

With respect to understanding the mechanisms regulating CGRP-mediated attenuations in leukocyte adhesion, there is work yet to be done. Initial experiments should be classically pharmacological in nature, and attempt to understand the relationship between concentration of CGRP and its ability to alter the leukocyte adhesion response. Thus, one would be able to determine the minimal concentration required to produce attenuations in adhesion and at which point this process becomes saturated. CGRP receptor experts may argue that the concentration of CGRP that we have used in this thesis is high with respect to affinity for other receptors for other peptides of the CGRP family (i.e. the AM_1 receptor) and therefore lower concentrations of peptide that still give a response may tell us something about the nature of the mechanism involved. Indeed, to further expand on this, experiments using selective antagonists for the CGRP receptor (such as BIBN4096BS) would definitively uncover whether or not the observed phenomenon is related to the CGRP receptor, or others. Finally, whilst it is generally accepted that CGRP can induce cAMP synthesis in ECs and many of the cellular mechanisms induced by CGRP are related to the cAMP-PKA axis, other signalling pathways may also be involved. I conjecture that CGRP may be increasing NO bioavailability and liberation from the EC and this may, in part, be driving the anti-inflammatory response. Thus, it would be exciting to investigate the relative contribution of this pathway to the phenotype, perhaps by making use of pharmacological NOS inhibitors and seeing if this alters the response in any way. Results from Chapter Four add credence to this hypothesis, where we showed

that eNOS activity was drastically altered in αCGRP WT and KO mice from juvenility through to middle-age.

With regards to unraveling molecular mechanisms, we have already shown that CGRP does not appear to influence gene transcription, despite taking six hours to achieve full effect. Thus, I believe that future experiments here should be carefully examining temporospatial protein dynamics within the EC. For instance, experiments should investigate the expression of cell adhesion molecules within the plasma membrane over time; perhaps by biotinylating membrane-bound proteins under control conditions and seeing if the expression profile of proteins within the membrane changes within this period. If any key proteins are uncovered, it may be useful to employ microscopy techniques to image the trafficking of these proteins to understand their fate. Indeed, it is known that some endothelial adhesion molecules are constantly being recycled from the plasma membrane, through endosomal compartments and back into the membrane again (Mamdouh et al., 2008), which may contribute towards part of the phenotype that we are observing.

7.2.5 Infusion of 1.1 mg/kg/day ATII into fifteen month-old αCGRP WT and KO mice did not result in the generation of a hypertensive phenotype, despite evidence of pharmacological activity by ATII.

Throughout our earlier *in vivo* studies, we appreciated that ageing in mice was sufficient to induce a degree of subclinical vascular inflammation that was largely unaffected by αCGRP gene deletion. This was valuable information that helped us understand some of the key biological processes underpinning vascular ageing. We then hypothesised that the addition of a more acute and inflammatory stress into aged mice might push the cardiovascular system further in order to precipitate an exacerbated phenotype that was regulated by αCGRP. Thus, we combined our ageing model with our existing ATII model of murine hypertension. We thought that this model would be more representative of the human condition, owing to the fact that most humans living in developed nations become hypertensive in middle-age(Franklin, 1999). We were surprised to learn, however, that aged mice did not develop hypertension following the infusion of 1.1mg/kg/day ATII. We went on to validate that ATII was being infused into these animals by analysing other known markers of ATII activity, specifically water consumption and cardiac hypertrophy. Following these analyses, we concluded that ATII was having a level of pharmacological activity in the absence of increasing blood pressure. Thus, we may have inadvertently generated a 'subpressor' model of cardiovascular stress, though our limited early studies did not see much evidence for aberrant vascular inflammation in these mice. Interestingly, studies from other laboratories have shown that aged mice develop a worsened hypertensive phenotype, in comparison to younger animals (Csiszar et al., 2013; Toth et al., 2013a; 2013b). With these and our results in mind, coupled with the relative dearth of publications associated with this new model, I would suggest that a repeat of this study with an increased sample size would be justifiable in order to evaluate the reproducibility of the model. A repetition of this model would be further justified by its increased relevance to human disease.

7.3 Summary & Conclusions

In summary, the results of this thesis are novel in that they for the first time show that CGRP is not protective of the cardiovascular system in a mouse model of ageing. However, CGRP may possess intrinsic anti-inflammatory properties when acting on the endothelium, which may work to limit vascular inflammation induced by aberrant leukocyte activity. Further work is required to elucidate the mechanisms by which CGRP might be able to afford this level of protection and, indeed, it will be important to see whether this protection can be translated back into *in vivo* models of CVD. However, it is hoped that the results presented in this thesis have advanced our knowledge of CGRP within the setting of age-induced vascular inflammation and in processes related to leukocyte recruitment.

8. | Chapter Eight: References

Adler, A.S., Sinha, S., Kawahara, T.L.A., Zhang, J.Y., Segal, E., and Chang, H.Y. (2007). Motif module map reveals enforcement of aging by continual NF- B activity. Genes & Development *21*: 3244–3257.

Aiyar, N., Rand, K., Elshourbagy, N.A., Zeng, Z., Adamou, J.E., Bergsma, D.J., et al. (1996). A cDNA encoding the calcitonin gene-related peptide type 1 receptor. J. Biol. Chem. *271*: 11325–11329.

Alexander, S.P.H., Mathie, A., and Peters, J.A. (2011). Guide to Receptors and Channels (GRAC), 5th edition. Br J Pharmacol *164 Suppl 1*: S1–324.

Alley, D.E., Crimmins, E., Bandeen-Roche, K., Guralnik, J., and Ferrucci, L. (2007). Three-year change in inflammatory markers in elderly people and mortality: the Invecchiare in Chianti study. J Am Geriatr Soc *55*: 1801–1807.

Alom-Ruiz, S.P., Anilkumar, N., and Shah, A.M. (2008). Reactive Oxygen Species and Endothelial Activation. Antioxid. Redox Signal. *10*: 1089–1100.

Amara, S.G., Arriza, J.L., Leff, S.E., Swanson, L.W., Evans, R.M., and Rosenfeld, M.G. (1985). Expression in brain of a messenger RNA encoding a novel neuropeptide homologous to calcitonin gene-related peptide. Science *229*: 1094–1097.

Amara, S.G., Evans, R.M., and Rosenfeld, M.G. (1983). Stimulation of noradrenergic sympathetic outflow by calcitonin gene-related peptide. Nature *305*: 534–536.

Amara, S.G., Rosenfeld, M.G., Ong, E.S., and Evans, R.M. (1982). Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. Nature *298*: 240–244.

Amerini, S., Mantelli, L., Filippi, S., and Ledda, F. (1994). Effects of aging and hypertension on vasorelaxant activity of calcitonin gene-related peptide: a comparison with other vasodilator agents. J. Cardiovasc. Pharmacol. *23*: 432–437.

Ando, K., Pegram, B.L., and Frohlich, E.D. (1990). Hemodynamic effects of calcitonin gene-related peptide in spontaneously hypertensive rats. Am. J. Physiol. *258*: R425–9.

Arulmani, U., Schuijt, M.P., Heiligers, J.P.C., Willems, E.W., Villalón, C.M., and Saxena, P.R. (2004). Effects of the calcitonin gene-related peptide (CGRP) receptor antagonist BIBN4096BS on alpha-CGRP-induced regional haemodynamic changes in anaesthetised rats. Basic Clin. Pharmacol. Toxicol. *94*: 291–297.

Assar, El, M., Angulo, J., and Rodríguez-Mañas, L. (2013). Oxidative stress and vascular inflammation in aging. Free Radic. Biol. Med. *65*: 380–401.

Assas, B.M. (2014). Calcitonin gene-related peptide is a key neurotransmitter in the neuro-immune axis. 1–9.

Bachschmid, M.M., Schildknecht, S., Matsui, R., Zee, R., Haeussler, D., A Cohen, R., et al. (2012). Vascular aging: Chronic oxidative stress and impairment of redox signaling-consequences for vascular homeostasis and disease. Ann Med.

Baird, L., and Dinkova-Kostova, A.T. (2011). The cytoprotective role of the Keap1-Nrf2 pathway.

Arch. Toxicol. *85*: 241–272.

Baker, D.J., Wijshake, T., Tchkonia, T., LeBrasseur, N.K., Childs, B.G., van de Sluis, B., et al. (2011). Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. Nature *479*: 232–236.

Balkan, W., Oates, E.L., Howard, G.A., and Roos, B.A. (1999). Testes exhibit elevated expression of calcitonin gene-related peptide receptor component protein. Endocrinology *140*: 1459–1469.

Barton, M., Cosentino, F., Brandes, R.P., Moreau, P., Shaw, S., and Lüscher, T.F. (1997). Anatomic heterogeneity of vascular aging: role of nitric oxide and endothelin. Hypertension *30*: 817–824.

Barwell, J., Miller, P.S., Donnelly, D., and Poyner, D.R. (2010). Mapping interaction sites within the N-terminus of the calcitonin gene-related peptide receptor; the role of residues 23-60 of the calcitonin receptor-like receptor. Peptides *31*: 170–176.

Bataller, R., Gäbele, E., Schoonhoven, R., Morris, T., Lehnert, M., Yang, L., et al. (2003). Prolonged infusion of angiotensin II into normal rats induces stellate cell activation and proinflammatory events in liver. Am. J. Physiol. Gastrointest. Liver Physiol. *285*: G642–51.

Bekaert, S., De Meyer, T., Rietzschel, E.R., De Buyzere, M.L., De Bacquer, D., Langlois, M., et al. (2007). Telomere length and cardiovascular risk factors in a middle-aged population free of overt cardiovascular disease. Aging Cell *6*: 639–647.

Bell, D., and McDermott, B.J. (1996). Calcitonin gene-related peptide in the cardiovascular system: characterization of receptor populations and their (patho)physiological significance. Pharmacol. Rev. *48*: 253–288.

Benetos, A., Gardner, J.P., Zureik, M., Labat, C., Xiaobin, L., Adamopoulos, C., et al. (2004). Short telomeres are associated with increased carotid atherosclerosis in hypertensive subjects. Hypertension *43*: 182–185.

Benetos, A., Okuda, K., Lajemi, M., Kimura, M., Thomas, F., Skurnick, J., et al. (2001). Telomere length as an indicator of biological aging: the gender effect and relation with pulse pressure and pulse wave velocity. Hypertension *37*: 381–385.

Bierhaus, A., Schiekofer, S., Schwaninger, M., Andrassy, M., Humpert, P.M., Chen, J., et al. (2001). Diabetes-associated sustained activation of the transcription factor nuclear factor-kappaB. Diabetes *50*: 2792–2808.

Bigal, M.E., Escandon, R., Bronson, M., Walter, S., Sudworth, M., Huggins, J.P., et al. (2013). Safety and tolerability of LBR-101, a humanized monoclonal antibody that blocks the binding of CGRP to its receptor: Results of the Phase 1 program. Cephalalgia *34*: 483–492.

Bivalacqua, T.J., Hyman, A.L., Kadowitz, P.J., Paolocci, N., Kass, D.A., and Champion, H.C. (2002). Role of calcitonin gene-related peptide (CGRP) in chronic hypoxia-induced pulmonary hypertension in the mouse. Influence of gene transfer in vivo. Regul. Pept. *108*: 129–133.

Blagosklonny, M.V., Campisi, J., and Sinclair, D.A. (2009). Aging: past, present and future. Aging (Albany NY) *1*: 1–5.

Bodkin, J.V., and Brain, S.D. (2011). Transient receptor potential ankyrin 1: emerging pharmacology and indications for cardiovascular biology. Acta Physiol (Oxf) *203*: 87–98.

Bodkin, J.V., Thakore, P., Aubdool, A.A., Liang, L., Fernandes, E.S., Nandi, M., et al. (2014). Investigating the potential role of TRPA1 in locomotion and cardiovascular control during hypertension. Pharmacol Res Perspect *2*: n/a–n/a.

Bowen, E.J., Schmidt, T.W., Firm, C.S., Russo, A.F., and Durham, P.L. (2006). Tumor necrosis factor-alpha stimulation of calcitonin gene-related peptide expression and secretion from rat trigeminal ganglion neurons. J. Neurochem. *96*: 65–77.

Bowers, M.C. (2005). Role of Calcitonin Gene-Related Peptide in Hypertension-Induced Renal Damage. Hypertension *46*: 51–57.

Bracci-Laudiero, L., Aloe, L., Caroleo, M.C., Buanne, P., Costa, N., Starace, G., et al. (2005). Endogenous NGF regulates CGRP expression in human monocytes, and affects HLA-DR and CD86 expression and IL-10 production. Blood *106*: 3507–3514.

Brain, S.D., and Williams, T.J. (1985). Inflammatory oedema induced by synergism between calcitonin gene-related peptide (CGRP) and mediators of increased vascular permeability. Br J Pharmacol *86*: 855–860.

Brain, S.D., and Williams, T.J. (1989). Interactions between the tachykinins and calcitonin gene-related peptide lead to the modulation of oedema formation and blood flow in rat skin. Br J Pharmacol *97*: 77–82.

Brain, S.D., Tippins, J.R., Morris, H.R., MacIntyre, I., and Williams, T.J. (1986). Potent vasodilator activity of calcitonin gene-related peptide in human skin. J. Invest. Dermatol. *87*: 533–536.

Brain, S.D., Williams, T.J., Tippins, J.R., Morris, H.R., and MacIntyre, I. (1985). Calcitonin gene-related peptide is a potent vasodilator. Nature *313*: 54–56.

Bruunsgaard, H., Andersen-Ranberg, K., Jeune, B., Pedersen, A.N., Skinhøj, P., and Pedersen, B.K. (1999). A high plasma concentration of TNF-alpha is associated with dementia in centenarians. J. Gerontol. a Biol. Sci. Med. Sci. *54*: M357–64.

Bucala, R., Tracey, K.J., and Cerami, A. (1991). Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. J. Clin. Invest. *87*: 432–438.

Burnstock, G. (2009). Autonomic Neurotransmission: 60 Years Since Sir Henry Dale. Annu. Rev. Pharmacol. Toxicol. *49*: 1–30.

Bühlmann, N., Leuthäuser, K., Muff, R., Fischer, J.A., and Born, W. (1999). A receptor activity modifying protein (RAMP)2-dependent adrenomedullin receptor is a calcitonin gene-related peptide receptor when coexpressed with human RAMP1. Endocrinology *140*: 2883–2890.

Cai, W.Q., Dikranian, K., Bodin, P., Turmaine, M., and Burnstock, G. (1993). Colocalization of vasoactive substances in the endothelial cells of human umbilical vessels. Cell Tissue Res. *274*: 533–538.

Carretero, O.A., and Oparil, S. (2000). Essential Hypertension : Part I: Definition and Etiology. Circulation *101*: 329–335.

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., and Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature *389*: 816–824.

Cave, A.C., Brewer, A.C., Narayanapanicker, A., Ray, R., Grieve, D.J., Walker, S., et al. (2006). NADPH oxidases in cardiovascular health and disease. Antioxid. Redox Signal. *8*: 691–728.

Cernadas, M.R., de Miguel, L.S., Garcia-Duran, M., Gonzalez-Fernandez, F., Millas, I., Monton, M., et al. (1998a). Expression of Constitutive and Inducible Nitric Oxide Synthases in the Vascular Wall of Young and Aging Rats. Circ. Res. *83*: 279–286.

Cernadas, M.R., Sánchez de Miguel, L., García-Durán, M., González-Fernández, F., Millás, I., Montón, M., et al. (1998b). Expression of constitutive and inducible nitric oxide synthases in the vascular wall of young and aging rats. Circ. Res. *83*: 279–286.

Chai, W., Mehrotra, S., Jan Danser, A.H., and Schoemaker, R.G. (2006). The role of calcitonin gene-related peptide (CGRP) in ischemic preconditioning in isolated rat hearts. Eur J Pharmacol *531*: 246–253.

Champion, H.C., Bivalacqua, T.J., Toyoda, K., Heistad, D.D., Hyman, A.L., and Kadowitz, P.J. (2000). In vivo gene transfer of prepro-calcitonin gene-related peptide to the lung attenuates chronic hypoxia-induced pulmonary hypertension in the mouse. Circulation *101*: 923–930.

Chan, G.H.H., and Fiscus, R.R. (2002). Severe impairment of CGRP-induced hypotension in vivo and vasorelaxation in vitro in elderly rats. Eur J Pharmacol *434*: 133–139.

Chan, S.L., and Fiscus, R.R. (2003). Vasorelaxations induced by calcitonin gene-related peptide, vasoactive intestinal peptide, and acetylcholine in aortic rings of endothelial and inducible nitric oxide synthase-knockout mice. J. Cardiovasc. Pharmacol. *41*: 434–443.

Chang, C.P., Pearse, R.V., O'Connell, S., and Rosenfeld, M.G. (1993). Identification of a seven transmembrane helix receptor for corticotropin-releasing factor and sauvagine in mammalian brain. Neuron *11*: 1187–1195.

Chiba, T., Yamaguchi, A., Yamatani, T., Nakamura, A., Morishita, T., Inui, T., et al. (1989). Calcitonin gene-related peptide receptor antagonist human CGRP-(8-37). Am. J. Physiol. *256*: E331–5.

Comi, P., Chiaramonte, R., and Maier, J.A. (1995). Senescence-dependent regulation of type 1 plasminogen activator inhibitor in human vascular endothelial cells. Exp. Cell Res. *219*: 304–308.

Connat, J.L., Busseuil, D., Gambert, S., Ody, M., Tébaldini, M., Gamboni, S., et al. (2001). Modification of the rat aortic wall during ageing; possible relation with decrease of peptidergic innervation. Anat. Embryol. *204*: 455–468.

Conner, A.C., Hay, D.L., Howitt, S.G., Kilk, K., Langel, U., Wheatley, M., et al. (2002). Interaction of calcitonin-gene-related peptide with its receptors. Biochem. Soc. Trans. *30*: 451–455.

Consonni, A., Morara, S., Codazzi, F., Grohovaz, F., and Zacchetti, D. (2011). Inhibition of lipopolysaccharide-induced microglia activation by calcitonin gene related peptide and adrenomedullin. Mol. Cell. Neurosci. *48*: 151–160.

Cottrell, G.S., Padilla, B., Pikios, S., Roosterman, D., Steinhoff, M., Grady, E.F., et al. (2007). Post-endocytic sorting of calcitonin receptor-like receptor and receptor activity-modifying protein 1. J. Biol. Chem. *282*: 12260–12271.

Cottrell, G.S., Roosterman, D., Marvizon, J.-C., Song, B., Wick, E., Pikios, S., et al. (2005). Localization of calcitonin receptor-like receptor and receptor activity modifying protein 1 in enteric neurons,

dorsal root ganglia, and the spinal cord of the rat. J. Comp. Neurol. *490*: 239–255.

Coughlan, M.T., Thorburn, D.R., Penfold, S.A., Laskowski, A., Harcourt, B.E., Sourris, K.C., et al. (2009). RAGE-induced cytosolic ROS promote mitochondrial superoxide generation in diabetes. J. Am. Soc. Nephrol. *20*: 742–752.

Coupe, M.O., Mak, J.C., Yacoub, M., Oldershaw, P.J., and Barnes, P.J. (1990). Autoradiographic mapping of calcitonin gene-related peptide receptors in human and guinea pig hearts. Circulation *81*: 741–747.

Covino, E., and C Spadaccio, C. (2011). Calcitonin gene related peptide: a new ally in cardiac ischemic disease. Minerva Anestesiol.

Cracknell, R. The ageing population.

Csiszar, A., Tucsek, Z., Toth, P., Sosnowska, D., Gautam, T., Koller, A., et al. (2013). Synergistic effects of hypertension and aging on cognitive function and hippocampal expression of genes involved in β-amyloid generation and Alzheimer's disease. AJP: Heart and Circulatory Physiology *305*: H1120–30.

Csiszar, A., Wang, M., Lakatta, E.G., and Ungvari, Z. (2008). Inflammation and endothelial dysfunction during aging: role of NF-kappaB. J. Appl. Physiol. *105*: 1333–1341.

Dai, D.-F., Rabinovitch, P.S., and Ungvari, Z. (2012). Mitochondria and cardiovascular aging. Circ. Res. *110*: 1109–1124.

Davis-Dusenbery, B.N., Wu, C., and Hata, A. (2011). Micromanaging vascular smooth muscle cell differentiation and phenotypic modulation. Arterioscler. Thromb. Vasc. Biol. *31*: 2370–2377.

Degnan, A.P., Chaturvedula, P.V., Conway, C.M., Cook, D.A., Davis, C.D., Denton, R., et al. (2008). Discovery of (R)-4-(8-fluoro-2-oxo-1,2-dihydroquinazolin-3(4H)-yl)-N-(3-(7-methyl-1H-indazol-5-yl)-1-oxo-1-(4-(piperidin-1-yl)piperidin-1-yl)propan-2-yl)piperidine-1-carboxamide (BMS-694153): a potent antagonist of the human calcitonin gene-related peptide receptor for migraine with rapid and efficient intranasal exposure. J. Med. Chem. *51*: 4858–4861.

Deng, P.-Y., Ye, F., Cai, W.-J., Deng, H.-W., and Li, Y.-J. (2004a). Role of calcitonin gene-related peptide in the phenol-induced neurogenic hypertension in rats. Regul. Pept. *119*: 155–161.

Deng, P.-Y., Ye, F., Cai, W.-J., Tan, G.-S., Hu, C.-P., Deng, H.-W., et al. (2004b). Stimulation of calcitonin gene-related peptide synthesis and release: mechanisms for a novel antihypertensive drug, rutaecarpine. J. Hypertens. *22*: 1819–1829.

Deng, P.-Y., Ye, F., Zhu, H.-Q., Cai, W.-J., Deng, H.-W., and Li, Y.-J. (2003). An increase in the synthesis and release of calcitonin gene-related peptide in two-kidney, one-clip hypertensive rats. Regul. Pept. *114*: 175–182.

Dennis, T., Fournier, A., Cadieux, A., Pomerleau, F., Jolicoeur, F.B., St Pierre, S., et al. (1990). hCGRP8-37, a calcitonin gene-related peptide antagonist revealing calcitonin gene-related peptide receptor heterogeneity in brain and periphery. J Pharmacol Exp Ther *254*: 123–128.

Dennis, T., Fournier, A., St Pierre, S., and Quirion, R. (1989). Structure-activity profile of calcitonin gene-related peptide in peripheral and brain tissues. Evidence for receptor multiplicity. J Pharmacol Exp Ther *251*: 718–725.

Dhall, U., Cowen, T., Haven, A.J., and Burnstock, G. (1986). Perivascular noradrenergic and peptide-containing nerves show different patterns of changes during development and ageing in the guinea-pig. J. Auton. Nerv. Syst. *16*: 109–126.

Diez Guerra, F.J., Zaidi, M., Bevis, P., MacIntyre, I., and Emson, P.C. (1988). Evidence for release of calcitonin gene-related peptide and neurokinin A from sensory nerve endings in vivo. Neuroscience *25*: 839–846.

Doi, Y., Kayashima, K., and Fujimoto, S. (2001). Synthesis of calcitonin gene-related peptide (CGRP) by rat arterial endothelial cells. Histol. Histopathol. *16*: 1073–1079.

Donato, A.J., Eskurza, I., Silver, A.E., Levy, A.S., Pierce, G.L., Gates, P.E., et al. (2007). Direct evidence of endothelial oxidative stress with aging in humans: relation to impaired endothelium-dependent dilation and upregulation of nuclear factor-kappaB. Circ. Res. *100*: 1659–1666.

Donato, A.J., Gano, L.B., Eskurza, I., Silver, A.E., Gates, P.E., Jablonski, K., et al. (2009). Vascular endothelial dysfunction with aging: endothelin-1 and endothelial nitric oxide synthase. AJP: Heart and Circulatory Physiology *297*: H425–32.

Dong, Y.-L., Chauhan, M., Green, K.E., Vegiraju, S., Wang, H.-Q., Hankins, G.D.V., et al. (2006). Circulating calcitonin gene-related peptide and its placental origins in normotensive and preeclamptic pregnancies. Am. J. Obstet. Gynecol. *195*: 1657–1667.

Donnerer, J., and Stein, C. (1992). Evidence for an increase in the release of CGRP from sensory nerves during inflammation. Ann. N. Y. Acad. Sci. *657*: 505–506.

Donnerer, J., Schuligoi, R., and Stein, C. (1992). Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: evidence for a regulatory function of nerve growth factor in vivo. Neuroscience *49*: 693–698.

Doods, H., Hallermayer, G., Wu, D., Entzeroth, M., Rudolf, K., Engel, W., et al. (2000). Pharmacological profile of BIBN4096BS, the first selective small molecule CGRP antagonist. Br J Pharmacol *129*: 420–423.

Drake, W.M., Ajayi, A., Lowe, S.R., Mirtella, A., Bartlett, T.J., and Clark, A.J. (1999). Desensitization of CGRP and adrenomedullin receptors in SK-N-MC cells: implications for the RAMP hypothesis. Endocrinology *140*: 533–537.

Drake, W.M., Lowe, S.R., Mirtella, A., Bartlett, T.J., and Clark, A.J. (2000). Desensitisation of calcitonin gene-related peptide responsiveness but not adrenomedullin responsiveness in vascular smooth muscle cells. J. Endocrinol. *165*: 133–138.

Durham, P.L., and Russo, A.F. (2003). Stimulation of the calcitonin gene-related peptide enhancer by mitogen-activated protein kinases and repression by an antimigraine drug in trigeminal ganglia neurons. J. Neurosci. *23*: 807–815.

Dvorakova, M., Haberberger, R.V., Hagner, S., McGregor, G.P., Slavikova, J., and Kummer, W. (2003). Expression and distribution of the calcitonin receptor-like receptor in the developing rat heart. Anat. Embryol. *207*: 307–315.

Edvinsson, L., Ekman, R., and Thulin, T. (1989). Reduced levels of calcitonin gene-related peptide (CGRP) but not substance P during and after treatment of severe hypertension in man. J Hum Hypertens *3*: 267–270.

Edvinsson, L., Sams, A., Jansen-Olesen, I., Tajti, J., Kane, S.A., Rutledge, R.Z., et al. (2001). Characterisation of the effects of a non-peptide CGRP receptor antagonist in SK-N-MC cells and isolated human cerebral arteries. Eur J Pharmacol *415*: 39–44.

Endtmann, C., Ebrahimian, T., Czech, T., Arfa, O., Laufs, U., Fritz, M., et al. (2011). Angiotensin II impairs endothelial progenitor cell number and function in vitro and in vivo: implications for vascular regeneration. Hypertension *58*: 394–403.

Evans, B.N., Rosenblatt, M.I., Mnayer, L.O., Oliver, K.R., and Dickerson, I.M. (2000). CGRP-RCP, a novel protein required for signal transduction at calcitonin gene-related peptide and adrenomedullin receptors. J. Biol. Chem. *275*: 31438–31443.

Fang, L., Chen, M.-F., Xiao, Z.-L., Liu, Y., Yu, G.-L., Chen, X.-B., et al. (2011). Calcitonin gene-related peptide released from endothelial progenitor cells inhibits the proliferation of rat vascular smooth muscle cells induced by angiotensin II. Mol Cell Biochem.

Farmer, D.G.S., and Kennedy, S. (2009). RAGE, vascular tone and vascular disease. Pharmacol. Ther. *124*: 185–194.

Feng, M., Whitesall, S., Zhang, Y., Beibel, M., Alecy, L.D., and DiPetrillo, K. (2008). Validation of Volume-Pressure Recording Tail-Cuff Blood Pressure Measurements. Am. J. Hypertens. *21*: 1288–1291.

Fernandes, E.S., Fernandes, M.A., and Keeble, J.E. (2012). The functions of TRPA1 and TRPV1: moving away from sensory nerves. Br J Pharmacol *166*: 510–521.

Fernandez, H.L., and Hodges-Savola, C.A. (1994). Axoplasmic transport of calcitonin gene-related peptide in rat peripheral nerve as a function of age. Neurochem. Res. *19*: 1369–1377.

Fleenor, B.S. (2013). Large elastic artery stiffness with aging: novel translational mechanisms and interventions. Aging Dis *4*: 76–83.

Fleg, J.L., and Strait, J. (2011). Age-associated changes in cardiovascular structure and function: a fertile milieu for future disease. Heart Fail Rev.

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

Flühmann, B., Muff, R., Hunziker, W., Fischer, J.A., and Born, W. (1995). A human orphan calcitonin receptor-like structure. Biochem. Biophys. Res. Commun. *206*: 341–347.

Fox, F.E., Kubin, M., Cassin, M., Niu, Z., Hosoi, J., Torii, H., et al. (1997). Calcitonin gene-related peptide inhibits proliferation and antigen presentation by human peripheral blood mononuclear cells: effects on B7, interleukin 10, and interleukin 12. J. Invest. Dermatol. *108*: 43–48.

Förstermann, U., and Sessa, W.C. (2012). Nitric oxide synthases: regulation and function. Eur. Heart J. *33*: 829–37– 837a–837d.

Franceschi, C., and Bonafè, M. (2003). Centenarians as a model for healthy aging. Biochem. Soc. Trans. *31*: 457–461.

Franklin, S.S. (1999). Ageing and hypertension: the assessment of blood pressure indices in predicting coronary heart disease. J. Hypertens. *17*: S29–S36.

Frayon, S., Cueille, C., Gnidéhou, S., de Vernejoul, M.C., and Garel, J.M. (2000). Dexamethasone increases RAMP1 and CRLR mRNA expressions in human vascular smooth muscle cells. Biochem. Biophys. Res. Commun. *270*: 1063–1067.

Freeland, K., Liu, Y.Z., and Latchman, D.S. (2000). Distinct signalling pathways mediate the cAMP response element (CRE)-dependent activation of the calcitonin gene-related peptide gene promoter by cAMP and nerve growth factor. Biochem. J. *345 Pt 2*: 233–238.

Furie, M.B., and McHugh, D.D. (1989). Migration of Neutrophils Across Endothelial Monolayers Is Stimulated by Treatment of the Monolayers with Interleukin-1 or Tumor Necrosis Factor-Alpha. J. Immunol. *143*: 3309–3317.

Gamble, J.R., HARLAN, J.M., KLEBANOFF, S.J., and VADAS, M.A. (1985). Stimulation of the Adherence of Neutrophils to Umbilical Vein Endothelium by Human Recombinant Tumor-Necrosis-Factor. Proc. Natl. Acad. Sci. U.S.a. *82*: 8667–8671.

Gamse, R., and Saria, A. (1985). Potentiation of tachykinin-induced plasma protein extravasation by calcitonin gene-related peptide. Eur J Pharmacol *114*: 61–66.

Gangula, P.R., Supowit, S.C., Wimalawansa, S.J., Zhao, H., Hallman, D.M., DiPette, D.J., et al. (1997). Calcitonin gene-related peptide is a depressor in NG-nitro-L-arginine methyl ester-induced hypertension during pregnancy. Hypertension *29*: 248–253.

Gangula, P.R., Zhao, H., Supowit, S.C., Wimalawansa, S.J., DiPette, D.J., Westlund, K.N., et al. (2000). Increased blood pressure in alpha-calcitonin gene-related peptide/calcitonin gene knockout mice. Hypertension *35*: 470–475.

Gangula, P.R.R., Chauhan, M., Reed, L., and Yallampalli, C. (2009). Age-related changes in dorsal root ganglia, circulating and vascular calcitonin gene-related peptide (CGRP) concentrations in female rats: effect of female sex steroid hormones. Neurosci. Lett. *454*: 118–123.

Garlick, R.L., and Mazer, J.S. (1983). The principal site of nonenzymatic glycosylation of human serum albumin in vivo. J. Biol. Chem. *258*: 6142–6146.

Gennari, C., and Fischer, J.A. (1985). Cardiovascular action of calcitonin gene-related peptide in humans. Calcif. Tissue Int. *37*: 581–584.

Gerhard, M., Roddy, M.A., Creager, S.J., and Creager, M.A. (1996). Aging progressively impairs endothelium-dependent vasodilation in forearm resistance vessels of humans. Hypertension *27*: 849–853.

Goel, A., Su, B., Flavahan, S., Lowenstein, C.J., Berkowitz, D.E., and Flavahan, N.A. (2010). Increased endothelial exocytosis and generation of endothelin-1 contributes to constriction of aged arteries. Circ. Res. *107*: 242–251.

Golias, C., Tsoutsi, E., Matziridis, A., Makridis, P., Batistatou, A., and Charalabopoulos, K. (2007). Review. Leukocyte and endothelial cell adhesion molecules in inflammation focusing on inflammatory heart disease. In Vivo *21*: 757–769.

Goltzman, D., and Mitchell, J. (1985). Interaction of calcitonin and calcitonin gene-related peptide at receptor sites in target tissues. Science *227*: 1343–1345.

Gomes, R.N., Castro-Faria-Neto, H.C., Bozza, P.T., Soares, M.B.P., Shoemaker, C.B., David, J.R., et al.

(2005). Calcitonin gene-related peptide inhibits local acute inflammation and protects mice against lethal endotoxemia. Shock *24*: 590–594.

Grant, A. (2001). Leukocytes and neurogenic inflammation. Inflammopharmacology *9*: 403–420.

Gupta, S., Amrutkar, D.V., Mataji, A., Salmasi, H., Hay-Schmidt, A., Sheykhzade, M., et al. (2010). Evidence for CGRP re-uptake in rat dura mater encephali. Br J Pharmacol *161*: 1885–1898.

Haar, ter, E., Koth, C.M., Abdul-Manan, N., Swenson, L., Coll, J.T., Lippke, J.A., et al. (2010). Crystal structure of the ectodomain complex of the CGRP receptor, a class-B GPCR, reveals the site of drug antagonism. Structure *18*: 1083–1093.

Hagner, S., Stahl, U., Knoblauch, B., McGregor, G.P., and Lang, R.E. (2002). Calcitonin receptor-like receptor: identification and distribution in human peripheral tissues. Cell Tissue Res. *310*: 41–50.

Hao, H., Fiscus, R.R., Wang, X., and Diana, J.N. (1994). N omega-nitro-L-arginine inhibits vasodilations and elevations of both cyclic AMP and cyclic GMP levels in rat aorta induced by calcitonin gene-related peptide (CGRP). Neuropeptides *26*: 123–131.

Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. J Gerontol *11*: 298–300.

Harris, T.B., Ferrucci, L., Tracy, R.P., Corti, M.C., Wacholder, S., Ettinger, W.H., et al. (1999). Associations of elevated interleukin-6 and C-reactive protein levels with mortality in the elderly. Am. J. Med. *106*: 506–512.

Hartmann, P., Varga, R., Zobolyák, Z., Héger, J., Csosz, B., Németh, I., et al. (2011). Anti-inflammatory effects of limb ischaemic preconditioning are mediated by sensory nerve activation in rats. Naunyn Schmiedebergs Arch. Pharmacol. *383*: 179–189.

Hartopo, A.B., Emoto, N., Vignon-Zellweger, N., Suzuki, Y., Yagi, K., Nakayama, K., et al. (2013). Endothelin-converting enzyme-1 gene ablation attenuates pulmonary fibrosis via CGRP-cAMP/EPAC1 pathway. Am. J. Respir. Cell Mol. Biol. *48*: 465–476.

Harzenetter, M.D., Novotny, A.R., Gais, P., Molina, C.A., Altmayr, F., and Holzmann, B. (2007). Negative regulation of TLR responses by the neuropeptide CGRP is mediated by the transcriptional repressor ICER. J. Immunol. *179*: 607–615.

Hasbak, P., Eskesen, K., Schifter, S., and Edvinsson, L. (2005). Increased alphaCGRP potency and CGRP-receptor antagonist affinity in isolated hypoxic porcine intramyocardial arteries. Br J Pharmacol *145*: 646–655.

Hasbak, P., Saetrum Opgaard, O., Eskesen, K., Schifter, S., Arendrup, H., Longmore, J., et al. (2003). Investigation of CGRP receptors and peptide pharmacology in human coronary arteries. Characterization with a nonpeptide antagonist. J Pharmacol Exp Ther *304*: 326–333.

Hasbak, P., Sams, A., Schifter, S., Longmore, J., and Edvinsson, L. (2001). CGRP receptors mediating CGRP-, adrenomedullin- and amylin-induced relaxation in porcine coronary arteries. Characterization with 'Compound 1' (WO98/11128), a non-peptide antagonist. Br J Pharmacol *133*: 1405–1413.

Hay, D.L., Harris, P.W.R., Kowalczyk, R., Brimble, M.A., Rathbone, D.L., Barwell, J., et al. (2013). Structure-activity relationships of the N-terminus of calcitonin gene-related peptide: key roles of alanine-5 and threonine-6 in receptor activation. Br J Pharmacol n/a–n/a.

Hay, D.L., Poyner, D.R., Quirion, R., International Union of Pharmacology (2008). International Union of Pharmacology. LXIX. Status of the calcitonin gene-related peptide subtype 2 receptor. Pharmacol. Rev. *60*: 143–145.

Hayflick, L., and Moorhead, P.S. (1961). The serial cultivation of human diploid cell strains. Exp. Cell Res. *25*: 585–621.

He, T., Joyner, M.J., and Katusic, Z.S. (2009). Aging decreases expression and activity of glutathione peroxidase-1 in human endothelial progenitor cells. Microvascular Research *78*: 447–452.

Heiss, C., Keymel, S., Niesler, U., Ziemann, J., Kelm, M., and Kalka, C. (2005). Impaired progenitor cell activity in age-related endothelial dysfunction. J. Am. Coll. Cardiol. *45*: 1441–1448.

Hemmeryckx, B., Hoylaerts, M.F., Deloose, E., Van Hove, C.E., Fransen, P., Bult, H., et al. (2013). Age-associated pro-inflammatory adaptations of the mouse thoracic aorta. Thromb Haemost *110*: 785–794.

Heymes, C., Silvestre, J.S., Llorens-Cortes, C., Chevalier, B., Marotte, F., Levy, B.I., et al. (1998). Cardiac senescence is associated with enhanced expression of angiotensin II receptor subtypes. Endocrinology *139*: 2579–2587.

Hilairet, S., Bélanger, C., Bertrand, J., Laperrière, A., Foord, S.M., and Bouvier, M. (2001a). Agonist-promoted internalization of a ternary complex between calcitonin receptor-like receptor, receptor activity-modifying protein 1 (RAMP1), and beta-arrestin. J. Biol. Chem. *276*: 42182–42190.

Hilairet, S., Foord, S.M., Marshall, F.H., and Bouvier, M. (2001b). Protein-protein interaction and not glycosylation determines the binding selectivity of heterodimers between the calcitonin receptor-like receptor and the receptor activity-modifying proteins. J. Biol. Chem. *276*: 29575–29581.

Hirata, Y., Takagi, Y., Takata, S., Fukuda, Y., Yoshimi, H., and Fujita, T. (1988). Calcitonin gene-related peptide receptor in cultured vascular smooth muscle and endothelial cells. Biochem. Biophys. Res. Commun. *151*: 1113–1121.

Hoare, S.R.J. (2005). Mechanisms of peptide and nonpeptide ligand binding to Class B G-protein-coupled receptors. Drug Discov. Today *10*: 417–427.

Hobara, N., Gessei-Tsutsumi, N., Goda, M., Takayama, F., Akiyama, S., Kurosaki, Y., et al. (2005). Long-term inhibition of angiotensin prevents reduction of periarterial innervation of calcitonin gene-related peptide (CGRP)-containing nerves in spontaneously hypertensive rats. Hypertens. Res. *28*: 465–474.

Hoffmann, J., Haendeler, J., Aicher, A., Rössig, L., Vasa, M., Zeiher, A.M., et al. (2001). Aging enhances the sensitivity of endothelial cells toward apoptotic stimuli: important role of nitric oxide. Circ. Res. *89*: 709–715.

Holzmann, B. (2011). Modulation of immune responses by the neuropeptide CGRP. Amino Acids *45*: 1–7.

Hong, K.W., Yu, S.S., Shin, Y.W., Kim, C.D., Rhim, B.Y., and Lee, W.S. (1997). Decreased CGRP level with increased sensitivity to CGRP in the pial arteries of spontaneously hypertensive rats. Life Sci. *60*: 697–705.

Hopkins, P.N. (2013). Molecular biology of atherosclerosis. Physiol. Rev. *93*: 1317–1542.

Hosoi, J., Murphy, G.F., Egan, C.L., Lerner, E.A., and Grabbe, S. (1993). Regulation of Langerhans cell function by nerves containing calcitonin gene-related peptide. Nature *363*: 159–163.

Hou, Q., Barr, T., Gee, L., Vickers, J., Wymer, J., Borsani, E., et al. (2011). Keratinocyte expression of calcitonin gene-related peptide β: Implications for neuropathic and inflammatory pain mechanisms. Pain.

Höppener, J.W., Steenbergh, P.H., Zandberg, J., Geurts van Kessel, A.H., Baylin, S.B., Nelkin, B.D., et al. (1985). The second human calcitonin/CGRP gene is located on chromosome 11. Hum. Genet. *70*: 259–263.

Hu, C.-P., Peng, J., Xiao, L., Ye, F., Deng, H.-W., and Li, Y.-J. (2002). Effect of age on alpha-calcitonin gene-related peptide-mediated delayed cardioprotection induced by intestinal preconditioning in rats. Regul. Pept. *107*: 137–143.

Hu, C.-P., Xiao, L., Deng, H.-W., and Li, Y.-J. (2003). The depressor and vasodilator effects of rutaecarpine are mediated by calcitonin gene-related peptide. Planta Med. *69*: 125–129.

Huang, J., Stohl, L.L., Zhou, X., Ding, W., and Granstein, R.D. (2011). Calcitonin gene-related peptide inhibits chemokine production by human dermal microvascular endothelial cells. Brain Behav. Immun. *25*: 787–799.

Hubbard, J.A., Martin, S.R., Chaplin, L.C., Bose, C., Kelly, S.M., and Price, N.C. (1991). Solution structures of calcitonin-gene-related-peptide analogues of calcitonin-gene-related peptide and amylin. Biochem. J. *275 (Pt 3)*: 785–788.

Husmann, K., Sexton, P.M., Fischer, J.A., and Born, W. (2000). Mouse receptor-activity-modifying proteins 1, -2 and -3: amino acid sequence, expression and function. Mol. Cell. Endocrinol. *162*: 35–43.

Huzen, J., Peeters, W., de Boer, R.A., Moll, F.L., Wong, L.S.M., Codd, V., et al. (2011). Circulating Leukocyte and Carotid Atherosclerotic Plaque Telomere Length: Interrelation, Association With Plaque Characteristics, and Restenosis After Endarterectomy. Arterioscler. Thromb. Vasc. Biol. *31*: 1219–1225.

Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E., and Chaudhuri, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc. Natl. Acad. Sci. U.S.a. *84*: 9265–9269.

Ishihata, A., and Katano, Y. (2006). Role of angiotensin II and endothelin-1 receptors in aging-related functional changes in rat cardiovascular system. Ann. N. Y. Acad. Sci. *1067*: 173–181.

Jani, B., and Rajkumar, C. (2006). Ageing and vascular ageing. Postgraduate Medical Journal *82*: 357–362.

Jansen-Olesen, I., Jørgensen, L., Engel, U., and Edvinsson, L. (2003). In-depth characterization of CGRP receptors in human intracranial arteries. Eur J Pharmacol *481*: 207–216.

Johnson, D.R., Douglas, I., Jahnke, A., Ghosh, S., and Pober, J.S. (1996). A sustained reduction in IkappaB-beta may contribute to persistent NF-kappaB activation in human endothelial cells. J. Biol. Chem. *271*: 16317–16322.

Jusek, G., Reim, D., Tsujikawa, K., and Holzmann, B. (2012). Deficiency of the CGRP receptor

component RAMP1 attenuates immunosuppression during the early phase of septic peritonitis. Immunobiology *217*: 761–767.

Kalehua, A.N., Taub, D.D., Baskar, P.V., Hengemihle, J., Muñoz, J., Trambadia, M., et al. (2000). Aged mice exhibit greater mortality concomitant to increased brain and plasma TNF-alpha levels following intracerebroventricular injection of lipopolysaccharide. Gerontology *46*: 115–128.

Kaliappan, G., Nagarajan, P., Moorthy, R., Kalai Gana Selvi, S., Avinash Raj, T., and Mahesh Kumar, J. (2012). Ang II induce kidney damage by recruiting inflammatory cells and up regulates PPAR gamma and Renin 1 gene: effect of β carotene on chronic renal damage. J Thromb Thrombolysis *36*: 277–285.

Kamada, K., Gaskin, F.S., Yamaguchi, T., Carter, P., Yoshikawa, T., Yusof, M., et al. (2006). Role of calcitonin gene-related peptide in the postischemic anti-inflammatory effects of antecedent ethanol ingestion. Am. J. Physiol. Heart Circ. Physiol. *290*: H531–7.

Kamitani, S., Asakawa, M., Shimekake, Y., Kuwasako, K., Nakahara, K., and Sakata, T. (1999a). The RAMP2/CRLR complex is a functional adrenomedullin receptor in human endothelial and vascular smooth muscle cells. FEBS Letters *448*: 111–114.

Kamitani, S., Asakawa, M., Shimekake, Y., Kuwasako, K., Nakahara, K., and Sakata, T. (1999b). The RAMP2/CRLR complex is a functional adrenomedullin receptor in human endothelial and vascular smooth muscle cells. FEBS Letters *448*: 111–114.

Kamiyoshi, A., Sakurai, T., Ichikawa-Shindo, Y., Iinuma, N., Kawate, H., Yoshizawa, T., et al. (2009). Endogenous α-calcitonin gene-related peptide mitigates liver fibrosis in chronic hepatitis induced by repeated administration of concanavalin A. Liver International *29*: 642–649.

Katayama, M., Nadel, J.A., Bunnett, N.W., Di Maria, G.U., Haxhiu, M., and Borson, D.B. (1991). Catabolism of calcitonin gene-related peptide and substance P by neutral endopeptidase. Peptides *12*: 563–567.

Katki, K.A., Supowit, S.C., and DiPette, D.J. (2001). Role of calcitonin gene-related peptide and substance P in Dahl-salt hypertension. Hypertension *38*: 679–682.

Kawasaki, H. (2002). Regulation of vascular function by perivascular calcitonin gene-related peptide-containing nerves. Jpn. J. Pharmacol. *88*: 39–43.

Kawasaki, H., and Takasaki, K. (1992). Age-related decrease of neurogenic release of calcitonin gene-related peptide from perivascular nerves in spontaneously hypertensive rats. Clin Exp Hypertens A *14*: 989–1001.

Kawasaki, H., Inaizumi, K., Nakamura, A., Hobara, N., and Kurosaki, Y. (2003). Chronic angiotensin II inhibition increases levels of calcitonin gene-related peptide mRNA of the dorsal root ganglia in spontaneously hypertensive rats. Hypertens. Res. *26*: 257–263.

Kawasaki, H., Nuki, Y., Yamaga, N., Kurosaki, Y., and Taguchi, T. (2000). Decreased depressor response mediated by calcitonin gene-related peptide (CGRP)-containing vasodilator nerves to spinal cord stimulation and levels of CGRP mRNA of the dorsal root ganglia in spontaneously hypertensive rats. Hypertens. Res. *23*: 693–699.

Kawasaki, H., Okazaki, M., Nakatsuma, A., Mimaki, Y., Araki, H., and Gomita, Y. (1999). Long-term treatment with angiotensin converting enzyme inhibitor restores reduced calcitonin gene-related

peptide-containing vasodilator nerve function in mesenteric artery of spontaneously hypertensive rats. Jpn. J. Pharmacol. *79*: 221–229.

Kawasaki, H., Saito, A., and Takasaki, K. (1990a). Age-related decrease of calcitonin gene-related peptide-containing vasodilator innervation in the mesenteric resistance vessel of the spontaneously hypertensive rat. Circ. Res. *67*: 733–743.

Kawasaki, H., Saito, A., and Takasaki, K. (1990b). Changes in calcitonin gene-related peptide (CGRP)-containing vasodilator nerve activity in hypertension. Brain Res. *518*: 303–307.

Kawasaki, H., Saito, A., Goto, K., and Takasaki, K. (1991). Age-related changes in calcitonin gene-related peptide (CGRP)-mediated neurogenic vasodilation of the mesenteric resistance vessel in SHR. Clin Exp Hypertens A *13*: 745–754.

Kawasaki, H., Takenaga, M., Araki, H., Futagami, K., and Gomita, Y. (1998). Angiotensin inhibits neurotransmission of calcitonin gene-related peptide-containing vasodilator nerves in mesenteric artery of spontaneously hypertensive rats. J Pharmacol Exp Ther *284*: 508–515.

Kawase, T., Okuda, K., and Burns, D.M. (2005). Immature osteoblastic MG63 cells possess two calcitonin gene-related peptide receptor subtypes that respond differently to [Cys(Acm)(2,7)] calcitonin gene-related peptide and CGRP(8-37). Am. J. Physiol., Cell Physiol. *289*: C811–8.

Kendall, C.H., Homer, C.E., Bishop, A.E., and Polak, J.M. (1986). Age-related peptide production by human thyroid C cells. An immunohistochemical study. Virchows Arch a Pathol Anat Histopathol *410*: 97–101.

Kim, Y.-G., Lone, A.M., Nolte, W.M., and Saghatelian, A. (2012). Peptidomics approach to elucidate the proteolytic regulation of bioactive peptides. Proc. Natl. Acad. Sci. U.S.a.

Kipling, D., and Cooke, H.J. (1990). Hypervariable ultra-long telomeres in mice. Nature *347*: 400–402.

Kirkwood, T.B. (1977). Evolution of ageing. Nature.

Kirkwood, T.B.L. (2002). Evolution of ageing. Mech. Ageing Dev. *123*: 737–745.

Kolovou, G.D., Kolovou, V., and Mavrogeni, S. (2014). We are ageing. Biomed Res Int *2014*: 808307.

Kossmann, S., Hu, H., Steven, S., Schönfelder, T., Fraccarollo, D., Mikhed, Y., et al. (2014). Inflammatory Monocytes Determine Endothelial Nitric-oxide Synthase Uncoupling and Nitro-oxidative Stress Induced by Angiotensin II. J. Biol. Chem. *289*: 27540–27550.

Koth, C.M., Abdul-Manan, N., Lepre, C.A., Connolly, P.J., Yoo, S., Mohanty, A.K., et al. (2010). Refolding and characterization of a soluble ectodomain complex of the calcitonin gene-related peptide receptor. Biochemistry *49*: 1862–1872.

Kroeger, I., Erhardt, A., Abt, D., Fischer, M., Biburger, M., Rau, T., et al. (2009). The neuropeptide calcitonin gene-related peptide (CGRP) prevents inflammatory liver injury in mice. J. Hepatol. *51*: 342–353.

Kubota, M., Moseley, J.M., Butera, L., Dusting, G.J., MacDonald, P.S., and Martin, T.J. (1985). Calcitonin gene-related peptide stimulates cyclic AMP formation in rat aortic smooth muscle cells. Biochem. Biophys. Res. Commun. *132*: 88–94.

Kuilman, T., Michaloglou, C., Mooi, W.J., and Peeper, D.S. (2010). The essence of senescence. Genes & Development *24*: 2463–2479.

Kurihara, H., Shindo, T., Oh-Hashi, Y., Kurihar, Y., and Kuwaki, T. (2003). Targeted disruption of adrenomedullin and alphaCGRP genes reveals their distinct biological roles. Hypertens. Res. *26 Suppl*: S105–8.

Kusano, S., Kukimoto-Niino, M., Akasaka, R., Toyama, M., Terada, T., Shirouzu, M., et al. (2008). Crystal structure of the human receptor activity-modifying protein 1 extracellular domain. Protein Sci. *17*: 1907–1914.

Kushner, E.J., Maceneaney, O.J., Weil, B.R., Greiner, J.J., Stauffer, B.L., and Desouza, C.A. (2011). Aging Is Associated with a Proapoptotic Endothelial Progenitor Cell Phenotype. J Vasc Res *48*: 408–414.

Kushner, E.J., Van Guilder, G.P., MacEneaney, O.J., Cech, J.N., Stauffer, B.L., and DeSouza, C.A. (2009). Aging and endothelial progenitor cell telomere length in healthy men. Clinical Chemistry and Laboratory Medicine *47*:.

Kuwasako, K., Shimekake, Y., Masuda, M., Nakahara, K., Yoshida, T., Kitaura, M., et al. (2000). Visualization of the calcitonin receptor-like receptor and its receptor activity-modifying proteins during internalization and recycling. J. Biol. Chem. *275*: 29602–29609.

Küng, C.F., and Lüscher, T.F. (1995). Different mechanisms of endothelial dysfunction with aging and hypertension in rat aorta. Hypertension *25*: 194–200.

Labinskyy, N., Csiszar, A., Orosz, Z., Smith, K., Rivera, A., Buffenstein, R., et al. (2006). Comparison of endothelial function, O2-* and H2O2 production, and vascular oxidative stress resistance between the longest-living rodent, the naked mole rat, and mice. Am. J. Physiol. Heart Circ. Physiol. *291*: H2698–704.

Lafont, A.-G., Dufour, S., and Fouchereau-Peron, M. (2007). Evolution of the CT/CGRP family: comparative study with new data from models of teleosts, the eel, and cephalopod molluscs, the cuttlefish and the nautilus. Gen. Comp. Endocrinol. *153*: 155–169.

Landmesser, U., Dikalov, S., Price, S.R., McCann, L., Fukai, T., Holland, S.M., et al. (2003). Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. J. Clin. Invest. *111*: 1201–1209.

Lang, M.G., Noll, G., and Lüscher, T.F. (1995). Effect of aging and hypertension on contractility of resistance arteries: modulation by endothelial factors. Am. J. Physiol. *269*: H837–44.

Le Grevès, P., Nyberg, F., Hökfelt, T., and Terenius, L. (1989). Calcitonin gene-related peptide is metabolized by an endopeptidase hydrolyzing substance P. Regul. Pept. *25*: 277–286.

Le Grevès, P., Nyberg, F., Terenius, L., and Hökfelt, T. (1985). Calcitonin gene-related peptide is a potent inhibitor of substance P degradation. Eur J Pharmacol *115*: 309–311.

Lemne, C.E., Lundeberg, T., Theodorsson, E., and de Faire, U. (1994). Increased basal concentrations of plasma endothelin in borderline hypertension. J. Hypertens. *12*: 1069–1074.

Lesniewski, L.A., Connell, M.L., Durrant, J.R., Folian, B.J., Anderson, M.C., Donato, A.J., et al. (2009). B6D2F1 Mice are a suitable model of oxidative stress-mediated impaired endothelium-dependent

dilation with aging. J. Gerontol. a Biol. Sci. Med. Sci. *64*: 9–20.

Lewis, K.N., Mele, J., Hayes, J.D., and Buffenstein, R. (2010). Nrf2, a guardian of healthspan and gatekeeper of species longevity. Integr. Comp. Biol. *50*: 829–843.

Ley, K. (2003). Arrest chemokines. Microcirculation *10*: 289–295.

Ley, K., Laudanna, C., Cybulsky, M.I., and Nourshargh, S. (2007). Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol *7*: 678–689.

Li, D., Chen, B.-M., Peng, J., Zhang, Y.-S., Li, X.-H., Yuan, Q., et al. (2009). Role of anandamide transporter in regulating calcitonin gene-related peptide production and blood pressure in hypertension. J. Hypertens. *27*: 1224–1232.

Li, D., Peng, J., Xin, H.-Y., Luo, D., Zhang, Y.-S., Zhou, Z., et al. (2008). Calcitonin gene-related peptide-mediated antihypertensive and anti-platelet effects by rutaecarpine in spontaneously hypertensive rats. Peptides *29*: 1781–1788.

Li, J., and Wang, D.H. (2005). Development of angiotensin II-induced hypertension: role of CGRP and its receptor. J. Hypertens. *23*: 113–118.

Li, J., Carnevale, K.A., Dipette, D.J., and Supowit, S.C. (2013a). Renal protective effects of α-calcitonin gene-related peptide in deoxycorticosterone-salt hypertension. Am. J. Physiol. Renal Physiol. *304*: F1000–8.

Li, J., Levick, S.P., Dipette, D.J., Janicki, J.S., and Supowit, S.C. (2013b). Regulatory Peptides. Regul. Pept. *185*: 20–28.

Li, J., Zhao, H., Supowit, S.C., Dipette, D.J., and Wang, D.H. (2004). Activation of the renin-angiotensin system in alpha-calcitonin gene-related peptide/calcitonin gene knockout mice. J. Hypertens. *22*: 1345–1349.

Li, Y.J., Song, Q.J., and Xiao, J. (2000). Calcitonin gene-related peptide: an endogenous mediator of preconditioning. Acta Pharmacol. Sin. *21*: 865–869.

Lind, H., and Edvinsson, L. (2002). Enhanced vasodilator responses to calcitonin gene-related peptide (CGRP) in subcutaneous arteries in human hypertension. J Hum Hypertens *16*: 53–59.

Lindstedt, I.H., Edvinsson, M.-L., and Edvinsson, L. (2006). Reduced responsiveness of cutaneous microcirculation in essential hypertension--a pilot study. Blood Press. *15*: 275–280.

Loghman-Adham, M. (2004). The intrarenal renin-angiotensin system in autosomal dominant polycystic kidney disease. AJP: Renal Physiology *287*: F775–F788.

López-Otín, C., Blasco, M.A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The hallmarks of aging. Cell *153*: 1194–1217.

Lu, J.T., Son, Y.J., Lee, J., Jetton, T.L., Shiota, M., Moscoso, L., et al. (1999). Mice lacking alpha-calcitonin gene-related peptide exhibit normal cardiovascular regulation and neuromuscular development. Mol. Cell. Neurosci. *14*: 99–120.

Lu, L.F., and Fiscus, R.R. (1999). Calcitonin gene-related peptide causes long-term inhibition of contraction in rat thoracic aorta through a nitric oxide-dependent pathway. Neuropeptides *33*: 145–

154.

Lu, R., Hu, C.P., Deng, H.W., and Li, Y.J. (2001a). Calcitonin gene-related peptide-mediated ischemic preconditioning in the rat heart: influence of age. Regul. Pept. *99*: 183–189.

Lu, R., Hu, C.P., Peng, J., Deng, H.W., and Li, Y.J. (2001b). Role of calcitonin gene-related peptide in ischaemic preconditioning in diabetic rat hearts. Clin. Exp. Pharmacol. Physiol. *28*: 392–396.

Lu, R., Zhu, H.-Q., Peng, J., Li, N.-S., and Li, Y.J. (2002). Endothelium-dependent vasorelaxation and the expression of calcitonin gene-related peptide in aged rats. Neuropeptides *36*: 407–412.

Luebke, A.E., Dahl, G.P., Roos, B.A., and Dickerson, I.M. (1996). Identification of a protein that confers calcitonin gene-related peptide responsiveness to oocytes by using a cystic fibrosis transmembrane conductance regulator assay. Proc. Natl. Acad. Sci. U.S.a. *93*: 3455–3460.

Lundberg, J.M., Franco-Cereceda, A., Hua, X., Hökfelt, T., and Fischer, J.A. (1985). Co-existence of substance P and calcitonin gene-related peptide-like immunoreactivities in sensory nerves in relation to cardiovascular and bronchoconstrictor effects of capsaicin. Eur J Pharmacol *108*: 315–319.

Lundberg, J.M., Hua, Y., and Fredholm, B.B. (1984). Capsaicin-induced stimulation of the guinea-pig atrium. Involvement of a novel sensory transmitter or a direct action on myocytes? Naunyn Schmiedebergs Arch. Pharmacol. *325*: 176–182.

Luo, D., Zhang, Y.-W., Peng, W.-J., Peng, J., Chen, Q.-Q., Li, D., et al. (2008a). Transient receptor potential vanilloid 1-mediated expression and secretion of endothelial cell-derived calcitonin gene-related peptide. Regul. Pept. *150*: 66–72.

Luo, X.-L., Yang, T.-L., Chen, X.-P., and Li, Y.-J. (2008b). Association of CALCA genetic polymorphism with essential hypertension. Chin. Med. J. *121*: 1407–1410.

Lynch, J.J., Detwiler, T.J., Kane, S.A., and Regan, C.P. (2010). Effect of calcitonin gene-related peptide receptor antagonism on the systemic blood pressure responses to mechanistically diverse vasomodulators in conscious rats. J. Cardiovasc. Pharmacol. *56*: 518–525.

Maggi, C.A., Rovero, P., Giuliani, S., Evangelista, S., Regoli, D., and Meli, A. (1990). Biological activity of N-terminal fragments of calcitonin gene-related peptide. Eur J Pharmacol *179*: 217–219.

Maier, J.A., Statuto, M., and Ragnotti, G. (1993). Senescence stimulates U937-endothelial cell interactions. Exp. Cell Res. *208*: 270–274.

Mallee, J.J., Salvatore, C.A., LeBourdelles, B., Oliver, K.R., Longmore, J., Koblan, K.S., et al. (2002). Receptor activity-modifying protein 1 determines the species selectivity of non-peptide CGRP receptor antagonists. J. Biol. Chem. *277*: 14294–14298.

Mamdouh, Z., Kreitzer, G.E., and Muller, W.A. (2008). Leukocyte transmigration requires kinesin-mediated microtubule-dependent membrane trafficking from the lateral border recycling compartment. J. Exp. Med. *205*: 951–966.

Mandela, P., and Ordway, G.A. (2006). The norepinephrine transporter and its regulation. J. Neurochem. *97*: 310–333.

Marshall, N.J., Liang, L., Bodkin, J., Dessapt-Baradez, C., Nandi, M., Collot-Teixeira, S., et al. (2013). A role for TRPV1 in influencing the onset of cardiovascular disease in obesity. Hypertension *61*: 246–

252.

Masuda, A., Shimamoto, K., and Mori, Y. (1992). Plasma calcitonin gene-related peptide levels patients with various hypertensive diseases. Journal of ….

Matsushita, H., Chang, E., Glassford, A.J., Cooke, J.P., Chiu, C.P., and Tsao, P.S. (2001). eNOS activity is reduced in senescent human endothelial cells: Preservation by hTERT immortalization. Circ. Res. *89*: 793–798.

Matteoli, M., Haimann, C., Torri-Tarelli, F., Polak, J.M., Ceccarelli, B., and De Camilli, P. (1988). Differential effect of alpha-latrotoxin on exocytosis from small synaptic vesicles and from large dense-core vesicles containing calcitonin gene-related peptide at the frog neuromuscular junction. Proc. Natl. Acad. Sci. U.S.a. *85*: 7366–7370.

Mármol, F., Sánchez, J., López, D., Martínez, N., Roselló-Catafau, J., Mitjavila, M.T., et al. (2007). Loss of adaptation to oxidative stress as a mechanism for aortic damage in aging rats. J. Physiol. Biochem. *63*: 239–247.

McCrann, D.J., Yang, D., Chen, H., Carroll, S., and Ravid, K. (2009). Upregulation of Nox4 in the aging vasculature and its association with smooth muscle cell polyploidy. Cell Cycle 1–15.

McKinley, M.J., and Johnson, A.K. (2004). The physiological regulation of thirst and fluid intake. News Physiol. Sci. *19*: 1–6.

McLatchie, L.M., Fraser, N.J., Main, M.J., Wise, A., Brown, J., Thompson, N., et al. (1998). RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature *393*: 333–339.

McNeish, A.J., Roux, B.T., Aylett, S.-B., Van Den Brink, A.M., and Cottrell, G.S. (2012). Endosomal proteolysis regulates calcitonin gene-related peptide responses in mesenteric arteries. Br J Pharmacol *167*: 1679–1690.

Medawar, P.B. (1952). An Unsolved Problem of Biology (H.K. Lewis and Company).

Meens, M.J.P.M.T., Mattheij, N.J.A., van Loenen, P.B., Spijkers, L.J.A., Lemkens, P., Nelissen, J., et al. (2012). G-protein βγ subunits in vasorelaxing and anti-endothelinergic effects of calcitonin gene-related peptide. Br J Pharmacol *166*: 297–308.

Meng, J., Wang, J., Lawrence, G., and Dolly, J.O. (2007). Synaptobrevin I mediates exocytosis of CGRP from sensory neurons and inhibition by botulinum toxins reflects their anti-nociceptive potential. J. Cell. Sci. *120*: 2864–2874.

Michel, J.B., Heudes, D., Michel, O., Poitevin, P., Philippe, M., Scalbert, E., et al. (1994). Effect of chronic ANG I-converting enzyme inhibition on aging processes. II. Large arteries. Am. J. Physiol. *267*: R124–35.

Miller, P.S., Barwell, J., Poyner, D.R., Wigglesworth, M.J., Garland, S.L., and Donnelly, D. (2010). Non-peptidic antagonists of the CGRP receptor, BIBN4096BS and MK-0974, interact with the calcitonin receptor-like receptor via methionine-42 and RAMP1 via tryptophan-74. Biochem. Biophys. Res. Commun. *391*: 437–442.

Miller, S.J., Watson, W.C., Kerr, K.A., Labarrere, C.A., Chen, N.X., Deeg, M.A., et al. (2007). Development of progressive aortic vasculopathy in a rat model of aging. AJP: Heart and Circulatory

Physiology *293*: H2634–H2643.

Mimeault, M., Quirion, R., Dumont, Y., St-Pierre, S., and Fournier, A. (1992). Structure-activity study of hCGRP8-37, a calcitonin gene-related peptide receptor antagonist. J. Med. Chem. *35*: 2163–2168.

Minamino, T., Miyauchi, H., Yoshida, T., Ishida, Y., Yoshida, H., and Komuro, I. (2002). Endothelial cell senescence in human atherosclerosis: role of telomere in endothelial dysfunction. Circulation *105*: 1541–1544.

Minamino, T., Yoshida, T., Tateno, K., Miyauchi, H., Zou, Y., Toko, H., et al. (2003). Ras induces vascular smooth muscle cell senescence and inflammation in human atherosclerosis. Circulation *108*: 2264–2269.

Mishima, T., Ito, Y., Hosono, K., Tamura, Y., Uchida, Y., Hirata, M., et al. (2011). Calcitonin gene-related peptide facilitates revascularization during hindlimb ischemia in mice. Am. J. Physiol. Heart Circ. Physiol. *300*: H431–9.

Moore, E.L., and Salvatore, C.A. (2012). Targeting a family B GPCR/RAMP receptor complex: CGRP receptor antagonists and migraine. Br J Pharmacol *166*: 66–78.

Moore, E.L., Gingell, J.J., Kane, S.A., Hay, D.L., and Salvatore, C.A. (2010). Mapping the CGRP receptor ligand binding domain: tryptophan-84 of RAMP1 is critical for agonist and antagonist binding. Biochem. Biophys. Res. Commun. *394*: 141–145.

Moreno, M.J., Terrón, J.A., Stanimirovic, D.B., Doods, H., and Hamel, E. (2002). Characterization of calcitonin gene-related peptide (CGRP) receptors and their receptor-activity-modifying proteins (RAMPs) in human brain microvascular and astroglial cells in culture. Neuropharmacology *42*: 270–280.

Morris, H.R., Panico, M., Girgis, S.I., and MacIntyre, I. (1984). Isolation and characterization of human calcitonin gene-related peptide. Nature *308*: 746–748.

Muff, R., Bühlmann, N., Fischer, J.A., and Born, W. (1999). An amylin receptor is revealed following co-transfection of a calcitonin receptor with receptor activity modifying proteins-1 or -3. Endocrinology *140*: 2924–2927.

Muff, R., Leuthäuser, K., Bühlmann, N., Foord, S.M., Fischer, J.A., and Born, W. (1998). Receptor activity modifying proteins regulate the activity of a calcitonin gene-related peptide receptor in rabbit aortic endothelial cells. FEBS Letters *441*: 366–368.

Mulderry, P.K., Ghatei, M.A., Rodrigo, J., Rosenfeld, M.G., Polak, J.M., and Bloom, S.R. (1985). Calcitonin gene-related peptide in cardiovascular tissues of the rat. Neuroscience *14*: 947–954.

Mulderry, P.K., Ghatei, M.A., Spokes, R.A., Jones, P.M., Pierson, A.M., Hamid, Q.A., et al. (1988). Differential expression of alpha-CGRP and beta-CGRP by primary sensory neurons and enteric autonomic neurons of the rat. Neuroscience *25*: 195–205.

Muñoz-Espín, D., and Serrano, M. (2014). Cellular senescence: from physiology to pathology. Nat Rev Mol Cell Biol *15*: 482–496.

Myśliwska, J., Bryl, E., Foerster, J., and Myśliwski, A. (1999). The upregulation of TNF alpha production is not a generalised phenomenon in the elderly between their sixth and seventh decades of life. Mech. Ageing Dev. *107*: 1–14.

Naghashpour, M., Rosenblatt, M.I., Dickerson, I.M., and Dahl, G.P. (1997). Inhibitory effect of calcitonin gene-related peptide on myometrial contractility is diminished at parturition. Endocrinology *138*: 4207–4214.

Nagoshi, Y., Kuwasako, K., Cao, Y.-N., Imamura, T., Kitamura, K., and Eto, T. (2004). Tumor necrosis factor-alpha downregulates adrenomedullin receptors in human coronary artery smooth muscle cells. Peptides *25*: 1115–1121.

Nakajima, M., Hashimoto, M., Wang, F., Yamanaga, K., Nakamura, N., Uchida, T., et al. (1997). Aging decreases the production of PGI2 in rat aortic endothelial cells. Exp Ger *32*: 685–693.

Naukkarinen, A., and Jaervikallio, A. (1996). Quantitative histochemical analysis of mast cells and sensory nerves in psoriatic skin. The Journal of ….

Nelson, M.T., Huang, Y., Brayden, J.E., Hescheler, J., and Standen, N.B. (1990). Arterial dilations in response to calcitonin gene-related peptide involve activation of K+ channels. Nature *344*: 770–773.

Neumann, J.-M., Couvineau, A., Murail, S., Lacapère, J.-J., Jamin, N., and Laburthe, M. (2008). Class-B GPCR activation: is ligand helix-capping the key? Trends Biochem. Sci. *33*: 314–319.

Nguyen Dinh Cat, A., Montezano, A.C., Burger, D., and Touyz, R.M. (2013). Angiotensin II, NADPH Oxidase, and Redox Signaling in the Vasculature. Antioxid. Redox Signal. *19*: 1110–1120.

Nikitenko, L.L., Blucher, N., Fox, S.B., Bicknell, R., Smith, D.M., and Rees, M.C.P. (2006). Adrenomedullin and CGRP interact with endogenous calcitonin-receptor-like receptor in endothelial cells and induce its desensitisation by different mechanisms. J. Cell. Sci. *119*: 910–922.

Njuki, F., Nicholl, C.G., Howard, A., Mak, J.C., Barnes, P.J., Girgis, S.I., et al. (1993). A new calcitonin-receptor-like sequence in rat pulmonary blood vessels. Clin. Sci. *85*: 385–388.

Novella, S., Dantas, A.P., Segarra, G., Novensa, L., Heras, M., Hermenegildo, C., et al. (2011). Aging enhances contraction to thromboxane A(2) in aorta from female senescence-accelerated mice. Age (Dordr).

Novella, S., Dantas, A.P., Segarra, G., Novensà, L., Bueno, C., Heras, M., et al. (2010). Gathering of aging and estrogen withdrawal in vascular dysfunction of senescent accelerated mice. Exp. Gerontol. *45*: 868–874.

Oh-hashi, Y., Shindo, T., Kurihara, Y., Imai, T., Wang, Y., Morita, H., et al. (2001). Elevated sympathetic nervous activity in mice deficient in alphaCGRP. Circ. Res. *89*: 983–990.

Oliver, K.R., Wainwright, A., Kinsey, A.M., Heavens, R.P., Sirinathsinghji, D.J., and Hill, R.G. (1999). Regional and cellular localization of calcitonin gene-related peptide-receptor component protein mRNA in the guinea-pig central nervous system. Brain Res. Mol. Brain Res. *66*: 205–210.

Ollivier, V., Parry, G., Cobb, R.R., deProst, D., and Mackman, N. (1996). Elevated cyclic AMP inhibits NF-kappa B-mediated transcription in human monocytic cells and endothelial cells. J. Biol. Chem. *271*: 20828–20835.

Orlandi, A., Bochaton-Piallat, M.-L., Gabbiani, G., and Spagnoli, L.G. (2006). Aging, smooth muscle cells and vascular pathobiology: Implications for atherosclerosis. Atherosclerosis *188*: 221–230.

Ostermann, G. (2005). Involvement of JAM-A in Mononuclear Cell Recruitment on Inflamed or

Atherosclerotic Endothelium: Inhibition by Soluble JAM-A. Arterioscler. Thromb. Vasc. Biol. *25*: 729–735.

Ozaka, T., Doi, Y., Kayashima, K., and Fujimoto, S. (1997). Weibel-Palade bodies as a storage site of calcitonin gene-related peptide and endothelin-1 in blood vessels of the rat carotid body. Anat. Rec. *247*: 388–394.

Pacher, P., Beckman, J.S., and Liaudet, L. (2008). Nitric Oxide and Peroxynitrite in Health and Disease. Physiol. Rev. 1–184.

Padilla, B.E., Cottrell, G.S., Roosterman, D., Pikios, S., Muller, L., Steinhoff, M., et al. (2007). Endothelin-converting enzyme-1 regulates endosomal sorting of calcitonin receptor-like receptor and beta-arrestins. J Cell Biol *179*: 981–997.

Palmer, R.M., Ferrige, A.G., and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature *327*: 524–526.

Paone, D.V., Shaw, A.W., Nguyen, D.N., Burgey, C.S., Deng, J.Z., Kane, S.A., et al. (2007). Potent, orally bioavailable calcitonin gene-related peptide receptor antagonists for the treatment of migraine: discovery of N-[(3R,6S)-6-(2,3-difluorophenyl)-2-oxo-1- (2,2,2-trifluoroethyl)azepan-3-yl]-4- (2-oxo-2,3-dihydro-1H-imidazo[4,5-b]pyridin- 1-yl)piperidine-1-carboxamide (MK-0974). J. Med. Chem. *50*: 5564–5567.

Parameswaran, N., and Spielman, W.S. (2006). RAMPs: The past, present and future. Trends Biochem. Sci. *31*: 631–638.

Petersen, K.A., Birk, S., Lassen, L.H., Kruuse, C., Jonassen, O., Lesko, L., et al. (2005). The CGRP-antagonist, BIBN4096BS does not affect cerebral or systemic haemodynamics in healthy volunteers. Cephalalgia *25*: 139–147.

Pin, S.S., and Bahr, B.A. (2008). Protein kinase C is a common component of CGRP receptor desensitization induced by distinct agonists. Eur J Pharmacol *587*: 8–15.

Pokabla, M.J., Dickerson, I.M., and Papka, R.E. (2002). Calcitonin gene-related peptide-receptor component protein expression in the uterine cervix, lumbosacral spinal cord, and dorsal root ganglia. Peptides *23*: 507–514.

Pollock, J., McFarlane, S.M., Connell, M.C., Zehavi, U., Vandenabeele, P., MacEwan, D.J., et al. (2002). TNF-alpha receptors simultaneously activate Ca2+ mobilisation and stress kinases in cultured sensory neurones. Neuropharmacology *42*: 93–106.

Portaluppi, F., Trasforini, G., Margutti, A., Vergnani, L., Ambrosio, M.R., Rossi, R., et al. (1992). Circadian rhythm of calcitonin gene-related peptide in uncomplicated essential hypertension. J. Hypertens. *10*: 1227–1234.

Prado, M.A., Evans-Bain, B., Oliver, K.R., and Dickerson, I.M. (2001). The role of the CGRP-receptor component protein (RCP) in adrenomedullin receptor signal transduction. Peptides *22*: 1773–1781.

Puca, A.A., Carrizzo, A., Villa, F., Ferrario, A., Casaburo, M., Maciąg, A., et al. (2013). Vascular ageing: the role of oxidative stress. Int. J. Biochem. Cell Biol. *45*: 556–559.

Qin, X.-P., Ye, F., Hu, C.-P., Liao, D.-F., Deng, H.-W., and Li, Y.-J. (2004). Effect of calcitonin gene-related peptide on angiotensin II-induced proliferation of rat vascular smooth muscle cells. Eur J

Pharmacol *488*: 45–49.

Qin, X.-P., Zeng, S.-Y., Li, D., Chen, Q.-Q., Luo, D., Zhang, Z., et al. (2007). Calcitonin gene-related Peptide-mediated depressor effect and inhibiting vascular hypertrophy of rutaecarpine in renovascular hypertensive rats. J. Cardiovasc. Pharmacol. *50*: 654–659.

Qin, Z. (2012). The use of THP-1 cells as a model for mimicking the function and regulation of monocytes and macrophages in the vasculature. Atherosclerosis *221*: 2–11.

Qing, X., and Keith, I.M. (2003). Targeted blocking of gene expression for CGRP receptors elevates pulmonary artery pressure in hypoxic rats. Am. J. Physiol. Lung Cell Mol. Physiol. *285*: L86–96.

Quayle, J.M., Bonev, A.D., Brayden, J.E., and Nelson, M.T. (1994). Calcitonin gene-related peptide activated ATP-sensitive K+ currents in rabbit arterial smooth muscle via protein kinase A. J Physiol (Lond) *475*: 9–13.

Rauscher, F.M. (2003). Aging, Progenitor Cell Exhaustion, and Atherosclerosis. Circulation *108*: 457–463.

Reckelhoff, J.F., Zhang, H., Srivastava, K., Roberts, L.J., Morrow, J.D., and Romero, J.C. (2000). Subpressor doses of angiotensin II increase plasma F(2)-isoprostanes in rats. Hypertension *35*: 476–479.

Richter, J., Andersson, R., Edvinsson, L., and Gullberg, U. (1992). Calcitonin gene-related peptide (CGRP) activates human neutrophils--inhibition by chemotactic peptide antagonist BOC-MLP. Immunology *77*: 416–421.

Rimoldi, S.F., Scherrer, U., and Messerli, F.H. (2014). Secondary arterial hypertension: when, who, and how to screen? Eur. Heart J. *35*: 1245–1254.

Rippe, C., Lesniewski, L., Connell, M., LaRocca, T., Donato, A., and Seals, D. (2010). Short-term calorie restriction reverses vascular endothelial dysfunction in old mice by increasing nitric oxide and reducing oxidative stress. Aging Cell *9*: 304–312.

Roh, J., Chang, C.L., Bhalla, A., Klein, C., and Hsu, S.Y.T. (2004). Intermedin is a calcitonin/calcitonin gene-related peptide family peptide acting through the calcitonin receptor-like receptor/receptor activity-modifying protein receptor complexes. J. Biol. Chem. *279*: 7264–7274.

Rojas, A., Romay, S., González, D., Herrera, B., Delgado, R., and Otero, K. (2000). Regulation of endothelial nitric oxide synthase expression by albumin-derived advanced glycosylation end products. Circ. Res. *86*: E50–4.

Roosterman, D., Cottrell, G.S., Padilla, B.E., Muller, L., Eckman, C.B., Bunnett, N.W., et al. (2007). Endothelin-converting enzyme 1 degrades neuropeptides in endosomes to control receptor recycling. Proc. Natl. Acad. Sci. U.S.a. *104*: 11838–11843.

Rosenblatt, M.I., Dahl, G.P., and Dickerson, I.M. (2000). Characterization and localization of the rabbit ocular calcitonin gene-related peptide (CGRP)-receptor component protein (RCP). *41*: 1159–1167.

Rubio, C., Moreno, A., Briones, A., Ivorra, M.D., D'Ocon, P., and Vila, E. (2002). Alterations by age of calcium handling in rat resistance arteries. J. Cardiovasc. Pharmacol. *40*: 832–840.

Russell, F.A., King, R., Smillie, S.J., Kodji, X., and Brain, S.D. (2014). Calcitonin gene-related Peptide: physiology and pathophysiology. Physiol. Rev. *94*: 1099–1142.

Sabharwal, R., Zhang, Z., Lu, Y., Abboud, F.M., Russo, A.F., and Chapleau, M.W. (2010). Receptor activity-modifying protein 1 increases baroreflex sensitivity and attenuates Angiotensin-induced hypertension. Hypertension *55*: 627–635.

Saetrum Opgaard, O., Hasbak, P., de Vries, R., Saxena, P.R., and Edvinsson, L. (2000). Positive inotropy mediated via CGRP receptors in isolated human myocardial trabeculae. Eur J Pharmacol *397*: 373–382.

Salmon, A.M., Damaj, I., Sekine, S., Picciotto, M.R., Marubio, L., and Changeux, J.P. (1999). Modulation of morphine analgesia in alphaCGRP mutant mice. Neuroreport *10*: 849–854.

Salvatore, C.A., Hershey, J.C., Corcoran, H.A., Fay, J.F., Johnston, V.K., Moore, E.L., et al. (2008). Pharmacological characterization of MK-0974 [N-[(3R,6S)-6-(2,3-difluorophenyl)-2-oxo-1-(2,2,2-trifluoroethyl)azepan-3-yl]-4-(2-oxo-2,3-dihydro-1H-imidazo[4,5-b]pyridin-1-yl)piperidine-1-carboxamide], a potent and orally active calcitonin gene-related peptide receptor antagonist for the treatment of migraine. J Pharmacol Exp Ther *324*: 416–421.

Salvatore, C.A., Mallee, J.J., Bell, I.M., Zartman, C.B., Williams, T.M., Koblan, K.S., et al. (2006). Identification and pharmacological characterization of domains involved in binding of CGRP receptor antagonists to the calcitonin-like receptor. Biochemistry *45*: 1881–1887.

Salvatore, C.A., Moore, E.L., Calamari, A., Cook, J.J., Michener, M.S., O'Malley, S., et al. (2010). Pharmacological properties of MK-3207, a potent and orally active calcitonin gene-related peptide receptor antagonist. J Pharmacol Exp Ther *333*: 152–160.

Sams-Nielsen, A., Orskov, C., and Jansen-Olesen, I. (2001). Pharmacological evidence for CGRP uptake into perivascular capsaicin sensitive nerve terminals. Br J Pharmacol *132*: 1145–1153.

Santos-Parker, J.R., LaRocca, T.J., and Seals, D.R. (2014). Aerobic exercise and other healthy lifestyle factors that influence vascular aging. AJP: Advances in Physiology Education *38*: 296–307.

Sardi, C., Zambusi, L., Finardi, A., Ruffini, F., Tolun, A.A., Dickerson, I.M., et al. (2014). Involvement of calcitonin gene-related peptide and receptor component protein in experimental autoimmune encephalomyelitis. J. Neuroimmunol. 1–12.

Sassone-Corsi, P. (1998). Coupling gene expression to cAMP signalling: role of CREB and CREM. Int. J. Biochem. Cell Biol. *30*: 27–38.

Sato, I., Morita, I., Kaji, K., Ikeda, M., Nagao, M., and Murota, S. (1993). Reduction of nitric oxide producing activity associated with in vitro aging in cultured human umbilical vein endothelial cell. Biochem. Biophys. Res. Commun. *195*: 1070–1076.

Saurwein-Teissl, M., Blasko, I., Zisterer, K., Neuman, B., Lang, B., and Grubeck-Loebenstein, B. (2000). An imbalance between pro- and anti-inflammatory cytokines, a characteristic feature of old age. Cytokine *12*: 1160–1161.

Savoia, C., Burger, D., Nishigaki, N., Montezano, A., and Touyz, R.M. (2011). Angiotensin II and the vascular phenotype in hypertension. Expert Rev Mol Med *13*: e11.

Schifter, S., Krusell, L.R., and Sehested, J. (1991). Normal serum levels of calcitonin gene-related

peptide (CGRP) in mild to moderate essential hypertension. Am. J. Hypertens. *4*: 565–569.

Schmitt, M.M.N., Fraemohs, L., Hackeng, T.M., Weber, C., and Koenen, R.R. (2014). Atherogenic mononuclear cell recruitment is facilitated by oxidized lipoprotein-induced endothelial junctional adhesion molecule-A redistribution. Atherosclerosis *234*: 254–264.

Schulz, E., Gori, T., and Münzel, T. (2011). Oxidative stress and endothelial dysfunction in hypertension. Hypertens. Res. *34*: 665–673.

Schutz, B., Mauer, D., Salmon, A.-M., Changeux, J.-P., and Zimmer, A. (2004). Analysis of the cellular expression pattern of b-CGRP in a-CGRP-deficient mice. J. Comp. Neurol. *476*: 32–43.

Seals, D.R. (2004). Collateral damage: cardiovascular consequences of chronic sympathetic activation with human aging. Am. J. Physiol. Heart Circ. Physiol. *287*: H1895–H1905.

Seals, D.R., Jablonski, K.L., and Donato, A.J. (2011). Aging and vascular endothelial function in humans. Clin. Sci. *120*: 357–375.

Sedger, L.M., and McDermott, M.F. (2014). TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants - past, present and future. Cytokine Growth Factor Rev. *25*: 453–472.

Sessa, W.C., Fulton, D., Gratton, J.-P., McCabe, T.J., Fontana, J., Fujio, Y., et al. (1999). Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature *399*: 597–601.

Sheykhzade, M., Gupta, S., Sørensen, T., Sørensen, O.A., Koch, H., Boonen, H.C.M., et al. (2011). Characterization of capsaicin induced responses in mice vas deferens: Evidence of CGRP uptake. Eur J Pharmacol *667*: 375–382.

Shi, R.-Z., Hu, C.-P., Luo, D., Li, D., Pan, W., Li, S.-X., et al. (2012). Decreased anandamide transporter activity and calcitonin gene-related peptide production in spontaneously hypertensive rats: role of angiotensin II. Eur J Pharmacol 1–7.

Siervo, M., Lara, J., Chowdhury, S., Ashor, A., Oggioni, C., and Mathers, J.C. (2014). Effects of the Dietary Approach to Stop Hypertension (DASH) diet on cardiovascular risk factors: a systematic review and meta-analysis. Br J Nutr 1–15.

Simm, A. (2013). Protein glycation during aging and in cardiovascular disease. J Proteomics *92*: 248–259.

Simms, J., Hay, D.L., Wheatley, M., and Poyner, D.R. (2006). Characterization of the structure of RAMP1 by mutagenesis and molecular modeling. Biophys. J. *91*: 662–669.

Sin, T.K., Yu, A.P., Yung, B.Y., Yip, S.P., Chan, L.W., Wong, C.S., et al. (2014). Modulating effect of SIRT1 activation induced by resveratrol on Foxo1-associated apoptotic signalling in senescent heart. J Physiol (Lond) *592*: 2535–2548.

Sinkler, S.Y., and Segal, S.S. (2014). Aging alters reactivity of microvascular resistance networks in mouse gluteus maximus muscle. AJP: Heart and Circulatory Physiology *307*: H830–9.

Sirén, A.L., and Feuerstein, G. (1988). Cardiovascular effects of rat calcitonin gene-related peptide in the conscious rat. J Pharmacol Exp Ther *247*: 69–78.

Smillie, S.-J., and Brain, S.D. (2011). Calcitonin gene-related peptide (CGRP) and its role in hypertension. Neuropeptides *45*: 93–104.

Smillie, S.-J., King, R., Kodji, X., Outzen, E., Pozsgai, G., Fernandes, E., et al. (2014). An ongoing role of α-calcitonin gene-related peptide as part of a protective network against hypertension, vascular hypertrophy, and oxidative stress. Hypertension *63*: 1056–1062.

Smith, A.R., Visioli, F., and Hagen, T.M. (2006). Plasma membrane-associated endothelial nitric oxide synthase and activity in aging rat aortic vascular endothelia markedly decline with age. Archives of Biochemistry and Biophysics *454*: 100–105.

Somasundaram, C., Bukoski, R.D., and Diz, D.I. (2012). Identification and Characterization of Novel Perivascular Adventitial Cells in the Whole Mount Mesenteric Branch Artery Using Immunofluorescent Staining and Scanning Confocal Microscopy Imaging. Int J Cell Biol *2012*: 1–6.

Somasundaram, C., Diz, D.I., Coleman, T., and Bukoski, R.D. (2006). Adventitial neuronal somata. J Vasc Res *43*: 278–288.

Soucy, K.G., Ryoo, S., Benjo, A., Lim, H.K., Gupta, G., Sohi, J.S., et al. (2005). Impaired shear stress-induced nitric oxide production through decreased NOS phosphorylation contributes to age-related vascular stiffness. J. Appl. Physiol. *101*: 1751–1759.

Spiecker, M., Darius, H., and Liao, J.K. (2000). A functional role of I kappa B-epsilon in endothelial cell activation. J. Immunol. *164*: 3316–3322.

Stead, R.H., Tomioka, M., Quinonez, G., Simon, G.T., Felten, S.Y., and Bienenstock, J. (1987). Intestinal Mucosal Mast-Cells in Normal and Nematode-Infected Rat Intestines Are in Intimate Contact with Peptidergic Nerves. Proc. Natl. Acad. Sci. U.S.a. *84*: 2975–2979.

Steenbergh, P.H., Höppener, J.W., Zandberg, J., Lips, C.J., and Jansz, H.S. (1985). A second human calcitonin/CGRP gene. FEBS Letters *183*: 403–407.

Steenbergh, P.H., Höppener, J.W., Zandberg, J., Visser, A., Lips, C.J., and Jansz, H.S. (1986). Structure and expression of the human calcitonin/CGRP genes. FEBS Letters *209*: 97–103.

Steiner, S., Born, W., Fischer, J.A., and Muff, R. (2003). The function of conserved cysteine residues in the extracellular domain of human receptor-activity-modifying protein. FEBS Letters *555*: 285–290.

Steinhoff, M., Vergnolle, N., Young, S.H., Tognetto, M., Amadesi, S., Ennes, H.S., et al. (2000). Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism. Nature Medicine *6*: 151–158.

Sun, D. (2004). Reduced release of nitric oxide to shear stress in mesenteric arteries of aged rats. Am. J. Physiol. Heart Circ. Physiol. *286*: H2249–H2256.

Sun, W., Guo, J., Tang, Y., and Wang, X. (1998). Alteration of capsaicin and endotoxin-induced calcitonin gene-related peptide release from mesenteric arterial bed and spinal cord slice in 18-month-old rats. J. Neurosci. Res. *53*: 385–392.

Sundlöf, G., and Wallin, B.G. (1978). Human muscle nerve sympathetic activity at rest. Relationship to blood pressure and age. J Physiol (Lond) *274*: 621–637.

Sung, C.P., Arleth, A.J., Aiyar, N., Bhatnagar, P.K., Lysko, P.G., and Feuerstein, G. (1992). CGRP

stimulates the adhesion of leukocytes to vascular endothelial cells. Peptides *13*: 429–434.

Supowit, S.C., Katki, K.A., Dickerson, I.M., and Dipette, D.J. (2011). Vascular reactivity to calcitonin gene-related peptide is enhanced in subtotal nephrectomy-salt induced hypertension. AJP: Heart and Circulatory Physiology *301*: H683–8.

Supowit, S.C., Ramana, C.V., Westlund, K.N., and DiPette, D.J. (1993). Calcitonin gene-related peptide gene expression in the spontaneously hypertensive rat. Hypertension *21*: 1010–1014.

Supowit, S.C., Rao, A., Bowers, M.C., Zhao, H., Fink, G., Steficek, B., et al. (2005). Calcitonin gene-related peptide protects against hypertension-induced heart and kidney damage. Hypertension *45*: 109–114.

Supowit, S.C., Zhao, H., and DiPette, D.J. (2001). Nerve growth factor enhances calcitonin gene-related peptide expression in the spontaneously hypertensive rat. Hypertension *37*: 728–732.

Supowit, S.C., Zhao, H., Hallman, D.M., and DiPette, D.J. (1997). Calcitonin gene-related peptide is a depressor of deoxycorticosterone-salt hypertension in the rat. Hypertension *29*: 945–950.

Supowit, S.C., Zhao, H., Hallman, D.M., and DiPette, D.J. (1998). Calcitonin gene-related peptide is a depressor in subtotal nephrectomy hypertension. Hypertension *31*: 391–396.

Supowit, S.C., Zhao, H., Wang, D.H., and DiPette, D.J. (1995). Regulation of neuronal calcitonin gene-related peptide expression. Role of increased blood pressure. Hypertension *26*: 1177–1180.

Şakul, A., Cumaoğlu, A., Aydın, E., Arı, N., Dilsiz, N., and Karasu, Ç. (2013). Age- and diabetes-induced regulation of oxidative protein modification in rat brain and peripheral tissues: Consequences of treatment with antioxidant pyridoindole. Exp Ger *48*: 476–484.

Taddei, S., Virdis, A., Mattei, P., Ghiadoni, L., Gennari, A., Fasolo, C.B., et al. (1995). Aging and endothelial function in normotensive subjects and patients with essential hypertension. Circulation *91*: 1981–1987.

Tanabe, T., Otani, H., Zeng, X.T., Mishima, K., Ogawa, R., and Inagaki, C. (1996). Inhibitory effects of calcitonin gene-related peptide on substance-P-induced superoxide production in human neutrophils. Eur J Pharmacol *314*: 175–183.

Tang, E.H.C., and Vanhoutte, P.M. (2007). Gene expression changes of prostanoid synthases in endothelial cells and prostanoid receptors in vascular smooth muscle cells caused by aging and hypertension. Physiol. Genomics *32*: 409–418.

Tang, E.H.C., and Vanhoutte, P.M. (2008). Gene expression changes of prostanoid synthases in endothelial cells and prostanoid receptors in vascular smooth muscle cells caused by aging and hypertension. Physiol. Genomics *32*: 409–418.

Tang, J.A., Xu, D., Yuan, Q.X., Meng, Z.H., Cheng, F.R., Chen, M.Z., et al. (1989). Calcitonin gene-related peptide in the pathogenesis and treatment of hypertension. Chin. Med. J. *102*: 897–901.

Terenghi, G. (1999). Peripheral nerve regeneration and neurotrophic factors. J. Anat. *194 (Pt 1)*: 1–14.

Tjen-A-Looi, S., Ekman, R., Lippton, H., Cary, J., and Keith, I. (1992). CGRP and somatostatin modulate chronic hypoxic pulmonary hypertension. Am. J. Physiol. *263*: H681–90.

Tjen-A-Looi, S., Kraiczi, H., Ekman, R., and Keith, I.M. (1998). Sensory CGRP depletion by capsaicin exacerbates hypoxia-induced pulmonary hypertension in rats. Regul. Pept. *74*: 1–10.

Torii, H., Hosoi, J., Beissert, S., Xu, S., Fox, F.E., Asahina, A., et al. (1997). Regulation of cytokine expression in macrophages and the Langerhans cell-like line XS52 by calcitonin gene-related peptide. J. Leukoc. Biol. *61*: 216–223.

Toth, P., Csiszar, A., Tucsek, Z., Sosnowska, D., Gautam, T., Koller, A., et al. (2013a). Role of 20-HETE, TRPC channels, and BKCa in dysregulation of pressure-induced Ca2+ signaling and myogenic constriction of cerebral arteries in aged hypertensive mice. AJP: Heart and Circulatory Physiology *305*: H1698–708.

Toth, P., Tucsek, Z., Sosnowska, D., Gautam, T., Mitschelen, M., Tarantini, S., et al. (2013b). Age-related autoregulatory dysfunction and cerebromicrovascular injury in mice with angiotensin II-induced hypertension. 1–11.

Traupe, T., Lang, M., Goettsch, W., Münter, K., Morawietz, H., Vetter, W., et al. (2002). Obesity increases prostanoid-mediated vasoconstriction and vascular thromboxane receptor gene expression. J. Hypertens. *20*: 2239–2245.

Tschopp, F.A., Henke, H., Petermann, J.B., Tobler, P.H., Janzer, R., Hökfelt, T., et al. (1985). Calcitonin gene-related peptide and its binding sites in the human central nervous system and pituitary. Proc. Natl. Acad. Sci. U.S.a. *82*: 248–252.

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., and Tada, K. (1980). Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int. J. Cancer *26*: 171–176.

Tsujikawa, K., Yayama, K., Hayashi, T., Matsushita, H., Yamaguchi, T., Shigeno, T., et al. (2007). Hypertension and dysregulated proinflammatory cytokine production in receptor activity-modifying protein 1-deficient mice. Proc. Natl. Acad. Sci. U.S.a. *104*: 16702–16707.

Ubersax, J.A., and Ferrell, J.E., Jr (2007). Mechanisms of specificity in protein phosphorylation. Nat Rev Mol Cell Biol *8*: 530–541.

Uddman, R., Edvinsson, L., Ekblad, E., Håkanson, R., and Sundler, F. (1986). Calcitonin gene-related peptide (CGRP): perivascular distribution and vasodilatory effects. Regul. Pept. *15*: 1–23.

Ungvari, Z., Bailey-Downs, L., Gautam, T., Sosnowska, D., Wang, M., Monticone, R.E., et al. (2011). Age-Associated Vascular Oxidative Stress, Nrf2 Dysfunction, and NF-{kappa}B Activation in the Nonhuman Primate Macaca mulatta. J. Gerontol. a Biol. Sci. Med. Sci. *66*: 866–875.

Ungvari, Z., Kaley, G., de Cabo, R., Sonntag, W.E., and Csiszar, A. (2010). Mechanisms of vascular aging: new perspectives. J. Gerontol. a Biol. Sci. Med. Sci. *65*: 1028–1041.

van der Loo, B., Labugger, R., Skepper, J.N., Bachschmid, M., Kilo, J., Powell, J.M., et al. (2000). Enhanced peroxynitrite formation is associated with vascular aging. J. Exp. Med. *192*: 1731–1744.

van der Loo, B., Schildknecht, S., Zee, R., and Bachschmid, M.M. (2009). Signalling processes in endothelial ageing in relation to chronic oxidative stress and their potential therapeutic implications in humans. Exp. Physiol. *94*: 305–310.

Van Vliet, B.N., and Montani, J.-P. (2008). The time course of salt-induced hypertension, and why it

matters. Int J Obes (Lond) *32 Suppl 6*: S35–47.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol *3*:.

Vasan, R.S., Demissie, S., Kimura, M., Cupples, L.A., Rifai, N., White, C., et al. (2008). Association of leukocyte telomere length with circulating biomarkers of the renin-angiotensin-aldosterone system: the Framingham Heart Study. Circulation *117*: 1138–1144.

Vause, C.V., and Durham, P.L. (2010). Calcitonin gene-related peptide differentially regulates gene and protein expression in trigeminal glia cells: findings from array analysis. Neurosci. Lett. *473*: 163–167.

Verdú, E., Ceballos, D., Vilches, J.J., and Navarro, X. (2000). Influence of aging on peripheral nerve function and regeneration. J. Peripher. Nerv. Syst. *5*: 191–208.

Virdis, A., Ghiadoni, L., Giannarelli, C., and Taddei, S. (2010). Endothelial dysfunction and vascular disease in later life. Maturitas *67*: 20–24.

Walker, C.S., Conner, A.C., Poyner, D.R., and Hay, D.L. (2010a). Regulation of signal transduction by calcitonin gene-related peptide receptors. Trends Pharmacol Sci *31*: 476–483.

Walker, C.S., Li, X., Whiting, L., Glyn-Jones, S., Zhang, S., Hickey, A.J., et al. (2010b). Mice lacking the neuropeptide alpha-calcitonin gene-related peptide are protected against diet-induced obesity. Endocrinology *151*: 4257–4269.

Walter, S., Alibhoy, A., Escandon, R., and Bigal, M.E. (2014). Evaluation of cardiovascular parameters in cynomolgus monkeys following IV administration of LBR-101, a monoclonal antibody against calcitonin gene-related peptide. MAbs *6*: 871–878.

Wang, M., Jiang, L., Monticone, R.E., and Lakatta, E.G. (2013). Proinflammation: the key to arterialaging. Trends Endocrinol. Metab. 1–8.

Wang, M., Takagi, G., Asai, K., Resuello, R.G., Natividad, F.F., Vatner, D.E., et al. (2003). Aging increases aortic MMP-2 activity and angiotensin II in nonhuman primates. Hypertension *41*: 1308–1316.

Wang, M., Zhang, J., Jiang, L.-Q., Spinetti, G., Pintus, G., Monticone, R., et al. (2007a). Proinflammatory profile within the grossly normal aged human aortic wall. Hypertension *50*: 219–227.

Wang, Z.-G., Liu, J.-L., Liu, Y., Wen, S.-J., Wen, J., Liu, Y.-P., et al. (2007b). [Increased plasma vasoactive substances and antioxidant enzymes levels in prehypertensive patients]. Zhonghua Xin Xue Guan Bing Za Zhi *35*: 719–722.

Watkins, H.A., Rathbone, D.L., Barwell, J., Hay, D.L., and Poyner, D.R. (2012). Structure-activity relationships for alpha calcitonin gene-related peptide. Br J Pharmacol.

Weihe, E., Müller, S., FINK, T., and ZENTEL, H.J. (1989). Tachykinins, Calcitonin Gene-Related Peptide and Neuropeptide-Y in Nerves of the Mammalian Thymus - Interactions with Mast-Cells in Autonomic and Sensory Neuroimmunomodulation. Neurosci. Lett. *100*: 77–82.

Weihe, E., Nohr, D., Michel, S., and Müller, S. (1991). Molecular anatomy of the neuro-immune connection. International Journal … *59*: 1–23.

Weissmann, A. (1891). Essays on Heredity and Kindred Biological Problems (Clarendon, London and New York: Oxford University press).

Wellman, G.C., Quayle, J.M., and Standen, N.B. (1998). ATP-sensitive K+ channel activation by calcitonin gene-related peptide and protein kinase A in pig coronary arterial smooth muscle. J Physiol (Lond) *507 (Pt 1)*: 117–129.

Werner, N., Kosiol, S., Schiegl, T., Ahlers, P., Walenta, K., Link, A., et al. (2005). Circulating endothelial progenitor cells and cardiovascular outcomes. N. Engl. J. Med. *353*: 999–1007.

Westcott, E.B., and Segal, S.S. (2013). Ageing alters perivascular nerve function of mouse mesenteric arteries in vivo. J Physiol (Lond) *591*: 1251–1263.

Westlund, K.N., DiPette, D.J., Carson, J., and Holland, O.B. (1991). Decreased spinal cord content of calcitonin gene-related peptide in the spontaneously hypertensive rat. Neurosci. Lett. *131*: 183–186.

Whittle, B.J., Lopez-Belmonte, J., and Moncada, S. (1992). Nitric oxide mediates rat mucosal vasodilatation induced by intragastric capsaicin. Eur J Pharmacol *218*: 339–341.

Williams, G.C. (1957). Pleiotropy, Natural-Selection, and the Evolution of Senescence. Evolution *11*: 398–411.

Wilson, P.W., D'Agostino, R.B., Levy, D., Belanger, A.M., Silbershatz, H., and Kannel, W.B. (1998). Prediction of coronary heart disease using risk factor categories. Circulation *97*: 1837–1847.

Wimalawansa, S.J. (1991). Age-related increase of calcitonin gene-related peptide in rat thyroid and circulation. Peptides *12*: 1143–1147.

Wittchen, E.S. (2009). Endothelial signaling in paracellular and transcellular leukocyte transmigration. Frontiers in Bioscience *14*: 2522–2545.

Woollard, K.J., and Geissmann, F. (2010). Monocytes in atherosclerosis: subsets and functions. Nature Publishing Group *7*: 77–86.

Wu, X., Zhou, Q., Huang, L., Sun, A., Wang, K., Zou, Y., et al. (2007). Ageing-exaggerated proliferation of vascular smooth muscle cells is related to attenuation of Jagged1 expression in endothelial cells. Cardiovasc. Res. *77*: 800–808.

Xiangdong, L., Yuanwu, L., Hua, Z., Liming, R., Qiuyan, L., and Ning, L. (2011). Animal models for the atherosclerosis research: a review. Protein Cell *2*: 189–201.

Xin, M.-G., Zhang, J., Block, E.R., and Patel, J.M. (2003). Senescence-enhanced oxidative stress is associated with deficiency of mitochondrial cytochrome c oxidase in vascular endothelial cells. Mech. Ageing Dev. *124*: 911–919.

Xu, D., Wang, X.A., Wang, J.P., Yuan, Q.X., Fiscus, R.R., Chang, J.K., et al. (1989). Calcitonin gene-related peptide (CGRP) in normotensive and spontaneously hypertensive rats. Peptides *10*: 309–312.

Xu, K.M., Tang, F., and Han, C. (1997). Alterations of mRNA levels of alpha 1-adrenoceptor subtypes with maturation and ageing in different rat blood vessels. Clin. Exp. Pharmacol. Physiol. *24*: 415–417.

Yamada, M., Ishikawa, T., Fujimori, A., Miyauchi, T., and Goto, K. (1998). Enhanced depressor and hyperemic responses to calcitonin gene-related peptide in spontaneously hypertensive rats. Peptides *19*: 697–701.

Yamaga, N., Kawasaki, H., Inaizumi, K., Shimizu, M., Nakamura, A., and Kurosaki, Y. (2001). Age-Related decrease in calcitonin gene-related peptide mRNA in the dorsal root ganglia of spontaneously hypertensive rats. Jpn. J. Pharmacol. *86*: 448–450.

Yamaguchi, A., Chiba, T., Okimura, Y., Yamatani, T., Morishita, T., Nakamura, A., et al. (1988). Receptors for calcitonin gene-related peptide on the rat liver plasma membranes. Biochem. Biophys. Res. Commun. *152*: 383–391.

Yan, L.Z., Johnson, K.W., Rothstein, E., Flora, D., Edwards, P., Li, B., et al. (2011). Discovery of potent, cyclic calcitonin gene-related peptide receptor antagonists. J. Peptide Sci. *17*: 383–386.

Yang, L., Sakurai, T., Kamiyoshi, A., Ichikawa-Shindo, Y., Kawate, H., Yoshizawa, T., et al. (2013). Endogenous CGRP protects against neointimal hyperplasia following wire-induced vascular injury. 1–12.

Yang, Y.-M., Huang, A., Kaley, G., and Sun, D. (2009). eNOS uncoupling and endothelial dysfunction in aged vessels. AJP: Heart and Circulatory Physiology *297*: H1829–36.

Ye, F., Deng, P.-Y., Li, D., Luo, D., Li, N.-S., Deng, S., et al. (2007). Involvement of endothelial cell-derived CGRP in heat stress-induced protection of endothelial function. Vascul. Pharmacol. *46*: 238–246.

Zaccolo, M. (2009). cAMP signal transduction in the heart: understanding spatial control for the development of novel therapeutic strategies. Br J Pharmacol *158*: 50–60.

Zarkovic, N. (2003). 4-Hydroxynonenal as a bioactive marker of pathophysiological processes. Molecular Aspects of Medicine *24*: 281–291.

Zeller, J., Poulsen, K.T., Sutton, J.E., Abdiche, Y.N., Collier, S., Chopra, R., et al. (2008). CGRP function-blocking antibodies inhibit neurogenic vasodilatation without affecting heart rate or arterial blood pressure in the rat. Br J Pharmacol *155*: 1093–1103.

Zhang, Y.-M., Peng, J., Hu, C.-P., Jiang, Q.-T., Jiang, G.-L., and Li, Y.-J. (2009). Clonidine induces calcitonin gene-related peptide expression via nitric oxide pathway in endothelial cells. Peptides *30*: 1746–1752.

Zhang, Z., and Russo, A.F. (2010). Receptor activity-modifying protein-1 augments cerebrovascular responses to calcitonin gene-related peptide and inhibits angiotensin II-induced vascular dysfunction. Stroke *41*: 2329–2334.

Zhao, F.-P., Guo, Z., and Wang, P.-F. (2010). Calcitonin gene related peptide (CGRP) inhibits norepinephrine induced apoptosis in cultured rat cardiomyocytes not via PKA or PKC pathways. Neurosci. Lett. *482*: 163–166.

Zhao, Q., Liu, Z., Wang, Z., Yang, C., Liu, J., and Lu, J. (2007). Effect of prepro-calcitonin gene-related peptide-expressing endothelial progenitor cells on pulmonary hypertension. Ann. Thorac. Surg. *84*: 544–552.

Zhao, Z., Pan, X., Liu, L., and Liu, N. (2014). Telomere length maintenance, shortening, and

lengthening. J. Cell. Physiol. *229*: 1323–1329.

Zheng, L.-R., Han, J., Yao, L., Sun, Y.-L., Jiang, D.-M., Hu, S.-J., et al. (2011). Up-regulation of calcitonin gene-related peptide protects streptozotocin-induced diabetic hearts from ischemia/reperfusion injury. Int J Cardiol.

Zhong, B., and Wang, D.H. (2007). TRPV1 gene knockout impairs preconditioning protection against myocardial injury in isolated perfused hearts in mice. Am. J. Physiol. Heart Circ. Physiol. *293*: H1791–8.

Zhou, X., Bohlen, H.G., Unthank, J.L., and Miller, S.J. (2009). Abnormal nitric oxide production in aged rat mesenteric arteries is mediated by NAD(P)H oxidase-derived peroxide. Am. J. Physiol. Heart Circ. Physiol. *297*: H2227–H2233.

Zhou, Z., Hu, C.-P., Wang, C.-J., Li, T.-T., Peng, J., and Li, Y.-J. (2010a). Calcitonin gene-related peptide inhibits angiotensin II-induced endothelial progenitor cells senescence through up-regulation of klotho expression. Atherosclerosis *213*: 92–101.

Zhou, Z., Peng, J., Wang, C.-J., Li, D., Li, T.-T., Hu, C.-P., et al. (2010b). Accelerated senescence of endothelial progenitor cells in hypertension is related to the reduction of calcitonin gene-related peptide. J. Hypertens. *28*: 931–939.

Zhu, Y., Qiu, H., Trzeciakowski, J.P., Sun, Z., Li, Z., Hong, Z., et al. (2012). Temporal analysis of vascular smooth muscle cell elasticity and adhesion reveals oscillation waveforms that differ with aging. Aging Cell *11*: 741–750.

Zimmerman, B.J., Anderson, D.C., and Granger, D.N. (1992). Neuropeptides promote neutrophil adherence to endothelial cell monolayers. Am. J. Physiol. *263*: G678–82.

(2003). Calcitonin gene related peptide and N-procalcitonin modulate CD11b upregulation in lipopolysaccharide activated monocytes and neutrophils. Intensive Care Med *29*: 923–928.