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The Relationship between Salt and Glucocorticoids: Implications for Salt-Sensitive Hypertension



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Doctor of Philosophy
The University of Edinburgh
2020

Declaration

I declare that the work carried out during this PhD was composed solely by myself and is entirely my own work, unless otherwise stated by reference or acknowledgement. This thesis has not previously been submitted for any other degree or professional qualification.

Signed:

Hannah Mhairi Costello

29.10.2020

Abstract

Salt-sensitive blood pressure (BP) reflects underlying renal salt excretion impairment, suggesting compromised pressure natriuresis (PN) relationship, and vascular (endothelial) dysfunction. People with abnormal glucocorticoid homeostasis, such as in Cushing's syndrome, are often salt-sensitive but the underlying mechanisms are not clearly defined. Evidence has suggested abnormal glucocorticoid activity, via dysregulation of hypothalamic pituitary adrenocortical (HPA) axis, may have a major contributory role in salt-sensitivity. Therefore, I hypothesised that glucocorticoid excess causes vascular and renal dysfunction, contributing to salt-sensitive hypertension. I tested this hypothesis in a mouse model of ACTH-dependent Cushing's syndrome. BP was measured longitudinally in adult male C57BL/6JCrI mice by radiotelemetry, examining the effect of high salt diet (3% sodium) before and after chronic ACTH treatment. I found underlying salt-sensitivity in C57BL/6JCrI mice: BP increased by ~12 mmHg following the transition from a control salt diet (0.3% sodium) to high salt diet. Following washout, ACTH was infused by osmotic minipump, which increased daytime BP (inactive phase), flattening the diurnal BP rhythm. Reintroduction of high salt diet amplified salt-sensitivity, increasing BP ~20 mmHg. To investigate underlying mechanisms of salt-sensitivity, the acute PN relationship and vascular function were assessed in ACTH-treated mice. The acute PN relationship was unaltered with glucocorticoid excess before and after a high salt challenge. The sensitivity and maximal contractile response to vasoconstrictor phenylephrine was significantly reduced in renal arteries following glucocorticoid excess before and after a high salt challenge, with no change in mesenteric arteries. The sensitivity and maximal dilatory response to both endothelium-dependent and -independent vasodilators was reduced in renal arteries, with no changes in mesenteric arteries. Messenger RNA (mRNA) levels of glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) were assessed in renal arteries. Chronic glucocorticoid excess decreased GR but not MR mRNA in the renal artery, suggesting that glucocorticoids are primarily acting via MR in the renal artery. Therefore, I hypothesised antagonism of the MR could be protective.

The effect of MR blockade (20 mg/kg/day spironolactone) on BP and vascular function following glucocorticoid excess and high salt was measured. MR blockade did not change BP but rescued the renal artery dysfunction. In other experiments, I assessed the effect of high salt diet on plasma glucocorticoid levels. High salt treatment in male C57BL/6JCrI mice increased plasma glucocorticoids. High salt also increased plasma copeptin levels, suggesting elevation of AVP. Activation of magnocellular AVP-secreting neurons could bypass glucocorticoid feedback and support sustained activation of the HPA axis. Additionally, high salt decreased hippocampal MR mRNA expression which could have implications on the tone of the HPA axis. Consistent with this, restraint test sensitivity was amplified in C57BL/6JCrI mice.

In conclusion, I found a reciprocal relationship between glucocorticoids and salt. Underlying glucocorticoid excess induces renal vasodysfunction and amplifies salt-sensitive BP response. Furthermore, high salt induces activation of the HPA axis. Together, this could have long-term implications on the stress response and salt-sensitivity.

Lay abstract

High blood pressure, or hypertension, is a major risk factor for heart disease, affecting ~30% of adults and ~5% of children worldwide. Lifestyle risk factors, such as high salt diet and high levels of stress hormone (glucocorticoids), are common risk factors for hypertension. As we live in a salt-saturated and stressed society, this can have a profound effect on the health of a large proportion of the population. Around a quarter of the population are more susceptible to salt-induced changes to blood pressure, defined as being salt-sensitive. People with abnormal glucocorticoid levels, such as in Cushing's syndrome, are often salt-sensitive but the mechanisms behind this are not fully understood. Theories of what causes salt-sensitivity are thought to be focussed on problems with the kidneys and/or blood vessels. Specifically, it is thought that following excessive salt, the kidneys reabsorb both salt and water back into the bloodstream instead of excreting it in the urine causing increases in blood volume to increase blood pressure. Alternatively, some think that following excessive salt, the blood vessel walls (specifically arteries) do not relax properly, resulting in maintenance of a higher blood pressure.

This thesis explored whether glucocorticoid excess caused complications in the kidney and arteries, causing salt-sensitivity. Firstly, we found that by measuring blood pressure in research mice with glucocorticoid excess, used to model Cushing's syndrome in humans, these mice had salt-sensitivity following feeding of a high salt diet. The mechanisms behind this salt-sensitivity were then investigated. To do this, the ability of the kidneys to excrete salt in response to rises in blood pressure was measured in mice with and without glucocorticoid excess. Both groups of mice increased the excretion of salt in the urine following increases in blood pressure at a similar rate. This suggests that the kidneys in mice with glucocorticoid excess appropriately excrete salt in response to rises in blood pressure. The artery of the kidney was investigated from these mice. The ability of the arteries to contract or relax to different drugs was measured. We found that the ability of these arteries from mice with glucocorticoid excess to contract or relax was

reduced. This suggests complications with the kidney artery could contribute to salt-sensitivity in glucocorticoid excess.

To test if glucocorticoids caused complications in kidney artery function to contribute to salt-sensitivity in glucocorticoid excess, blood pressure and kidney artery function was assessed following a drug treatment that blocks glucocorticoid action. To do this, mice with glucocorticoid excess were fed high salt diet and treated with a drug, used in humans known as spironolactone. Spironolactone caused no changes to blood pressure but improved the ability of the kidney arteries to relax. This suggests another mechanism behind salt-sensitivity is involved but still remains unclear.

Previously, we were measuring how stress impacted responses to salt, but the final aim of the thesis was to investigate the impact of salt on stress. A high salt diet increased glucocorticoids in the blood in mice. Furthermore, a high salt diet increased the sensitivity to stress in mice.

In conclusion, I found a relationship between salt and glucocorticoids. Glucocorticoid excess caused complications with kidney artery function and salt-sensitivity. Furthermore, high salt increases glucocorticoids. Together, this could have long-term implications on salt-sensitivity and the ability to handle stressful situations.

List of achievements

- Lead of the University of Edinburgh Cardiovascular Research Centre BHF Fundraising Group
- British Heart Foundation Youth and Schools Speaker
- Lead of the British Heart Foundation/STEM Schools Outreach Programme
- Part of a team that organised featured Open Sessions for the public in collaboration with ASCUS (a non-profit organisation that is bringing the art, science and design communities together)

Presentation of the work in this thesis

Published abstracts

- Costello, H.M., Stewart, K, Jones, N.K., Grenier, C, Angel, S, Ralph, A.F., Holmes, M.C., Livingstone, D.E.W., Dhaun, N, Bailey, M.A. Glucocorticoid excess induces renal vascular dysfunction and amplifies salt-sensitive hypertension. *APS/ASN Conference: Control of Renal Function in Health and Disease, Charlottesville, USA 2019* (oral presentation)
- Costello, H.M., Stewart, K, Jones, N.K., Grenier, C, Angel, S, Ralph, A.F., Holmes, M.C., Livingstone, D.E.W., Dhaun, N, Bailey, M.A. Glucocorticoid excess induces renal vascular dysfunction and amplifies salt-sensitive hypertension. *Early career researcher oral presentation award, CVS Symposium, Edinburgh, UK 2019* (oral presentation)
- Costello, H.M., Stewart, K, Jones, N.K., Grenier, C, Livingstone, D.E.W., Dhaun, N, Bailey, M.A. Mechanisms behind salt-sensitivity in a mouse model of ACTH-dependent Cushing's syndrome. *Experimental Biology, Orlando, USA 2019* (oral presentation and a poster presentation)
- Costello, H.M., Stewart, K, Jones, N.K., Grenier, C, Livingstone, D.E.W., Dhaun, N, Bailey, M.A. Mechanisms behind salt-sensitivity in a mouse model of ACTH-dependent Cushing's syndrome. *Pre-EB*

Epithelial Transport meeting, Orlando, USA 2019 (oral presentations and a poster presentation)

- Costello, H.M., Stewart, K, Jones, N.K., Livingstone, D.E.W., Dhaun, N, Bailey, M.A. Impairment of pressure natriuresis in a mouse model of ACTH-dependent Cushing's Syndrome. *ESAC Symposium, Erlangen, Germany 2018* (poster presentation)
- Costello, H.M., Stewart, K, Jones, N.K., Livingstone, D.E.W., Dhaun, N, Bailey, M.A. Impairment of pressure natriuresis in a mouse model of ACTH-dependent Cushing's Syndrome. *Europhysiology, London, UK 2018* (poster presentation)

Awards and funding

- Selected for an early career researcher oral presentation award at Edinburgh CVS Symposium 2019
- Awarded a BHF Heart for Heroes' award and VisitScotland Young Legend of Education for the work I have done for the BHF during my PhD. This has included setting up a school's outreach programme where BHF-funded scientists, who are all STEM ambassadors, visit schools (both primary and secondary) to engage and enthuse young people and explain the risk factors of CVD. Furthermore, explaining the important work the BHF do and teaching CPR.
- Completion of Edinburgh Teaching Award Category 1
- Completion of an application for the Deanery of Clinical Sciences Funding Challenge for a small volume electrolyte analyser. Unfortunately, I was not successful. However, since I obtained half the funding before the submission of the application (via BHF CoRE and CVS Lab Management), the remaining cost was covered by various PIs.
- Physiological Society Travel award - £500 to attend Experimental Biology 2019
- Biochemical Society Travel award - £550 to attend APS/ASN Conference: Control of Renal Function in Health and Disease 2019

- University of Edinburgh Go Abroad Fund Travel award - £350 to attend APS/ASN Conference: Control of Renal Function in Health and Disease 2019

Publications

- Czopek, A, Moorhouse, R, Guyonnet, L, Farrah, T, Lenoir, O, Owen, E, van Bragt, J, **Costello, H.M.**, Menolascina, F, Baudrie, V, Webb, D.J., Kluth, D.C., Bailey, M.A., Tharaux, PL & Dhaun, N (2019): A novel role for myeloid endothelin-B receptors in hypertension. *European Heart Journal* **40** (9), 768-784.
- Culshaw, G.J., **Costello, H.M.**, Binnie, D, Stewart, K.R., Czopek, A, Dhaun, N, Hadoke, P.W.F., Webb, D.J. & Bailey, M.A. (2019): Impaired Pressure Natriuresis and Non-Dipping Blood Pressure in Rats with Early Type 1 Diabetes Mellitus: Pressure natriuresis and BP in T1DM. *Journal of Physiology* **597** (3), 767-780.
- Ivy, J.R., Jones, N.K., **Costello, H.M.**, Mansley, M.K., Peltz, T.S., Flatman, P.W., Bailey, M.A. (2019): Glucocorticoid receptor activation stimulates the Na-Cl co-transporter and influences the diurnal rhythm of its phosphorylation. *Am J Physiol Renal Physiol*, DOI 10.1152/ajprenal.00372.2019.
- Ralph, A.F., Grenier C, **Costello, H.M.**, Stewart, K, Ivy, J.R., Dhaun, N, Bailey, M.A. (2021): Activation of the sympathetic nervous system promotes blood pressure salt-sensitivity in C57BL6 mice. *Hypertension* **77**, 158-168.

Acknowledgements

First and foremost, an immense amount of thanks goes to my primary supervisor, Professor Matthew Bailey, who has shown me nothing but support and guidance through the entirety of my PhD. Your pep talks are legendary and inspired me to continue in academia. I cannot thank you enough for everything you have done for me! I would also like to thank my secondary supervisor, Dr Neeraj Dhaun (Bean), for all his support and advice the past few years. To Dr Dawn Livingstone, thank you so much for being a mentor to me in research and in teaching - you are an inspiration! Finally, I would like to thank Professor Megan Holmes for her guidance while on my thesis committee.

An honourable mention must go to Mr Kevin Stewart for the time you spent performing surgeries and for teaching me pressure natriuresis. I am extremely grateful for your endless support. Thank you to all the members of Bailey and BDD group – amazing group of supportive scientists who have helped me so much. I am also so grateful to the staff in LF2, with particular mention to Sandra for going above and beyond for me, and to Hollie, Will and Duncan for keeping a smile on my face after my long days in the animal unit. Finally, thanks to Mr Dave Binnie for your superior animal expertise.

A special thanks to Morag. You have been there supporting me since day 1 and I cannot thank you enough. Thank you to Natalie (our golden girl and Team Bailey PA) who has done so much for me in and out of the lab over the last few years. To the past and present CVS PhD students, particularly Olivia, Kathleen (my desk and Starbucks buddy), Ailsa, ML, Jess, Bonnie, Teo, Rachel, Lisa and Clare, thank you for making the last 3 years so memorable.

A massive thanks must go to my partner Euan who has been through my highs and lows of my PhD with me – I couldn't have done it without you. Also, to my family – especially my parents and sister – for their support and love. Thanks to my wonderful best friends Lindsay and Joanne for fun thesis-free days.

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Abbreviations

A	11 β -HSDs	11 β -hydroxysteroid dehydrogenases
	ACh	Acetylcholine
	ACTH	Adrenocorticotrophic hormone
	AME	Apparent mineralocorticoid excess
	ANOVA	Analysis of variance
	ASDN	Aldosterone-sensitive distal nephron
	AU	Arbitrary unit
B	AVP	Arginine vasopressin
	BP	Blood pressure
C	bw	Body weight
	cAMP	Cyclic adenosine monophosphate
	CBG	Corticosteroid-binding globulin
	CD	Collecting duct
	cDNA	Complementary DNA
	CKD	Chronic kidney disease
	CNT	Connecting tubule
	CO	Cardiac output
	CRH	Corticotropin-releasing hormone
	CVD	Cardiovascular disease
	DBP	Diastolic blood pressure
D	DCT	Distal convoluted tubule
	EC50	Half maximal effective concentration
E	ECFV	Extracellular fluid volume
	ENaC	Epithelial sodium channel
	eNOS	Endothelial nitric oxide synthase
F	Fe	Fractional excretion
	FITC	Fluorescein isothiocyanate
	FKBP5	FK506 binding protein
G	GFR	Glomerular filtration rate
	GR	Glucocorticoid receptor
H	h	hours
	Hct	Haematocrit
	HPA	Hypothalamic-pituitary-adrenocortical
	HR	Heart rate

I	IC50	Half maximal inhibitory concentration
	KPSS	High potassium physiological salt solution
	MABP	Mean arterial blood pressure
	MC2R	Melanocortin type 2 receptor
	MESOR	Midline estimating statistic of rhythm
K	min	Minutes
	MR	Mineralocorticoid receptor
	mRNA	Messenger ribonucleic acid
	Na ⁺	Sodium
	NaCl	Sodium chloride
M	NCC	Thiazide-sensitive sodium chloride cotransporter
	NHE3	Sodium-hydrogen antiporter 3
	NO	Nitric oxide
	NTS	Nucleus of the solitary tract
	PE	Phenylephrine
N	PKA	Protein kinase A
	PN	Pressure natriuresis
	POMC	Pro-opiomelanocortin
	PSS	Physiological salt solution
	PVAT	Perivascular adipose tissue
P	PVN	Paraventricular nucleus
	qPCR	Quantitative real time polymerase chain reaction
	RAAS	Renin-angiotensin-aldosterone system
	RAP	Renal arterial pressure
	RBF	Renal blood flow
Q	RVR	Renal vascular resistance
	SBP	Systolic blood pressure
	SCN	Suprachiasmatic nuclei
	SD	Standard deviation
	SNP	Sodium nitroprusside
R	SNS	Sympathetic nervous system
	StAR	Steroidogenic acute regulatory protein
	SVR	Systemic vascular resistance
	THF	Tetrahydrocortisol
	TPR	Total peripheral resistance
S		
T		

U	UPL	Universal probe library
V	VSM	Vascular smooth muscle
W	WHO	World Health Organisation
Z	ZT	Zeitgeber time

Chapter 1: Introduction

1.1 Hypertension

Hypertension is defined as chronic elevation of blood pressure (BP), when average systolic BP (SBP) is 140 mmHg or higher and diastolic BP (DBP) is 90 mmHg or higher. Hypertension is the leading modifiable risk factor for cardiovascular disease (CVD) and all-cause mortality, with the global burden estimated in 2010 at approximately 1.4 billion (or 31% of the global adult population)¹⁻³. Lifestyle risk factors such as poor diets (high sodium and/or low potassium intake) and lack of physical activity are causing the prevalence of essential hypertension to rise further^{4,5}.

1.1.1 The relationship between sodium intake and elevated blood pressure

Salt (sodium chloride, NaCl) accounts for approximately 95% of daily sodium intake. Tight regulation of sodium is essential for maintenance of osmotic pressure and water homeostasis in the extracellular space⁶. The vast majority of salt (> 85%) is excreted by the kidneys. Historically, dietary salt intake was < 0.25 g/day (0.1 g/day sodium) until about 5000 years ago when its usefulness in preservation was discovered. Nowadays, there has been a shift towards a salt-preference related intake, as the average salt intake in most countries is approximately 9-12 g/day (3.6-4.8 g/day sodium), with many Asian countries exceeding 12 g/day^{7,8}. This is predominately due to increases in production of processed food in the developed world⁹. Interestingly, this is not the case in Asian countries with the highest salt intake. Dietary salt mainly arises from that added to home-cooked meals, condiments (e.g. soy and fish sauces) and consumption of salt-preserved foods¹⁰. This increased dietary salt intake challenges the kidneys with excreting these large amounts of salt. The notion that this large and habitual excess in dietary salt intake could be detrimental to human health has only emerged in the last 50 years. Evidence has shown that elevated dietary salt has been associated with an increased risk of hypertension, CVD and stroke, and decreasing dietary salt may reduce BP and these risks. Due to this, the World Health Organisation (WHO) has recommended a reduction in dietary salt to 5 g/day (2 g/day sodium) or less

for the adult population¹¹. However, almost the entire adult population (99.2%) exceed these recommendations¹².

Evidence from animal studies, observation studies and clinical trials shows a causal relationship between salt intake and elevated BP^{5,6,13–17} but underlying mechanisms are not resolved, likely complex and contentious. Back in the 1960s, Lewis K Dahl proposed that there was a possible relationship between chronic excess of salt consumption and hypertension¹⁸. This led to larger observational studies investigating this relationship. The INTERSALT study demonstrated a positive association between 24 h sodium excretion and BP in 10,079 participants (both men and women aged 29-59) from 52 population samples across 32 countries¹⁹. However, 4 of the 52 population samples were outliers and excluding these populations resulted in no association between salt intake and BP. It also reported widespread population variations in 24 h sodium excretion, from the Yanomami Indians in Brazil excreting 0.46 g/day sodium to a population in Northern China excreting up to 6.0 g/day²⁰. Studies with fewer participants also reported no association, such as The Scottish Health Study ($n = 7354$)²¹. It was suggested by Mente et al (2014) that a larger study was necessary to explore the relationship between salt intake and BP and whether this association differs among different populations and regions around the world. The PURE study aimed to investigate this in 102,216 adults from 18 countries. This study reported a positive association between salt intake and BP in all 18 countries in the study. Furthermore, there was a stronger association in participants who were hypertensive, in those who consumed a low potassium diet and in the older participants²². Clinical studies reducing salt intake have been shown to lower BP in both hypertensive and normotensive individuals^{15,16,23–25}. A recent meta-analysis of 34 clinical trials ($n = 3230$) showed that a reduction in salt intake (approximately to 4.4 g/day) was associated with SBP and DBP reduction of 4.2 and 2.1 mmHg, respectively. This BP reduction was greater in hypertensive participants¹⁶. Therefore, a reduction in salt intake has been recommended for the prevention and control of hypertension^{5,9}. Although, the TOHP trial has shown that salt

reduction over a longer period of time (> 6months) has less of an effect of lowering BP, which could be due to compliance or the possible attenuation of the antihypertensive effects of reduced salt intake over time²⁶.

Despite the extensive body of evidence for the causal relationship between salt intake and elevated BP, scepticism remains. This is based on variations in the results from observational studies and clinical trials as BP responses to changes in dietary salt are individualised across populations, demonstrating the heterogeneity of the physiological responses to salt. Furthermore, some have suggested that studies have shown only modest BP changes with salt reduction and these reductions are difficult to maintain due to societies salt preferences²⁷. Nevertheless, epidemiological observations have established a curvilinear relationship between dietary salt, the occurrence of hypertension and risk of CVD and there is a global consensus that the general population needs to reduce dietary salt intake^{9,27,28}.

1.2 Salt-sensitive hypertension

The response of BP to changes in dietary salt varies among the population; some individuals can effectively excrete an increased salt load without an associated increase in BP, whereas those defined as salt-sensitive cannot²⁹. This heterogeneity was first suggested by Kawasaki et al in a study that measured BP in 19 hypertensive participants who consumed their baseline salt diet, then a low salt diet (approx. 0.5 g/day) followed by a high salt diet (approx. 14.3 g/day). BP was significantly reduced during salt restriction and then increased back to baseline with salt loading. Although on analysis, 9 participants had a larger increase in BP (at least 10%) from low to high, deemed to be salt-sensitive. The remaining participants ($n = 10$) were considered salt-resistant with little to no rise in BP with sodium loading (Figure 1.1)²⁹. Based on this small study, it was estimated approximately 50% of hypertensive patients are salt-sensitive. Further studies, particularly from Weinberger et al, have investigated responsiveness of BP to dietary salt in

both normotensive and hypertensive individuals. In one study, they concluded that 26% of normotensive individuals were salt-sensitive (BP increase greater or equal to 10 mmHg), and 58% salt resistant (BP increase less than or equal to 5 mmHg). In hypertensive individuals, 51% were salt-sensitive and 33% salt-resistant, illustrating hypertensive individuals were more likely to be salt-sensitive than normotensive individuals. Those subjects with changes between 6 and 9 mmHg were considered indeterminate³⁰. These findings were reported as highly reproducible^{31,32}, leading to the global estimation of the population inherently salt-sensitive at 25-30% and greater than 50% in hypertensive individuals³³.

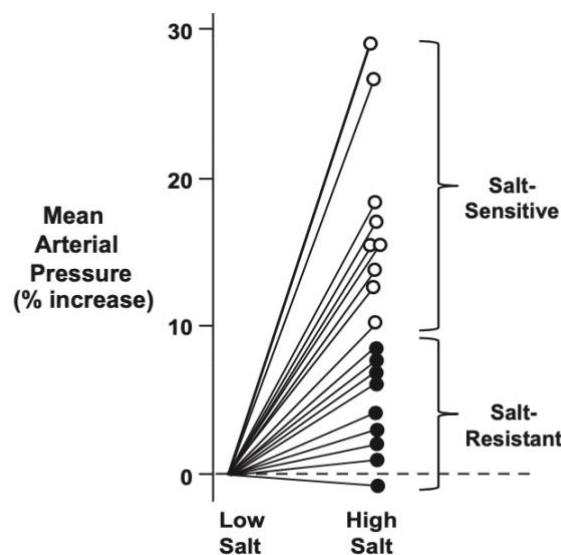


Figure 1.1: Responsiveness of blood pressure to increased dietary salt in salt-resistant and salt-sensitive hypertensive subjects.

Percentage increase in mean arterial blood pressure in salt-resistant ($n = 10$) and salt-sensitive ($n = 9$) hypertensive patients when their diet was changed from a low salt (0.5 g/day) to high salt (14.3 g/day) diet for 7 days. Data are from Kawasaki et al²⁹ and graph from Hall³⁴.

1.2.1 Populations at risk of salt-sensitivity

Salt-sensitivity prevalence appears to be associated, at varying degrees, with the aging population, race-ethnicity, gender, body mass index, diet and co-morbidities (hypertension, diabetes mellitus, chronic kidney disease (CKD) and metabolic syndrome)^{33,35}. Salt-sensitivity has been shown to be most prevalent in normotensive and hypertensive subjects of African descent. It has

been suggested that enhanced tubular renal sodium reabsorption may be responsible for ethnic salt-sensitivity susceptibility. Salt-sensitive individuals have been suggested to be more likely female, although a weak association has been reported between gender and salt-sensitivity³⁶. Moreover, obesity (particularly visceral obesity), stress and a low potassium diet have all been linked with salt-sensitivity^{37,38}.

1.2.2 Genetic components of salt-sensitivity

Data have shown a high prevalence of salt-sensitivity in certain races, including the African American population³⁹. There is substantial evidence from different racial and ethnic groups to support that a genetic component is involved in the BP response to salt. Genes encoding the epithelial sodium channel (ENaC), WNKs, mineralocorticoid receptor (MR) and 11 β -hydroxysteroid dehydrogenases (11 β -HSDs), all involved in renal sodium regulation, can cause rare forms of salt-sensitive human monogenic hypertension^{40,41}. Due to the rarity of these variants, this could not explain the high prevalence of salt-sensitivity in the population. Therefore, there is continued interest in identifying allelic variants of candidate genes in salt-sensitivity. Rodent strains and generated transgenic models have been useful in the identification of susceptible genes. These include genes involved in the renin-angiotensin-aldosterone system (RAAS)^{42–45}, sympathetic nervous system⁴⁶, endothelin system⁴⁷, oxidative stress⁴⁸, metabolic factors⁴⁹, inflammation⁵⁰, renal transporters and its regulatory molecules^{51,52}, CYP450 enzymes⁵³ and the renal dopaminergic natriuretic system⁵⁴. This has led to the conception that salt-sensitivity involves multiple systems.

1.2.3 Salt-sensitivity and disease

Studies have also demonstrated that salt-sensitive patients are more at risk of both adverse cardiovascular and renal events. Total cardiovascular events, both fatal and non-fatal, have been shown to occur more frequently in salt-sensitive hypertensive patients over salt-resistant hypertensive individuals (Figure 1.2 A). Interestingly, it has also been reported that salt-sensitivity is a

cardiovascular risk factor independent of changes in BP. Renal function has also been shown to be impaired in salt-sensitive hypertension⁵⁵. Furthermore, salt-sensitive hypertensive individuals or salt-sensitive CKD patients are more likely to have a blunted nocturnal reduction in BP (“non-dipper”; less than 10% decline compared to mean daytime BP). This disturbed circadian BP rhythm can also increase the risk of cardiovascular events⁵⁶. Interestingly, this non-dipping pattern can be shifted to dipping (10-20% decline of daytime BP) in hypertensive salt-sensitive subjects by dietary salt restriction, restoring the nocturnal decline. The dipping pattern was present in hypertensive salt-resistant subjects and was maintained following dietary salt restriction⁵⁷.

A positive relationship has been observed between salt-sensitivity and the risk factors associated with metabolic syndrome, including a higher waist circumference, hypertriglyceridemia, insulin resistance and hypertension³⁸. The mechanisms behind the BP rise in these patients are not fully understood. A study investigating the response of BP to dietary salt in metabolic syndrome patients demonstrated a correlation between severity of the disease and the responsiveness of BP to dietary salt. This study proposed that dietary salt could be a major determinant of the increase in BP associated with metabolic syndrome⁵⁸.

With salt-sensitivity increasing the risk of adverse cardiovascular and renal events, it is no surprise that salt-sensitivity also has been associated with an increased risk in mortality in hypertensive individuals. However, it has also been shown to increase the risk of mortality in normotensive individuals (aged >25 years old; Figure 1.2 B)⁵⁹. This is thought to be due to salt-sensitive normotensive subjects being at a higher risk of age-related hypertension. This is supported by a study showing normotensive salt-sensitive individuals BP rose significantly more than the BP of those who were salt-resistant over a 10 year follow-up, suggesting that normotensive salt-sensitive subjects are more likely to become hypertensive⁶⁰. Therefore, salt-sensitivity is a risk factor for

the predisposition of essential hypertension, which are both risk factors of cardiovascular and renal events, increasing the likelihood of mortality.

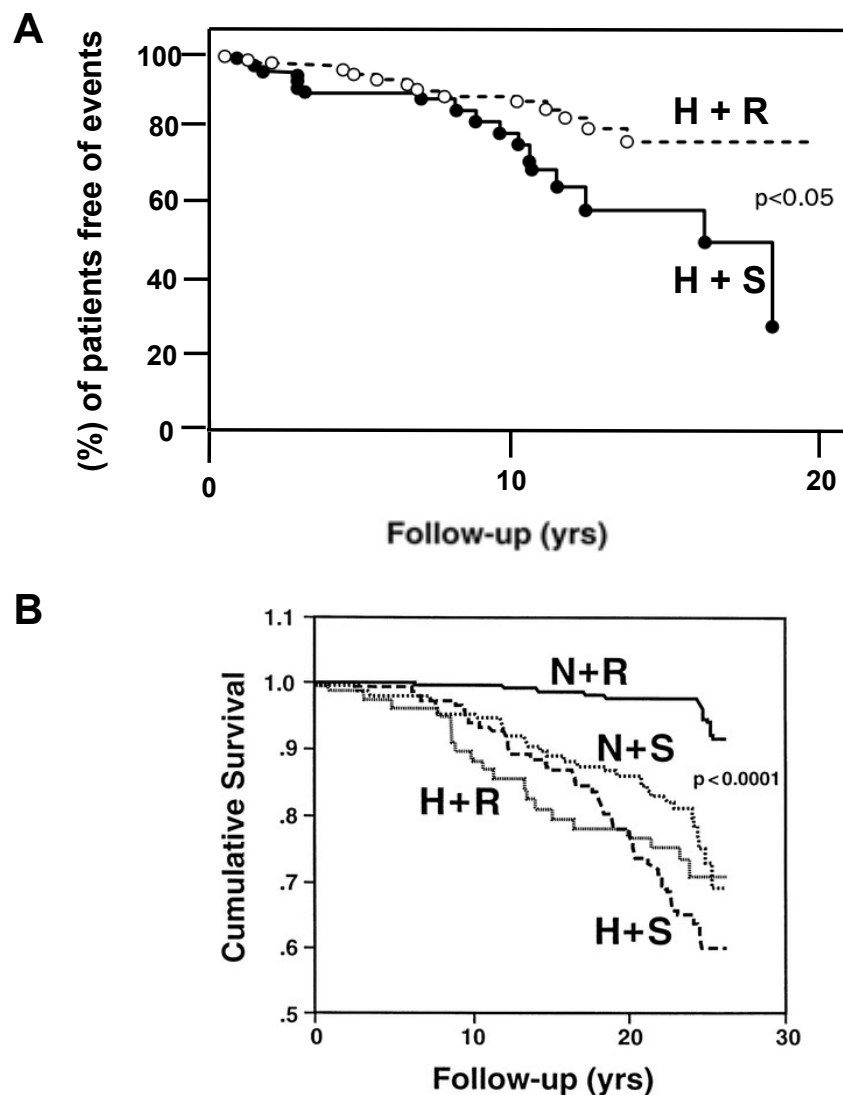


Figure 1.2: Implications of salt-sensitivity on mortality and total cardiovascular events.

(A) Kaplan-Meier plots of total cardiovascular events of hypertensive salt-resistant (H+R) and hypertensive salt-sensitive subjects (H+S) patients over the follow-up period. Graph adapted from Morimoto et al⁵⁵. (B) Kaplan-Meier survival plots for normotensive salt-resistant subjects (N+R), normotensive salt-sensitive subjects (N+S), H+R and H+S over the follow-up period. Graph from Weinberger et al⁵⁹.

1.2.4 Challenges of investigating salt-sensitivity

A major research challenge is the lack of a universally accepted definition of salt-sensitivity. As previously mentioned, studies defined salt-sensitivity

differently. Kawasaki et al reported salt-sensitivity as at least a 10% increase in BP in response to a high salt diet and salt-resistant less than 10%²⁹. The use of two distinct categories could lead to misclassification of salt-sensitivity as Figure 1.1 shows the variation of BP responses to dietary salt intake, again illustrating the heterogeneity of salt-sensitivity. Therefore, defining an individual as salt-sensitive can then depend on the selection of the BP change cut-off. Furthermore, Weinberger et al stated certain subjects were indeterminate, emphasising the lack of consensus on classifying salt-sensitivity. Defining salt-sensitivity became even more complex as studies classified salt-sensitivity differently during office and 24 h ambulatory BP monitoring, classifying salt-sensitivity as an increase in office BP measurement between 5-10% (or at least 5 mmHg)⁶⁰ and 24 h ambulatory BP monitoring of at least 4 mmHg⁶¹.

The second challenge is testing salt-sensitivity in humans. There are two different methods to diagnose salt-sensitivity. The first method is a dietary test involving patients consuming a low (1.2 g/day) and high (15 g/day) salt intake over a 14 day period. Another method is an intravenous test, measuring BP following an infusion of 2 L of normal physiological saline (0.9% NaCl) over 4 h, compared to BP measured in the morning after a low sodium diet (0.5 g/day) and administration of a diuretic (furosemide, 40 mg/8 h orally)⁵⁹. This full protocol has been described to last 5 days to include acclimatisation⁶¹. Therefore, alongside the lack of clarity in defining salt sensitivity, there is variation in the testing of salt-sensitivity.

For this thesis, salt sensitivity was assessed by a dietary test and defined as a 10% rise in BP.

1.2.5 Potential mechanisms underlying salt-sensitivity

BP regulation is determined by multiple systems, including cardiovascular, renal, central and peripheral nervous systems. These systems regulate blood volume and the release of neurohormonal controllers of BP (Figure 1.3). BP dysregulation involves complex crosstalk between these multiple systems and neurohormonal controllers. Although salt-sensitivity is a well-established

phenomenon, the mechanisms underpinning salt-sensitive hypertension are not fully understood due to the complexity of BP regulation. As seen in Figure 1.3, dysregulation of these systems, including RAAS and the endothelium, have been reported to lead to salt-sensitivity⁶². Although the mechanisms remain unclear, this supports the involvement of two fundamental mechanisms thought to be behind this rise in BP with salt intake - impaired renal salt excretion and vascular (endothelial) dysfunction^{63,64}.

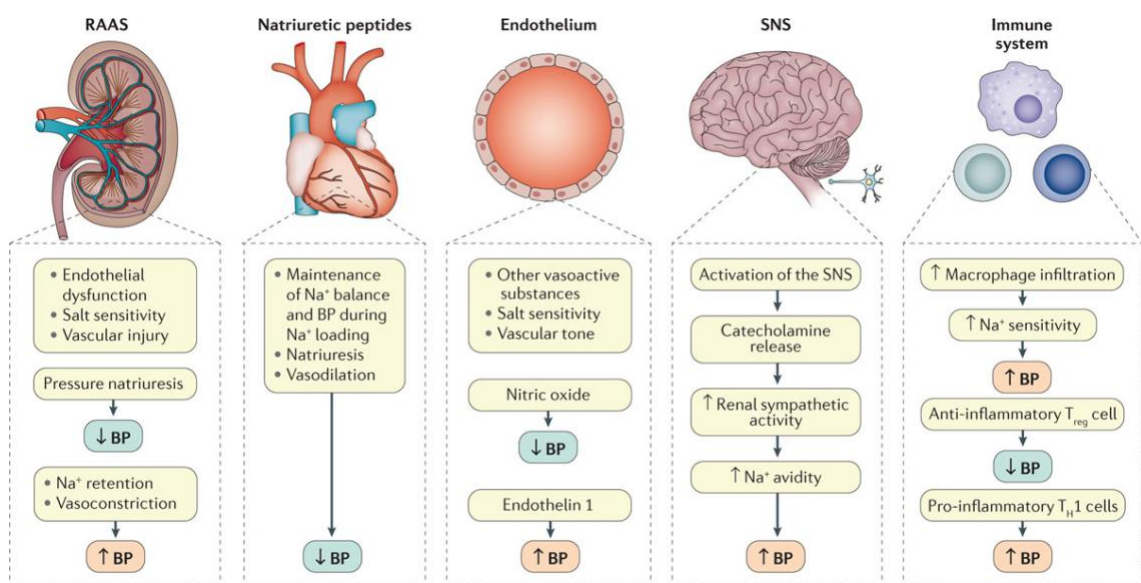


Figure 1.3: Multiple systems involved in blood pressure regulation.

Schematic diagram from Oparil et al⁶² of the major systems involved in the maintenance of blood pressure (BP) and their associations with salt-sensitivity. Na⁺, sodium; RAAS, renin-angiotensin-aldosterone system; SNS, sympathetic nervous system; T_{reg}, regulatory T; T_H, T helper.

1.2.5.1 Impaired renal salt excretion

BP is the product of cardiac output (CO, representing total blood flow) and total peripheral resistance (TPR). CO and arterial BP were widely accepted to be controlled by the heart and TPR, respectively. Guyton, Coleman and Granger introduced new theories on long-term BP regulation using a large computer model of circulatory control, which centred around effective blood volume in BP regulation, instead of TPR. Extracellular fluid volume (ECFV), regulated by

salt and water balance, and vascular tone are major determinants of effective blood volume. The kidney regulates salt balance through the pressure natriuresis (PN) response, which is the relationship between arterial pressure and urinary sodium excretion. Figure 1.4 illustrates the renal-body fluid mechanism proposed by Guyton, Coleman and Granger showing any imbalance between salt intake and output will result in a cascade of events to bring the system back to equilibrium, acting as a negative feedback loop. For example, increases in salt intake cause ECFV expansion and increases in blood volume. This leads to rises in mean circulatory filling pressure (relating blood volume to vascular capacity), yielding an increase in venous return. This increases CO to increase BP transiently. If BP rises, so too does renal arterial pressure (RAP). To restore this equilibrium, the kidney responds by increasing sodium excretion to reduce ECFV and in turn RAP (acute PN response)^{64–66}. Figure 1.5 A illustrates a typical acute PN relationship, exclusively from anaesthetised animals and isolated perfused kidney, where the equilibrium point (long-term set point) of mean arterial pressure is predicted at the intercept of acute PN curve and daily salt intake. The chronic PN relationship (represented by the solid red line in Figure 1.5 B) is generated by measuring the effect of differing salt intake on sodium excretion and systemic arterial pressure and defined as the relationship between steady-state sodium output and arterial pressure. From this theory, Guyton et al proposed that hypertension, including salt-sensitive hypertension, can only develop if the PN response is shifted to the right at a higher arterial pressure^{66–68}.

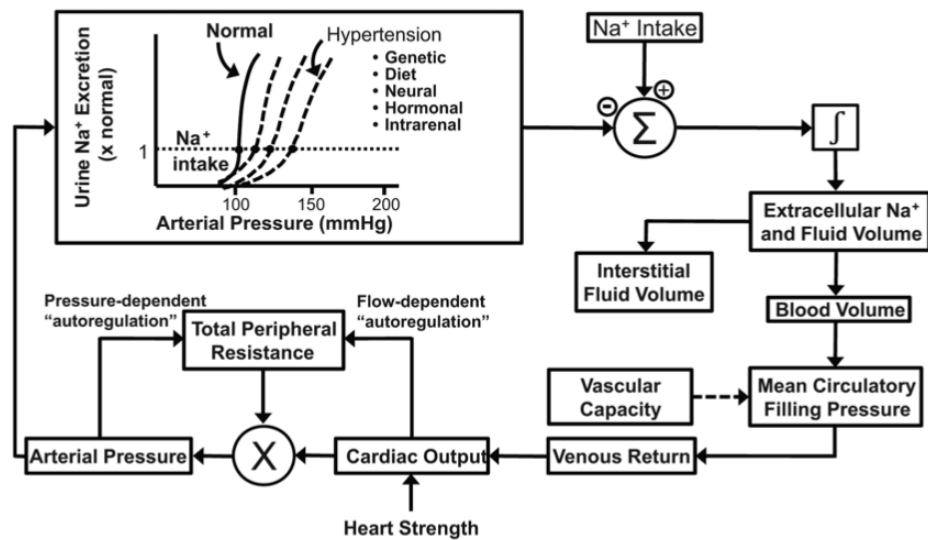


Figure 1.4: Renal-body fluid feedback mechanism for long term blood pressure regulation.

Schematic diagram by Hall³⁴ illustrating Guyton and Coleman's proposed theory for long-term control of blood pressure by the renal-body fluid feedback and cardiovascular, neural and hormonal mechanisms.

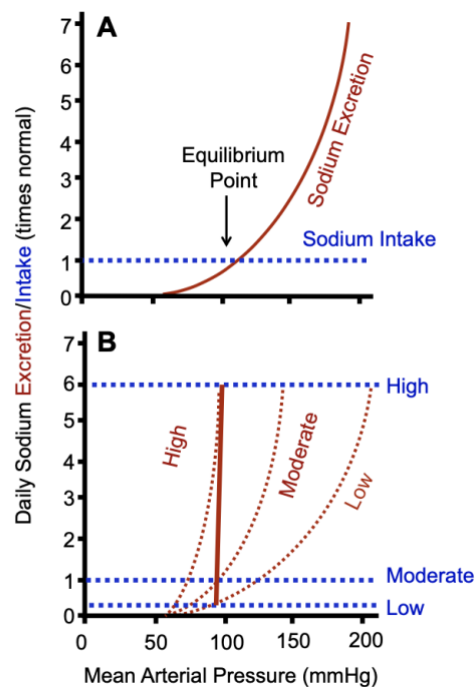


Figure 1.5: Pressure natriuresis and long-term control of arterial pressure.

(A) Acute pressure natriuresis (PN) relationship. The equilibrium point represents the long-term set point of arterial pressure. (B) Chronic PN relationship (solid red line) with changes in sodium intake from low to high (dashed blue lines) after the PN relationship (red dashed lines). Figure from Evans & Bie⁶⁷ which was adapted from Guyton et al⁶⁶.

This PN response previously described has been reported to occur in subjects who are considered salt-resistant. However, in salt-sensitive individuals, the increase in BP with salt intake contributes to increased salt/water excretion through PN. As illustrated in Figure 1.4, the dashed PN lines show a shift in the PN curve to the right, impairing PN response and increasing salt-sensitivity in hypertension. The steepness of this curve is the basis of the theory from Guyton and Coleman, and adopted more recently by Hall, that chronic salt-sensitive hypertension is caused by renal dysfunction, characterised by impaired PN, leading to decreased sodium excretion at a given pressure^{34,69}. This shift is thought to be caused by either reductions in glomerular filtration rate (GFR), increases in tubular reabsorption, increases in sympathetic nervous system (SNS) activity or arginine vasopressin (AVP) excess to reduce the kidneys' ability to excrete salt and water. This has been supported as salt-sensitivity has been reported to be effectively treated with drugs, such as diuretics and RAAS blockers, that increase GFR/reduce renal salt reabsorption³⁴. Therefore, impaired renal salt excretion, suggesting compromised PN relationship, could be a fundamental mechanism underlying salt-sensitivity.

1.2.5.2 Vascular dysfunction

This concept has been challenged by the “vasodysfunction” theory for initiation of salt-sensitivity by Morris et al (Figure 1.6). This theory proposes that an abnormal vascular resistance response to increased salt intake, in the absence of a substantial rise in renal sodium reabsorption, predominately initiates salt-sensitivity. In salt-resistant normotensive individuals, an increased salt intake stimulates sodium retention and changes blood volume to increase CO. This results in a decrease in systemic vascular resistance (SVR; also referred to as TPR) and renal vascular resistance (RVR), instead of an increase in renal sodium excretion, to maintain BP. However, in salt-sensitive individuals, SVR and RVR fail to decrease, remaining above baseline levels. This is thought to be the initial haemodynamic abnormality leading to

salt-induced rises in BP. This may include unaltered or rises in RVR but decreases in vascular resistance of other vascular beds⁷⁰.

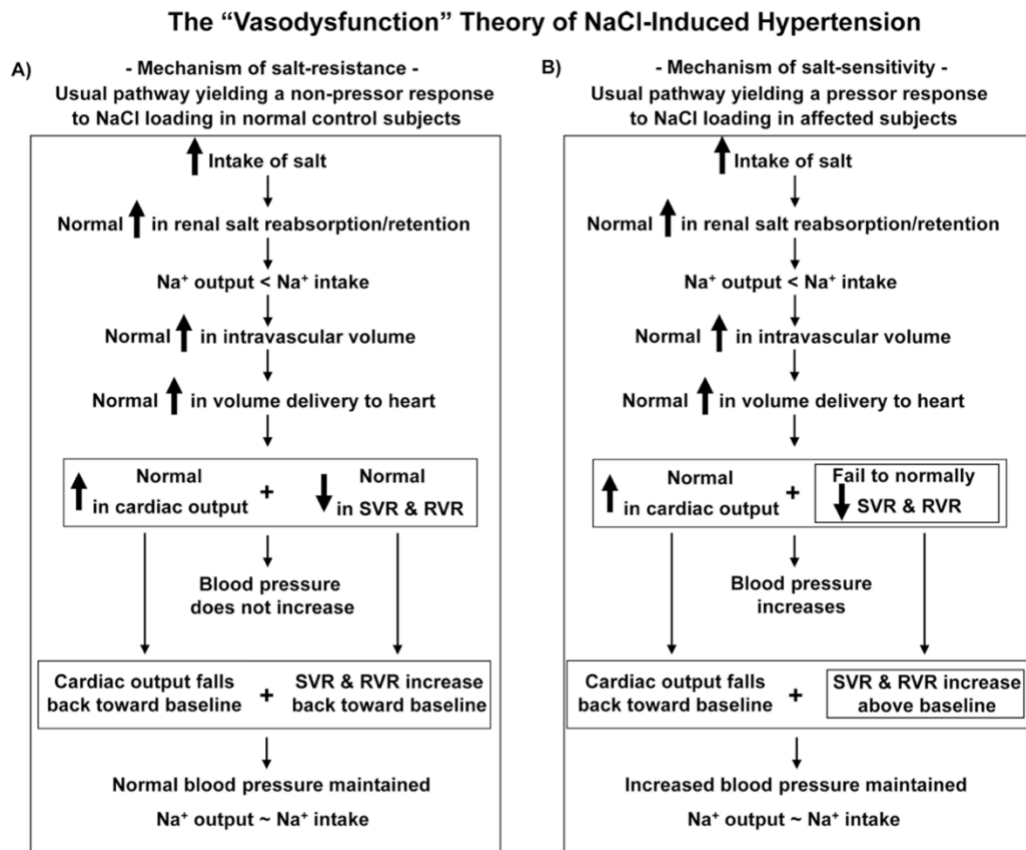


Figure 1.6: The vasodysfunction theory for initiation of salt-sensitivity.

Schematic diagram from Morris et al⁷⁰. (A) Proposed pathway for mechanism of salt-resistance depicting initial sodium balance and haemodynamic changes in response to increased salt intake. (B) Proposed pathway for mechanism of salt-sensitivity depicting initial sodium balance and haemodynamic changes in response to increased salt intake but failure to normally decrease systemic vascular resistance (SVR) and renal vascular resistance (RVR) mediating increases in blood pressure.

1.2.5.3 The neurogenic hypothesis

The neurogenic hypothesis argues that sympathetic overactivity, particularly to the vasculature and kidney, contributes to salt-sensitivity. The neurogenic model proposes that in salt-resistant individuals increased dietary salt suppresses plasma angiotensin II, reducing osmotically driven sympathoexcitatory pathways in the brain. This reduces sympathetic activity

and enhances both venous and arterial vasodilation, adapting through blood volume expansion to maintain BP. However, in salt-sensitive individuals, this response is impaired leading to sympathetic overactivity^{71–73}.

1.2.5.4 An emerging theory – abnormal glucocorticoid signalling

Evidence has emerged over recent years that abnormal glucocorticoid activity, via dysregulation of hypothalamic-pituitary-adrenocortical (HPA) axis, may have a major contributory role in salt-sensitivity^{74,75}. This has been supported by studies showing salt-sensitive humans having enhanced stress-induced activation of the HPA axis and reduced glucocorticoid clearance^{76,77}. Although, the mechanisms behind this are not fully understood. This thesis focusses on the relationship between salt and glucocorticoids and implications for understanding salt-sensitivity

1.3 Glucocorticoids

Glucocorticoids (cortisol in humans; corticosterone in rodents) are released in response to stressors and modulate many genes involved in glucose and fatty acid metabolism, immune function, inflammation and growth, to maintain homeostasis. They exert beneficial effects on the immune system and cardiovascular function to restore balance during stress⁷⁸. Under basal conditions, the HPA axis is under control of circadian and ultradian cues. Circulating glucocorticoids fluctuate with a diurnal rhythm in which the nadir occurs during sleep. Furthermore, food intake and acute stress result in ultradian rhythm of pulsatile release of glucocorticoids⁷⁹.

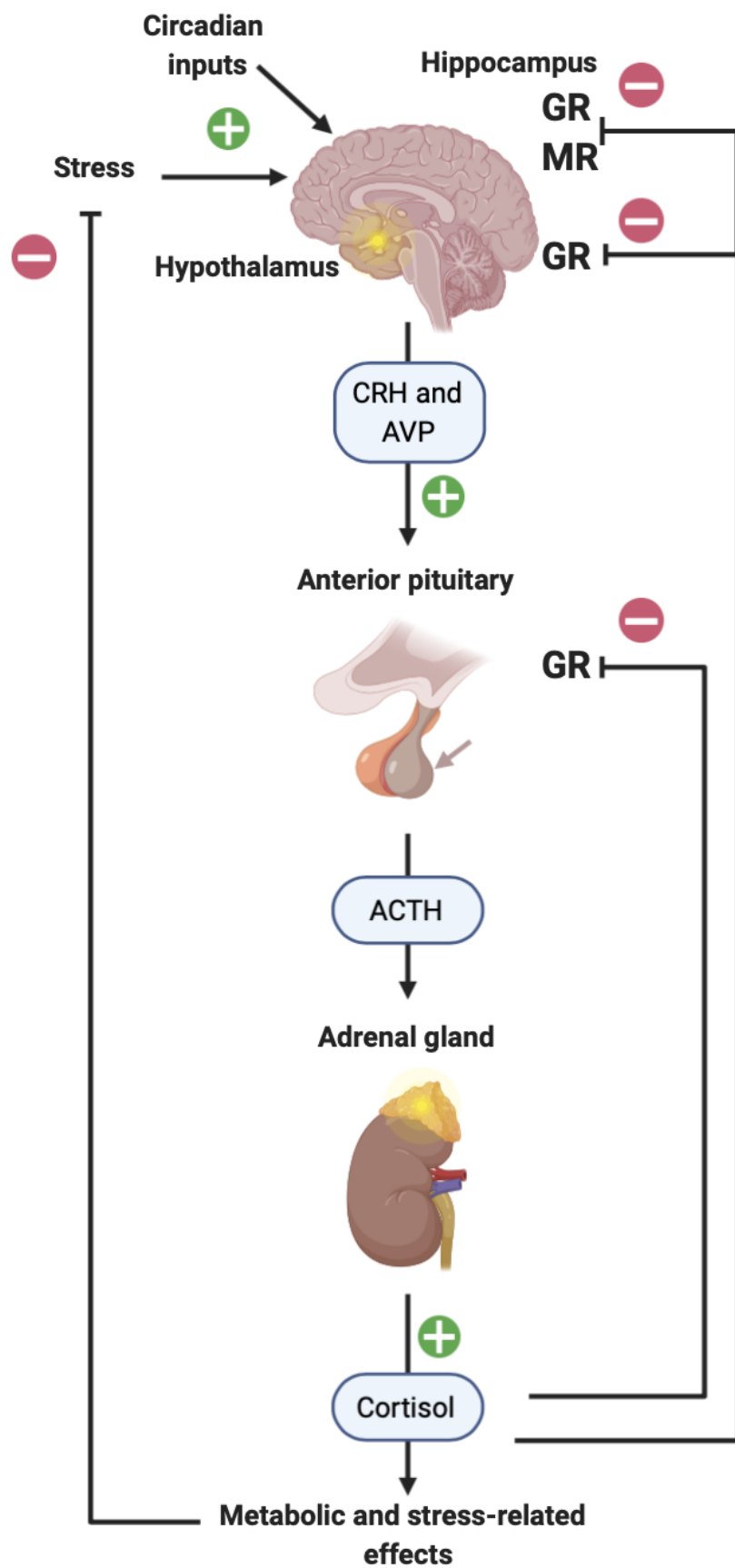
1.3.1 Glucocorticoid synthesis

Humans secrete 10-20 mg/day of cortisol. The HPA axis (Figure 1.7) controls the synthesis of glucocorticoids and other adrenal hormones. The HPA axis is activated by environmental, physical or physiological stressors (including circadian input from the suprachiasmatic nuclei (SCN) and stress inputs from the brainstem and limbic regions), resulting in the stimulation of

hypophysiotropic neurons, localised in the paraventricular nucleus (PVN) of the hypothalamus⁸⁰. The PVN projects to the median eminence to stimulate the synthesis and release of corticotropin-releasing hormone (CRH) and AVP into the hypophyseal portal vessels. CRH acts on CRH type 1 receptors on anterior pituitary corticotrophs, which stimulates the activation of pro-opiomelanocortin (POMC) transcription for the secretion of POMC-encoded adrenocorticotrophic hormone (ACTH). Similarly, AVP via activation of the V_{1b} receptor can trigger the release of ACTH^{81,82}. ACTH levels fluctuate with circadian rhythm, reaching its peak in the morning and declining throughout the day⁷⁸. ACTH enters the systemic circulation and binds to the G-protein-coupled receptor melanocortin type 2 receptors (MC2R) in the *zona fasciculata* of the adrenal cortex. This binding induces activation of adenylyl cyclase, leading to increased intracellular cyclic adenosine monophosphate (cAMP) to activate protein kinase A (PKA). PKA activates cAMP response element binding protein to induce the transcription of steroidogenic acute regulatory protein (StAR). PKA also phosphorylates StAR to increase its ability to mobilise cholesterol for its transfer into the mitochondria. Once cholesterol has entered the mitochondria, a cascade of processes both within the mitochondria and smooth endoplasmic reticulum trigger the synthesis of glucocorticoids from cholesterol via the intermediate pregnenolone (Figure 1.8). In rodents, the glucocorticoid is corticosterone which shares the same biosynthetic pathway as aldosterone. However, diverges at 11-deoxycorticosterone to synthesise corticosterone via *CYP11B1*. As well as glucocorticoids, cholesterol triggers the synthesis of the mineralocorticoids (aldosterone) in the *zona glomerulosa*. Although, compared with glucocorticoids, low concentrations of aldosterone are secreted (100-150 µg/day). Synthesis of aldosterone is predominately under the control of angiotensin II (RAAS), potassium and to a lesser extent ACTH⁸³.

Figure 1.7 : The hypothalamic-pituitary-adrenocortical axis

The hypothalamic paraventricular nucleus (PVN) receives circadian input from the suprachiasmatic nuclei (SCN) and stress inputs from the brainstem and limbic regions (including the hippocampus). The PVN projects to the median eminence to stimulate the release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the hypophyseal portal vessels. CRH and AVP acts on anterior pituitary corticotrophs to release adrenocorticotrophic hormone (ACTH) into the systemic circulation. ACTH activates the synthesis and secretion of glucocorticoids (cortisol in humans) to stimulate metabolic and stress-related effects. Glucocorticoids regulate the activity of the HPA axis through feedback mechanisms by binding to its receptors, glucocorticoid receptor (GR) and/or mineralocorticoid receptor (MR), in the anterior pituitary, hypothalamus and hippocampus. Diagram created with BioRender.com.



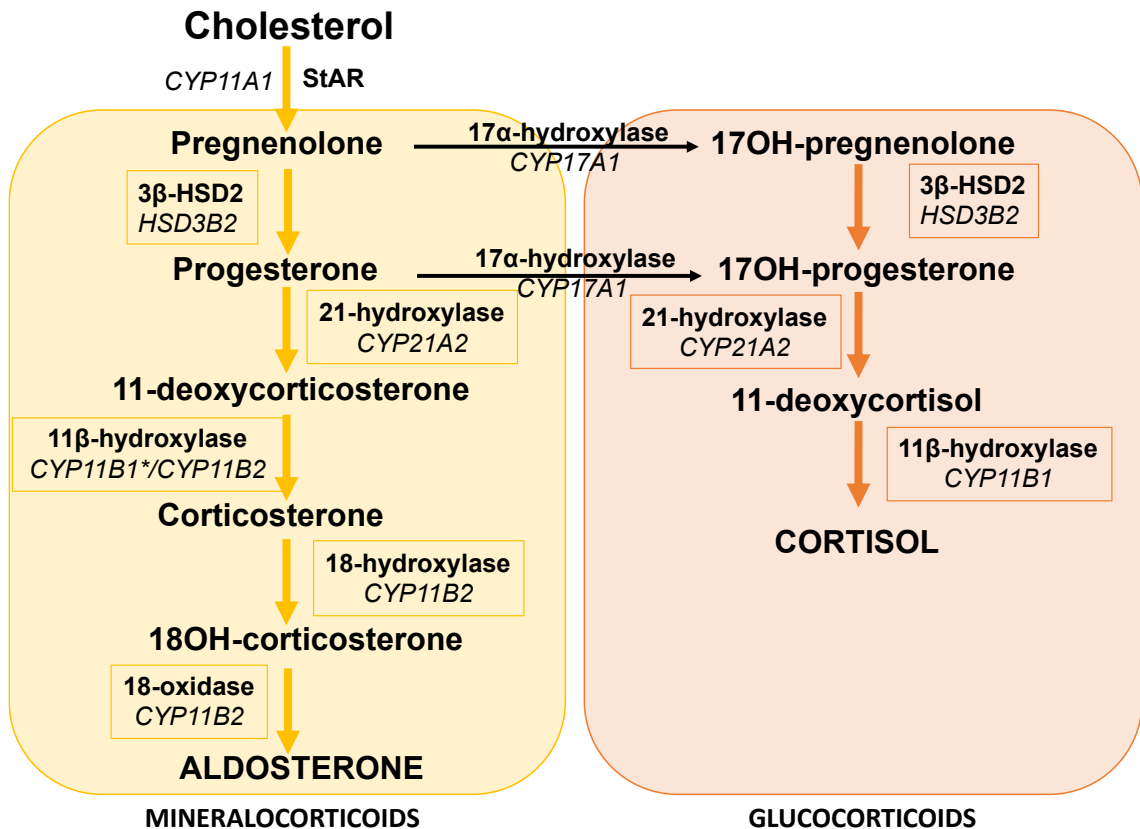


Figure 1.8: Steroidogenesis from cholesterol in the adrenal cortex.

Synthesis of cortisol and aldosterone in the human adrenal gland. *In rodents adrenal gland, synthesis of corticosterone is via the same pathway as aldosterone but diverges at 11-deoxycorticosterone where *CYP11B1*, instead of *CYP11B2* in humans, stimulates the production of corticosterone.

1.3.2 Glucocorticoid genomic and nongenomic responses

In the circulation, glucocorticoids bind predominately with corticosteroid-binding globulin (CBG) at high affinity but low capacity as CBG can only bind one glucocorticoid molecule⁸⁴. CBG plays a critical role in regulating the bioavailability of plasma glucocorticoid levels and their access to target tissues. To a lesser extent, glucocorticoids also bind to albumin with high capacity but low affinity⁸⁵. Over 90% of circulating glucocorticoids are bound. The free fraction of circulating glucocorticoids (approximately 3-5%) is biologically active and act to regulate physiological changes through ubiquitously distributed intracellular receptors. Specifically, they activate the low affinity, high capacity glucocorticoid receptor (GR) and high affinity, low

capacity MR⁸⁶. When inactive, these receptors reside in the cytoplasmic compartment, stabilised by co-chaperone molecules, 90-kDa heat-shock proteins (hsp-90), hsp70, hsp23 and FK506 binding protein 51 (FKBP51; encoded by *Fkbp5*) and FKBP52 (encoded by *Fkbp4*)⁸⁷. Heat-shock proteins are involved in trafficking, folding and activation of GR and MR. Whereas, FKBP51 and FKBP52 regulate affinity of GR and MR for glucocorticoids and the ligand-receptor complex translocation into the nucleus. As glucocorticoids binds to either GR or MR, FKBP51 dissociates from the complex, allowing for the binding of FKBP52 and the nuclear transporter dynein. This promotes the translocation of the ligand-receptor complex into the nucleus where it acts as a transcription factor⁸⁸. Glucocorticoids can bind both GR and MR but bind with different affinities as previously mentioned. Therefore, increases in circulating glucocorticoids can shift the balance between MR and GR activation. However, this is dependent on the tissue as only some tissues express both receptor types. Unlike GR, which is ubiquitously expressed, MR is localised to specific organs including the kidney, heart and regions of the brain. Importantly, both receptors are often abundantly co-expressed in regions regulating the HPA axis including the hippocampus and anterior pituitary⁸⁹.

Given the rapid stressor response of glucocorticoids, they can modulate cellular activity through non-genomic actions involving non-specific interactions with the cell membrane, or with cytosolic or membrane-bound GRs⁹⁰.

1.3.3 Glucocorticoid regulation

In addition to free plasma concentration and relative tissue density of receptors, glucocorticoid actions are also influenced by cell enzyme pathways in some tissues. The 11 β -HSDs are important - 11 β -HSD1 re-activates glucocorticoids in peripheral tissues including liver, adipose, skeletal muscle and kidney. Conversely, 11 β -HSD2 catalyses the reverse reaction *in vivo* and converts active glucocorticoids (cortisol/corticosterone) into inactive metabolites (cortisone/11-dehydro-corticosterone). Importantly,

cortisol/corticosterone can activate MR whereas their metabolites cannot. The action of 11 β -HSD2 is a major factor in conferring aldosterone specificity to cells such as the principal cells of the renal distal tubule and salt-seeking neurones in the nucleus of the solitary tract (NTS)^{91,92,93}.

Glucocorticoids regulate their own secretion via a negative feedback mechanism controlled by glucocorticoids binding to predominately GR, but also MR, in brain regions including the hypothalamus, hippocampus and anterior pituitary⁸⁰. This process is crucial for glucocorticoid circadian oscillations and termination of the HPA axis response to stress. Studies have identified both a fast nongenomic feedback, involving inhibition of CRH and ACTH secretion, and a delayed genomic feedback response, involving inhibition of CRH and POMC transcription from the hypothalamus and anterior pituitary, respectively. Furthermore, glucocorticoids can also act on the hippocampus for the rapid inhibition of the CRH neurons within the PVN. It has also been suggested that glucocorticoids regulate its own synthesis by local feedback within the adrenal gland⁸⁸. *In vitro* and *in vivo* studies have shown that glucocorticoid exposure to the adrenal gland reduces glucocorticoid synthesis via ACTH^{94,95}. The mechanisms behind glucocorticoid adrenal negative feedback is thought to be via inhibition of StAR transcription. Nongenomic mechanisms are thought to be involved as well but the mechanisms remain elusive⁸⁸.

FKBP5 regulates GR sensitivity as when this is bound to the receptor complex, glucocorticoids bind with low affinity. However, as previously mentioned, when glucocorticoids bind to GR, FKBP5 is replaced with FKBP4 increasing affinity. Glucocorticoids also regulate the sensitivity of GR, via a short negative feedback loop induced by glucocorticoid stimulation of *Fkbp5* transcription which, in turn, reduces ligand binding and delays the nuclear translocation of GR therefore, decreasing GR-dependent transcription^{96,97}.

Complications can arise if glucocorticoids are chronically elevated due to prolonged stress or genetic disorders. This can lead to obesity, hypertension and increased risk of CVD⁹⁸. Therefore, glucocorticoids have to be tightly regulated.

1.3.4 Glucocorticoid clearance

The circulating half-life of free cortisol varies between 70 and 120 minutes, with reports of corticosterone in rodents being between 30 and 60 minutes^{83,99,100}. The major routes of glucocorticoid metabolism involve the inactivation of cortisol to cortisone (corticosterone to dehydrocorticosterone in rodents) by 11 β -HSD2 as previously mentioned or cortisol reduction by hepatic 5 α - or 5 β -reductase to 5 α -tetrahydrocortisol (THF) or 5 β -THF, respectively. These products are conjugated and excreted via bile or urine⁸³.

1.3.5 Abnormal glucocorticoid signalling in disease

During stressful stimuli, activation of the HPA axis to increase glucocorticoid synthesis and secretion is an essential physiological response. However, chronic elevation of glucocorticoids occurs in Cushing's syndrome, which is most commonly caused by extensive use of exogenous glucocorticoids. Furthermore, chronically elevated glucocorticoids have been associated with obesity, CVD, type 2 diabetes mellitus and mood disorders. There is also evidence for higher circulating glucocorticoid levels in individuals with hypertension and glucose intolerance^{101,102}.

For this thesis, there will be particular focus on glucocorticoid excess. However, it is worth noting that treatment for diseases characterised by glucocorticoid deficiency, such as congenital adrenal hyperplasia (a group of autosomal recessive diseases caused by mutations in genes involved in steroidogenesis), is often glucocorticoid replacement therapy. Treatment is particularly challenging since glucocorticoids exhibit a diurnal rhythm. Therefore, there is a risk of glucocorticoid overtreatment in these patients¹⁰³.

1.3.5.1 Cushing's syndrome

As previously mentioned, Cushing's syndrome results from prolonged exposure to high concentrations of circulating free glucocorticoids¹⁰⁴. Severe glucocorticoid excess was first described by Dr Harvey Cushing over a century ago¹⁰⁵. There are several subtypes of Cushing's syndrome, including endogenous (or spontaneous) and exogenous (or iatrogenic). Endogenous Cushing's syndrome is a rare condition with an estimated incidence of 0.7-2.4 per million of the population every year¹⁰⁶. Endogenous Cushing's syndrome can either be ACTH-dependent (80-85% of cases) or ACTH-independent. MC2R within the adrenal cortex is overstimulated in ACTH-dependent Cushing's syndrome, resulting in inappropriate glucocorticoid secretion. This is predominately caused by an ACTH-secreting pituitary adenoma (Cushing's disease). However, a small percentage is caused by ectopic ACTH secretion via nonpituitary tumours. ACTH-independent Cushing's syndrome, as the name states, involves glucocorticoid production without ACTH stimulation, often caused by a benign adrenocortical adenoma. However, the most common cause of glucocorticoid excess is by long exposure of administered exogenous glucocorticoids, as glucocorticoids are often used to treat inflammatory, autoimmune and neoplastic disorders^{104,106,107}.

Cushing's syndrome is difficult to diagnose as its clinical features develop gradually over time and it shares many features with metabolic syndrome, which is considerably more prevalent. These pathophysiological features include obesity or weight gain (present in 95% of adult cases of Cushing's syndrome), hypertension (75%), depression (70%) and glucose intolerance (60%)¹⁰⁴.

1.3.5.2 Metabolic syndrome

Metabolic syndrome displays many of the same features as Cushing's syndrome, leading to the hypothesis that excess glucocorticoids may also play a critical role in the pathogenesis of metabolic syndrome. Elevated circulating cortisol levels are rare in metabolic syndrome patients. However, recent

studies have proposed increases in generation of active cortisol from cortisone by 11 β -HSD1 within tissues¹⁰¹.

Metabolic syndrome is increasingly prevalent in Western societies, affecting approximately 20% of the adult population. Each feature of metabolic syndrome is an independent risk factor for CVD. Hypertension in metabolic syndrome, again like in Cushing's syndrome, is not fully understood. However, a unifying factor is that hypertension in metabolic syndrome is salt-sensitive^{58,92}. This led to questions arising if abnormal glucocorticoid signalling could contribute to salt-sensitivity.

1.4 Hypertension in systemic glucocorticoid excess

The association between glucocorticoids and hypertension is complex as glucocorticoids have pathophysiological actions on multiple organ systems, including the kidney and vasculature. This can ultimately lead to increases in BP, with impacts on the diurnal rhythm of BP, inducing non-dipping BP likely to be linked to a disruption in glucocorticoid circadian rhythm^{108–110}. Studies using administration of ACTH in humans and rodents, to model ACTH-dependent Cushing's syndrome, has been shown to increase BP, CO and RVR with no change in TPR. It has been suggested that RVR plays a crucial role in development of ACTH-induced hypertension as studies have shown reductions in CO could not prevent ACTH- or glucocorticoid-induced hypertension in rats^{111–113}. The underlying mechanisms behind glucocorticoid-induced hypertension are thought to be centred around direct and indirect effects on the vasculature and the kidney^{114,115}.

1.4.1 Regulation of vascular and haemodynamic changes by glucocorticoids

Glucocorticoids regulate BP by having direct effects on the vasculature, mediated by both GR and MR. Both 11 β -HSD1 and 11 β -HSD2 are present in endothelial cells and vascular smooth muscle (VSM) cells, suggesting they play a role in modulating local access of glucocorticoids to the vasculature to

influence vascular tone^{116–118}. In the VSM, glucocorticoids have been shown to enhance the vascular reactivity to vasoconstrictors (endothelin 1, angiotensin II, catecholamines and AVP) and suppress the vasodilatory system (nitric oxide synthase) in the endothelium^{117–121}. Enhanced vascular responsiveness to various vasoconstrictors is thought to be due, in part at least, to glucocorticoids increasing intracellular calcium to cause contraction, via downregulation of the sodium calcium exchanger in the VSM¹²². Therefore, systemic glucocorticoid excess can lead to increased vasoconstriction and vascular resistance, leading to increases in BP.

Glucocorticoid-induced vascular and haemodynamic changes help maintain renal blood flow (RBF) and GFR¹²³. The effects of glucocorticoid excess on RBF and RVR are conflicting. In humans, glucocorticoid-induced RBF changes are variable^{124,125}. Furthermore, glucocorticoid excess, or exogenous administration, acting via GR, can induce natriuresis thought to be due to glucocorticoids impairing RBF autoregulation, increasing GFR to promote both diuresis, natriuresis and kaliuresis. Although, glucocorticoids acting via MR can have opposing effects on renal haemodynamics¹²⁶. For example, chronic ACTH infusion in mice increased urinary sodium excretion, even though MR and GR activation induced sodium reabsorption in the distal tubule^{127,128}. These alterations have been suggested to be involved in glucocorticoid-induced hypertension¹²⁹.

1.4.2 Regulation of renal sodium transport by glucocorticoids

RAAS is activated by volume depletion to stimulate the production of angiotensin II, which in turn stimulates the release of aldosterone. Aldosterone promotes renal sodium retention via ENaC, regulated by MR in the connecting tubule (CNT) and principal cells of the collecting duct (CD), defined as the aldosterone-sensitive distal nephron (ASDN). Thus, RAAS plays a critical role in BP control. The ASDN is defined by the co-localisation of MR and 11 β -HSD2. MR is expressed in the distal convoluted tubule (DCT). However, the expression of 11 β -HSD2 in the DCT has only been shown to be at very low

levels around the end of the DCT transitioning to the CNT^{130,131}. It has been proposed that this intermediate segment of the nephron has a role in sodium transport with MR activated by aldosterone under basal conditions or by glucocorticoids when in excess¹³². Glucocorticoids have previously not been thought of as major regulators of sodium transport, even though GRs are expressed throughout the kidney, including the ASDN. Glucocorticoids have been shown to impact sodium transport along the length of the renal tubule, including sodium-hydrogen antiporter 3 (NHE3) in the proximal tubule¹³³ and thiazide-sensitive NaCl cotransporter (NCC) in the DCT^{46,134}. Glucocorticoid stimulates ENaC activation in the CD via GR *in vitro* but still remains to be established *in vivo*¹³². This highlights a role for glucocorticoids in renal sodium transport and could have implications for hypertension and salt-sensitivity.

The relationship between glucocorticoids and hypertension has been explored in mouse models of abnormal HPA axis activation that induce systemic glucocorticoid excess, including chronic ACTH infusion and corticosterone infusion. These models clamped plasma glucocorticoid levels at a mid-physiological range but the diurnal rhythm of corticosterone was attenuated therefore, excess was achieved at certain times of the day^{128,134}. Chronic ACTH infusion caused hypertension and enhanced sodium reabsorption, via ENaC, in the distal nephron, involving both GR and MR pathways¹²⁸. Furthermore, chronic corticosterone infusion flattened the diurnal BP rhythm due to enhanced sodium reabsorption via NCC¹³⁴. This suggests in situations of glucocorticoid excess, glucocorticoids can impair renal salt excretion. Therefore, hypertension can develop as the HPA axis escapes negative feedback mechanisms through volume/electrolyte status.

Taken together, glucocorticoids regulate renal sodium transport all along the nephron, having implications when in excess on hypertension, with the expression of 11 β -HSDs influencing this.

1.5 Abnormal glucocorticoid signalling and salt-sensitivity

Overexpression of adipose 11 β -HSD1 in mice causes salt-sensitive hypertension and loss of the BP dip, similarly found in models of glucocorticoid excess, which is proposed as a mechanism underpinning the BP profile in metabolic syndrome patients¹³⁵. Furthermore, failure to downregulate renal medullary 11 β -HSD1 expression contributed to sodium retention and salt-sensitivity in Dahl salt-sensitive rats. However, the mechanisms behind this are not fully understood¹³⁶.

Reduced activity of the glucocorticoid metabolising enzyme, 11 β -HSD2, has also been shown to contribute to salt-sensitivity^{137–139}. Null mutations in the 11 β -HSD2 gene, *Hsd11b2*, causes Apparent Mineralocorticoid Excess (AME), characterized by sodium retention and severe salt-sensitive hypertension. Global knockout of *Hsd11b2* in mice recapitulates AME with heterozygous knockout mice being normotensive^{114,140}. Polymorphisms in *Hsd11b2* are associated with salt-sensitivity in normotensive individuals¹³⁷. Heterozygous *Hsd11b2* mice, with 50% of the renal enzyme activity, were salt-sensitive driven by salt-induced elevations in circulating glucocorticoids. The mechanisms behind salt-sensitivity in these mice was impaired renal sodium excretion, specifically failure to downregulate ENaC upon salt loading which was GR mediated. Blockade of either ENaC or GR prevented salt-sensitivity in heterozygous *Hsd11b2* mice^{44,141}. Expression of 11 β -HSD2 is predominately in the NTS and distal nephron of the kidney. Knock out models of *Hsd11b2* in either the brain or the kidney were generated to further explore the link between central/renal 11 β -HSD2 and salt-sensitive hypertension. Reduced activity of 11 β -HSD2 in the brain promoted MR-mediated salt appetite and a transition to salt-sensitivity without systemic sodium retention¹³⁹. Reduced 11 β -HSD2 activity in the kidney also lead to salt-sensitivity but was driven by increased renal membrane ENaC expression, as seen in the heterozygous mice, and increased phosphorylation of NCC. However, this study suggested this was MR mediated¹⁴².

The role of MR was supported further in a study modelling glucocorticoid resistance, using heterozygous GR knockout mice. This study suggested that global reduction in GR expression induced salt-sensitivity in mice, most likely reflecting sustained MR activation by glucocorticoids¹⁴³.

Further studies have demonstrated a role of MR and GR in salt-sensitivity, via impaired renal salt excretion. Shibata et al and Mu et al identified crucial roles of two novel pathways, the Rac1-MR-NCC/ENaC pathway and the renal β -adrenergic stimulant GR-WNK4-NCC pathway, in salt-sensitive hypertension (Figure 1.9)^{46,144}. Rac1, a member of the Rho-guanine trisphosphate hydroxylases family, has been shown as an upstream regulator of MR and regulates salt-sensitive BP. In Dahl salt-sensitive rats, salt loading stimulated renal Rac1, activating MR to cause sodium retention and BP to rise, despite reduced plasma aldosterone levels¹⁴⁴. Furthermore, WNK4, a serine-threonine kinase, is a negative regulator of NCC, leading to decreased sodium reabsorption to maintain BP. However, dietary salt intake induced increases in the renal SNS, resulting in norepinephrine activating its β -adrenergic receptor to downregulate WNK4. This results in the upregulation of NCC, increasing sodium reabsorption and elevating BP. Glucocorticoids and GR in the distal nephron have been shown to be required for this WNK4 downregulation⁴⁶. These studies support the potential contribution of both glucocorticoids, GR and MR in salt-sensitivity.

Collectively, this supports glucocorticoids having a major role in the salt-sensitive phenotype. The suggested molecular mechanisms whereby glucocorticoids contribute to salt-sensitive hypertension focus around stimulation of renal sodium transport with a potential role of both MR and GR in some capacity^{46,141}. However, there are limited studies exploring the potential contribution of the vasculature in glucocorticoid-induced salt-sensitive hypertension.

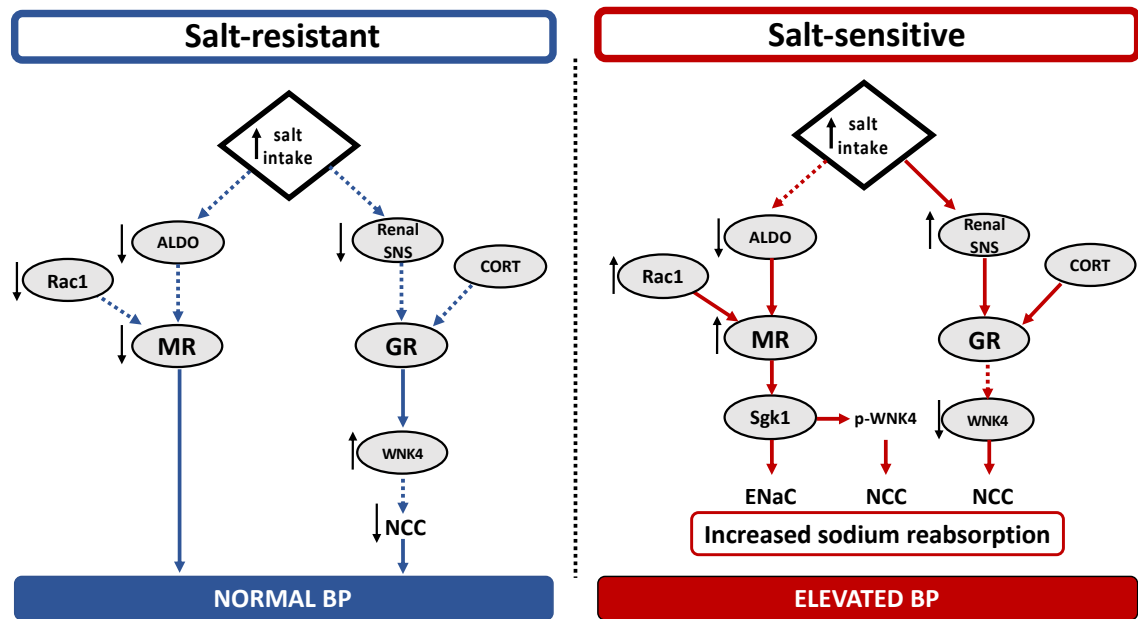


Figure 1.9: Proposed Rac1-MR-NCC/ENaC and GR-WNK4-NCC pathway involved in salt-sensitive hypertension.

MR and GR, stimulated by Rac1 and renal SNS overactivity, are involved in increasing renal sodium reabsorption via NCC and ENaC in the distal nephron. Arrowhead solid lines and arrowhead dashed lines indicate stimulation and suppression, respectively. Adapted from Fujita 2014⁷². Aldo, aldosterone; CORT, cortisol/corticosterone; p-WNK4, phosphorylation of WNK4.

1.5.1 A link between salt and glucocorticoids?

Interestingly, in heterozygous *Hsd11b2* knockout mice previously mentioned, salt induced an elevation in circulating corticosterone signalling, driving a rise in BP. This has been shown in rodent and human studies where high salt increased urinary glucocorticoid excretion, suggesting an increase in overall daily production^{141,145,146}. Ivy et al have also reported that high salt increases corticosterone activity in heterozygous GR knockout mice¹⁴³. Salt activating the HPA axis has been suggested but further research is needed and the potential mechanisms behind this phenomenon is of interest in a salt-saturated society.

1.6 Hypothesis and aims

The overarching hypothesis of my thesis is:

Glucocorticoid excess causes vascular and renal dysfunction, contributing to salt-sensitive hypertension.

To address this hypothesis, I aim to determine the effect of glucocorticoid excess on salt and BP homeostasis in C57BL/6JCrI mice using ACTH-dependent Cushing's syndrome as a model of an extreme phenotype of glucocorticoid excess. This mouse strain was selected as it has been described in the *American Heart Association* Scientific Statement on Animal Models in Hypertension as salt-resistant¹⁴⁷. The desire of the animal model selected was to resemble a disease state associated with glucocorticoid excess, such as Cushing's syndrome or metabolic syndrome. There are various types of metabolic syndrome animal models, including diet-induced, drug/chemical-induced and genetic models (ZF rat, DS/obese rat, POUND mouse). However, diet and drug-induced models have a delayed onset of metabolic syndrome and genetic models do not resemble all the criteria of metabolic syndrome in humans¹⁴⁸. Therefore, the ACTH-dependent Cushing's syndrome model was selected, involving infusion of ACTH via minipumps over a minimum of 12 days. Secondly, I aim to assess renal and vascular function in ACTH-treated mice before and after a high salt challenge. Finally, to explore the effect of high dietary salt on HPA activity, I will systematically profile the HPA axis in C57BL/6JCrI mice before and after a high- salt challenge.

Chapter 2: Materials and methods

2.1 Source of animals and husbandry

All animal experiments described were performed in line with United Kingdom Home Office regulations and in accordance with the Animals (Scientific procedures) Act 1986. Mice were maintained under conditions of controlled temperatures of $21^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in stock-holding rooms or $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in procedural rooms; all rooms were maintained at $50\% \pm 10\%$ humidity and operated on a 12 h light dark cycle (lights on 7 AM to 7 PM). This is also stated in zeitgeber time (ZT), where ZT0 is lights on and ZT12 is lights off. Mice were housed in groups, unless otherwise stated, and given *ad libitum* access to food of a standard control salt diet (0.3% Na^+ and 0.7% K^+ by weight: RM1, SDS Diets, Essex, UK) and water. Where stated, mice were also placed on a high salt diet (3% Na^+ and 0.6% K^+ by weight: RM 3% Na^+ SY, SDS Diets). This percentage of high salt intervention was chosen as this was ten-fold higher than the standard control salt diet. The minimum physiological need for sodium is estimated around 0.5 g/day, with adequate intake estimated at 1.5 g/day⁹. Therefore, consumption is roughly ten-fold higher than adequate intakes, justifying the percentage of high salt intervention chosen.

Male C57BL/6JCrI mice used for experiments were purchased from Charles River Laboratories (Tranent, UK), between 8-10 weeks old, and allowed at least a week between transportation and the start of any experiment to acclimatise and to minimise stress. Mice were randomised into treatment groups and experiments performed with a single blind to group allocations.

2.2 Minipump implantation

2.2.1 Minipump preparation

Osmotic minipumps (Alzet model 2002 for 14-day infusion, 2004 for 28-day infusion or 2006 for 42-day infusion; Charles River Laboratories) were filled with ACTH (Adrenocorticotrophic Hormone Fragment 1-24 human, rat; Sigma, MO, USA) at the desired concentration of 2.5 $\mu\text{g}/\text{day}$ using a syringe and blunt-tipped filling tube. Filling was carried out slowly to prevent the introduction of

air bubbles which can affect pumping rates. Minipumps were “primed” in sterile saline at 37°C overnight for model 2002, 40 h for model 2004 or 60 h for model 2006, as per manufacturer’s instructions. Priming was necessary as minipumps used had a start-up gradient where the pump soaks up fluid, so this allowed immediate pumping when implanted into each mouse. Minipump preparation was carried out using aseptic techniques to prevent contamination of both the solution and minipump.

2.2.2 Minipump implantation

Osmotic minipumps were implanted subcutaneously (s/c) into adult male C57BL/6JCrI mice under isoflurane anaesthesia, 4% induction then 2-3% maintenance during the surgical procedure. Mice received a Vetergesic analgesic injection (s/c) to reduce post-operative pain, along with 0.5 mL 0.9% sterile saline to aid recovery. Surgery was performed aseptically and on a heat pad. A small incision was made in the nape of the neck and, using tissue separating scissors, a s/c pocket was created. The minipump was placed in the pocket on the flank of the animal and incision closed with 5-0 absorbing suture silk.

2.3 BP measurements by radio telemetry

Adult male C57BL/6CrI mice were implanted with radio telemeter devices (PA C10, DSI, MN, USA) into the thoracic aorta via the left carotid artery, which were electronically coupled to receiver pads. Prior to implantation, the offset of each device was assessed and recorded. The mice were single-housed one week prior to surgery with free access to standard salt diet and water.

2.3.1 Radio telemeter implantation

Radio telemeter implantation was carried out by Mr Kevin Stewart. Devices were implanted into mice under isoflurane anaesthesia in oxygen, with initial induction at 4% and reduced to 2-3% to maintain unconsciousness during the surgical procedure via a nose cone. Buprenorphine (0.1 mg/kg; Vetergesic)

was administered s/c along with 1 mL bolus injection of 0.9% sterile saline to aid in recovery. Ocular lubricant (Lactri-lube) was applied to the mouse's eyes to prevent them drying out during the surgery. The ventral neck area was shaved and iodine scrub (Vetasept) applied. A small midline ventral cervical incision was made and, using tissue separating scissors, a s/c pocket was made to house the DSI transmitter. The soft fascia tissue in the neck was gently separated to isolate the carotid artery. Once isolated from the vagus nerve, a cranial ligature was tied around the carotid artery and a small section of the artery occluded, using a micro-haemoclamp, to allow cannulation. A bent 23-gauge blue needle was used to make a small incision into the occluded section of the vessel and the anti-thrombotic gel-filled catheter tip of the device was inserted into the artery. Two ligatures were tied around both the vessel and catheter, with a small volume of tissue adhesive (3M™ Vetbond™) applied for extra security of catheter placement. A radio was used to ensure the device was transmitting. The transmitter was placed inside the s/c pocket created and incision closed with absorbable 5-0 suture silk (Vicryl). Oxygen was fed through the nose cone to allow the animal to regain consciousness and then transferred to a hot box until mobility was fully regained before returning to its cage. For 48 h post-surgery, mice were allowed ad libitum access to Vetergesic jelly (Hartley's, UK) and mash (RM1 diet soaked in drinking water). If jelly was uneaten, the Vetergesic was administered by injection s/c.

2.3.2 Data collection and analysis

BP were recorded over the experimental period. Data were acquired at a frequency of 1 Hz over a 5 min period every 30 min. Initial analysis was a calculation of an average 24 h SBP and DBP for each mouse, used in turn to calculate the group mean and standard deviation (SD).

Sections of data were subjected to cosinor analysis. Cosinor analysis is used to calculate circadian periods including midline estimating statistic of rhythm (MESOR), period, amplitude, acrophase and robustness. Since BP is rhythmic, MESOR provides a more appropriate estimate of central tendency as it takes circadian rhythm into account. The period is the time taken for a full

cycle or from one peak of the oscillation to the next. Amplitude is the difference between the peak and the MESOR. Acrophase is the time at which the peak of a rhythm occurs. Robustness is the strength of the rhythm and calculated as the percentage of variance accounted for by the cosinor model, with levels below 20% likely to be biological noise instead of signal¹⁴⁹. Data were smoothed using a moving average over 5 h and smoothed data for each mouse over 3 consecutive days and fitted to a cosinor curve using cosinor software designed by Refinetti et al¹⁴⁹ (as illustrated in figure 2.1).

Activity was acquired by a parameter of the DSI software. To calculate activity, the telemetry signal is transmitted to the receiver in varying strengths dependent on the animal's orientation or distance of the animal from the receiver. When this signal strength changes by a certain amount, the software can calculate an activity count. Total activity was presented as counts per min.

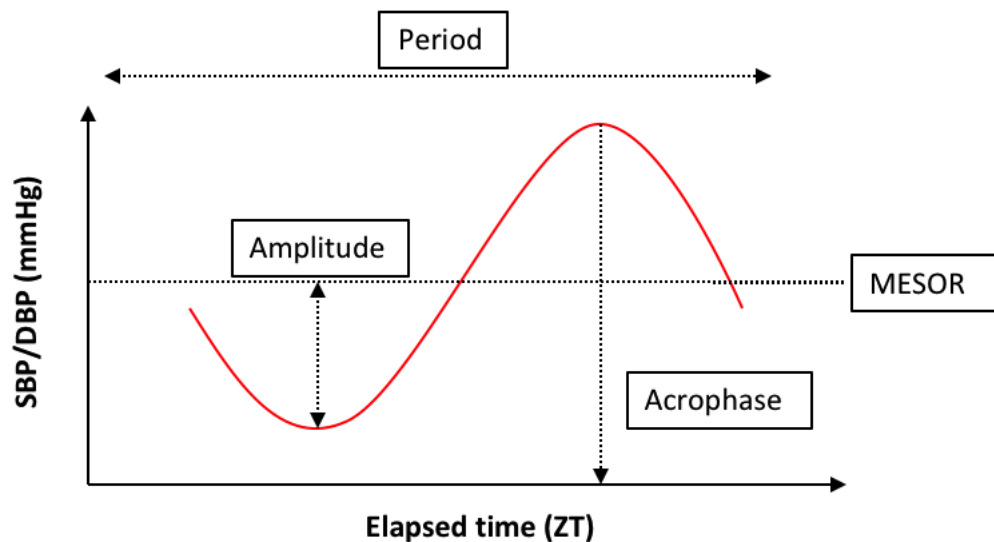


Figure 2.1: Representative diagram of cosinor fit.

Cosinor fit for visualisation of midline estimating statistic of rhythm (MESOR), period, amplitude and acrophase acquired from the cosinor curve fit.

2.4 Metabolic cage protocol

Mice were single housed and placed in metabolic cages for 24 h, with 2x 12 h urine collections between 7 AM and 7 PM, and 7 PM and 7 AM, at the end of each treatment. Metabolic cage experimental designs are outlined in the methods sections (Chapter 4).

2.4.1 Urinary electrolyte analysis

Electrolytes were measured in the urine, with samples diluted 1:1 with deionised H₂O, using an electrolyte analyser (SpotChem EL, Arkray, Amstelveen, Netherlands).

2.5 Acute pressure natriuretic response

2.5.1 Surgical protocol

Mice were anaesthetised with 120 mg/kg intraperitoneal barbiturate (Inactin, Sigma) and placed in a hot box until unconscious. Body temperature was controlled with a homoeothermic blanket system (AD instruments, Oxford, UK) to ensure their core temperature was maintained at 37°C. Lidocaine (1% w/w in 0.9% sterile saline; Sigma) was administered at each incision site as a local anaesthetic. During the surgery, unconsciousness was assessed by blink and/or toe pinch reflexes. Small volumes, a maximum of 10 µL, of anaesthetic were administered intravenously, via the jugular vein, to maintain unconsciousness.

2.5.1.1 Jugular vein cannulation

The right jugular vein was cannulated for infusion of physiological saline (100 mM NaCl, 15 mM NaHCO₃, 5 mM KCl, 0.25% fluorescein isothiocyanate (FITC)-inulin (Sigma), pH 7.4) and administration of anaesthetic. The infusate was to replace loss of fluid during the surgery. Furthermore, the infusate contained FITC-inulin to allow measurement of GFR, as FITC-inulin is freely filtered and is neither secreted or reabsorbed¹⁵⁰. The left jugular vein was isolated and a cranial ligature (5-0 non-absorbable suture silk; Fine Science Tools) was tied. Tension was applied, using a bulldog clamp, to elevate the vein. A small incision was made caudal to the ligature and a cannula (P10 tubing, Smiths medical, Kent, UK), containing 0.9% sterile saline connected to a 1 mL syringe, was inserted into the jugular vein and secured in place with a second ligature. To confirm the cannula was in place, the syringe was pulled back to ensure blood was present in the cannula tubing. The cannula was connected to an infusion pump (AL2000, World Precision Instruments, FL,

USA) for continuous administration of the infusate at a rate of 0.25 mL/10g bw/h.

2.5.1.2 Tracheostomy

A tracheostomy was performed to relieve any obstruction to breathing. The trachea was exposed and isolated to allow insertion of tubing (P50, Smiths medical) underneath the trachea. An incision was made between the cartilage rings of the raised portion of the trachea. The trachea was internally cleared, using thin tissue twists.

2.5.1.3 Carotid artery cannulation

Mean arterial BP (MABP), SBP and DBP were measured (Powerlab, AD Instruments, Sydney, Australia) and blood samples collected via the right carotid artery. The carotid artery was exposed, carefully isolated from the adjacent vagus nerve and cranially ligated. Tension was applied, using a bulldog clamp, to elevate the artery. A small section of the artery was occluded, using a micro-haemoclamp, to allow cannulation. A small incision was made in the occluded section of the artery close to the cranial ligature. A cannula (P50 with P10 tubing at the tip) containing sterile heparin saline connected to a 1 mL syringe was inserted into the carotid artery and secured with a second ligature. The cannula was carefully turned so the point of the bevel of the cannula was facing upwards to ensure blood flow and secured with a third ligature. The micro-haemoclamp was removed and cannula connected to a FE221 Bridge Amp (AD Instruments) for continuous arterial BP measurements through PowerLab data acquisition software.

2.5.1.4 Bladder catheter

The bladder was catheterised to collect urine and measure urinary flow rate. An abdominal incision was made, and the bladder was located. Tissue was placed around the bladder to soak up any urine as a small hole was made in the bladder, using a cauterizer to mitigate bleeding and damage. A catheter, made from P50 tubing, was inserted into the bladder and secured with a

ligature.

2.5.1.5 Abdominal arterial ligations

The incision was extended from the bladder incision up to the diaphragm. A swab was rolled and soaked in sterile saline. The intestines were carefully moved to the left side with cotton buds to expose the right kidney. The swab was placed in the incision to ensure the intestines were kept moist and out the way to expose the right kidney, vena cava and liver. The distal aorta was isolated away from the vena cava and a loose ligature placed around it. The mesenteric and coeliac arteries were located, and a loose ligature was placed around both arteries.

2.5.1.6 Renal blood flow measurements

The right renal artery was carefully isolated from the renal vein and the perivascular adipose tissue (PVAT) surrounding the artery was removed gently using cotton buds. Pulse-wave Doppler ultrasound probe (VisualSonics, Toronto, Canada) was hooked around the right renal artery and ensured it was running smoothly over the probe to achieve an accurate reading of RBF.

2.5.2 Pressure natriuresis protocol

Following the surgery, a 30 min equilibrium period was carried out. At the end of the equilibrium period, blood samples were collected for plasma (P1-P4) before and after 3 urine collections (U1-U3), as illustrated in Figure 2.2. At the end of the 30 min U1 period, the mesenteric and coeliac arteries were ligated. At the end of the 20 min U2 period, the distal aorta artery was ligated. At the end of the 20 min U3 period, mice were euthanised following a final plasma sample collection.

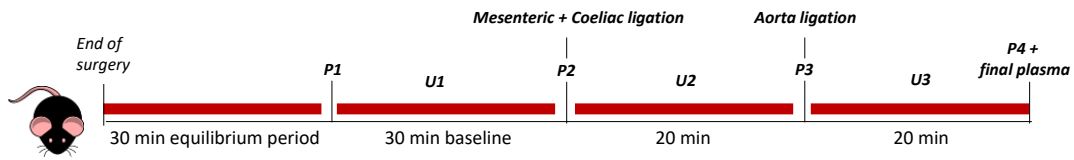


Figure 2.2: Schematic diagram of pressure natriuresis protocol post-surgery. Blood samples collected for plasma (**P1-P4**) before and after 3 urine collections (**U1-U3**). At the end of U1, mesenteric and coeliac arteries were ligated. At the end of U2, aorta was ligated.

2.5.3 Blood pressure and renal blood flow acquisition and analysis

Figure 2.3 illustrates representative BP and RBF traces during baseline, following mesenteric and coeliac artery ligation (Lig 1) and distal aorta artery ligation (Lig 2). Average cyclic measurements from each period (dotted boxes in Figure 2.3) were calculated in LabChart 7.3.2 to determine SBP, DBP and MABP.

Pulse-wave Doppler ultrasound probe, via a nano-flow probe (Transonic Systems, Netherlands) was used to measure RBF through the right renal artery, with measurements calculated as mean flow (mL/min) for each period in LabChart. Renal vascular resistance (RVR) could then be calculated as illustrated below:

$$\text{RVR (mmHg/mL.min}^{-1}\text{)} = \frac{\text{MABP}}{\text{RBF}}$$

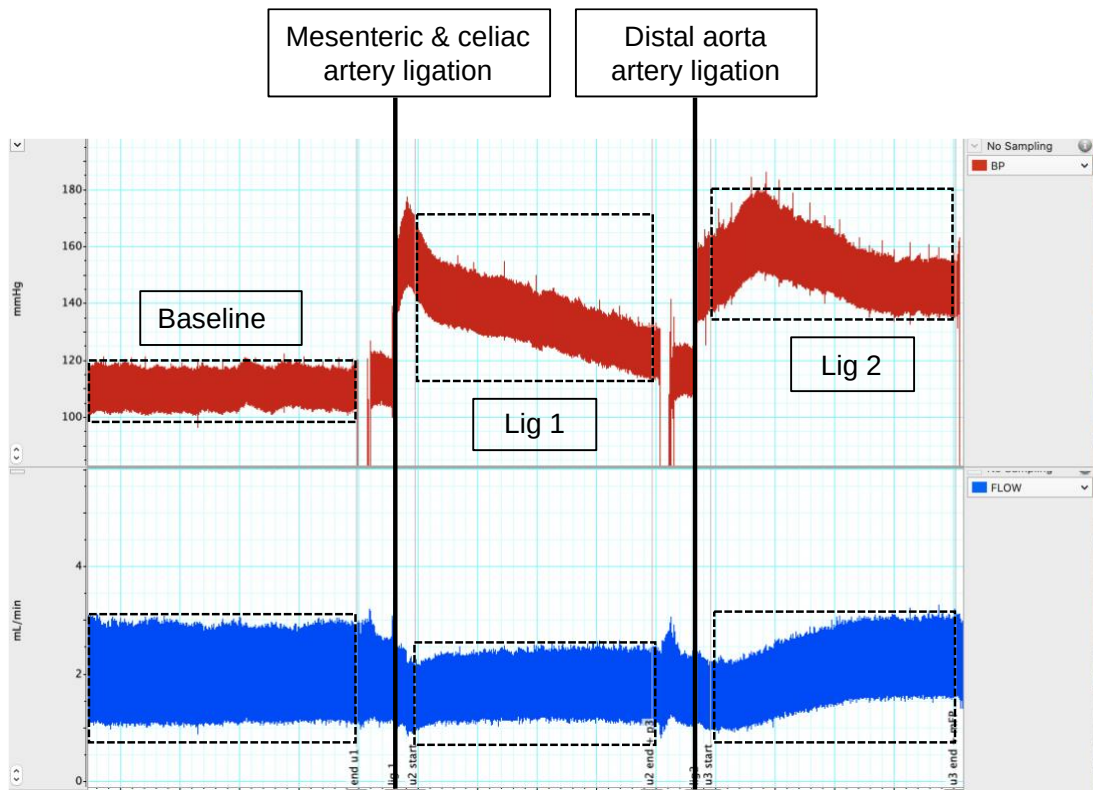


Figure 2.3: Schematic representative blood pressure and renal blood flow trace on LabChart.

A representative blood pressure (BP) trace, measured via the carotid artery, and renal blood flow (RBF), measured via a Doppler flow probe, in C57BL/6J mice during the pressure natriuresis protocol was acquired using LabChart 7.3.2. BP (red trace) and RBF (blue trace) for each period (baseline, Lig 1 and Lig 2) collected for further analysis is illustrated in the dotted boxes.

2.5.4 Sampling analysis

2.5.4.1 FITC-inulin assay

GFR is the gold standard for estimating renal function. GFR was calculated by measuring the FITC-inulin present in the urine and plasma and measuring the urine flow rate.

$$\text{GFR (mL/min)} = \frac{(\text{FITC-inulin in urine} \times \text{urine flow rate})}{\text{FITC-inulin in plasma}}$$

To calculate FITC inulin in urine and plasma, samples were diluted 1:10000 and 1:100 respectively in HEPES buffer (10 mM HEPES, pH 7.4) as FITC-inulin is pH dependent. The FITC fluorescence in urine and plasma samples

was measured. A 9 point 2-fold dilution series was set up for the standard curve with a top standard of 1 mg/mL FITC-inulin, before being diluted 1:100 in HEPES buffer. Diluted urine, plasma and standards were loaded (190 μ L) in duplicate in a Corning $\text{\textcircled{R}}$ 96 well black polystyrene microplate (Sigma). The plate was excited at 485 nm and read at 538 nm on a microplate reader (infinite M1000, TECAN, Männedorf, Switzerland).

2.5.4.2 Electrolyte analysis

Electrolytes were measured in the plasma and urine using an electrolyte analyser (SpotChem EL, Arkray). Plasma samples were run undiluted and urine samples diluted 1:1 with deionised H₂O.

2.6 *Ex vivo* wire myography

2.6.1 Isolation and mounting of arteries on wire myograph

Mice were culled by asphyxiated with CO₂, or via decapitation, prior to isolation of the renal and second order mesenteric arteries. Arteries were stored in fresh physiological salt solution (PSS; 119.0 mM NaCl, 3.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 25.0 mM NaHCO₃, 1.2 mM KH₂PO₄, 27.0 μ M EDTA, and 5.5 mM D-glucose) on ice. Vessels were cleaned of PVAT before mounting onto a wire myograph (Danish MyoTech, Aarhus, Denmark). Wells of the myograph were filled with PSS (6 mL) and vessels perfused with 95% O₂ and 5% CO₂ at 37°C.

2.6.2 Myography protocol

Each vessel mounted on the myograph was normalised using the DMT Normalisation Module on LabChart (AD Instruments) to calculate and set the optimal pre-tension conditions by determining its internal circumference (μ m). Each vessel was measured to calculate length in mm and wire diameter set to 40 μ m. A standard curve was generated by calculating the internal circumference at 2 mN increments of force, until an effective pressure (calculated from measured force divided by wall length i.e. resting wall tension)

exceeded 100 mmHg (13.3 kPa). The micrometer (Micrometer X1) generated was the calculated desired pre-tension condition and each vessel was set to this. A representative normalisation of a vessel is shown in Figure 2.4.

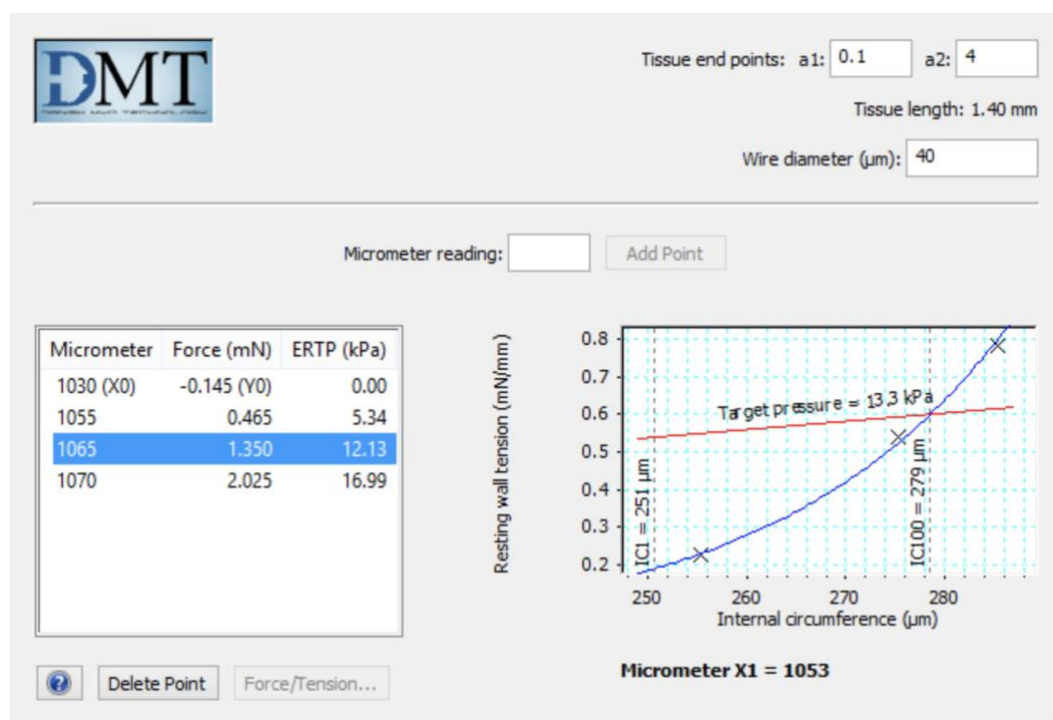


Figure 2.4: Representative normalisation using DMT Normalisation Module on LabChart.

A 30 min equilibrium period was carried out before a maximum depolarising contraction was measured by replacing the PSS with a high potassium physiological salt solution (KPSS; 125 mM KCl) at the beginning and end of the experiment. Vessels were treated with phenylephrine (1 nM to 30 μM PE; Sigma), acetylcholine (1nM to 30 μM ACh; Sigma) and sodium nitroprusside (1nM to 30 μM SNP; Sigma). ACH and SNP treatment followed a preconstriction with a required dose of PE for a half maximal contraction for each vessel.

2.6.3 Data analysis

Contractility data are presented as a percentage of the maximal KPSS response (shown as %KPSS) and dilatory data as a percentage of the vessel's preconstriction with PE (shown as %preconstriction) both against $-\log$ of the

molar drug concentration. Data are mean $\pm$ SD. PE, ACh and SNP data were fit with a non-linear regression to provide concentration response curves for each drug. EC50/IC50 and %Emax or %Preconstriction were calculated for each concentration response curve. Concentration responses between groups were compared using two-way analysis of variance (ANOVA), with ANOVA interaction reported, and multiple comparisons carried out assessing the effect of treatment at each drug concentration. Enhanced/reduced vascular reactivity was reported when taking into account the statistical significance of the ANOVA interaction, EC50/IC50 and %Emax or %Preconstriction for each concentration response curve.

2.7 HPA axis measurements

2.7.1 Tail venesection

Blood collection by tail nick technique was performed just after the lights came on at ~ 7.15 AM, when diurnal plasma corticosterone should be at its lowest, and just before the lights went off at ~6.30 PM, when diurnal plasma corticosterone should be at its highest, pre and post treatment. It was imperative that blood was collected within 1 min of disturbing the mouse's cage to acquire baseline measurements. Mice were removed from their home cages and placed on top of an empty clean cage. The tip of the tail (<1 mm) was snipped off using sharp sterile scissors. Mice were minimally restrained during blood sample collection and allowed to walk around the top of the cage until ~20 μ L of blood was collected into an EDTA coated tube and placed on wet ice. Blood was centrifuged at 2000 x g for 5 min at 4°C and plasma transferred to a fresh eppendorf to be stored at -80°C until all samples were collected for subsequent plasma corticosterone assays.

2.7.2 Terminal plasma sampling

A large plasma sample was needed to quantify aldosterone and copeptin concentrations, measure electrolytes and osmolality, and measure CBG

binding capacity. Blood was taken post-mortem via decapitation, centrifuged at 2000 x g for 5 min and stored at -80°C until all samples were collected for subsequent assays.

2.7.3 Plasma corticosterone assay

Plasma corticosterone concentration was quantified using a corticosterone ELISA kit (Enzo Life Sciences, Exeter, UK). Corticosterone was extracted from plasma by mixing 10 µL of plasma with 10 µL of diluted steroid displacement reagent (SDR; 1:100 dilution with deionised water) in sterile glass tubes for 5 min at room temperature. Following this, 480 µL assay buffer 15 (AB15) was added to give a final dilution of 1:50 for plasma sample. Samples were transferred to a fume hood where 3 mL ethyl acetate (Sigma) was added to glass tubes and vortexed thoroughly. The clear upper organic layer was transferred to a fresh glass tube. The ethyl acetate was evaporated under nitrogen at 60°C. Samples and standards were prepared and assayed according to the manufacturer's instructions. The plate was read on a microplate reader (Magellan, TECAN) with optical density measurements at 405 nm, and corrected with 550 nm.

The extraction efficiency was assessed for each extraction by calculating the corticosterone concentration of a known sample following extraction. The average extraction efficiency throughout the experiments carried out was 82 ± 7%.

2.7.4 Plasma aldosterone assay

Plasma aldosterone concentration was quantified using an aldosterone ELISA kit (Enzo Life Sciences). Plasma (25 µL) was diluted with 175 µL AB15 previously mentioned. Samples and standards were prepared and assayed according to the manufacturer's instructions. The plate was read on a microplate reader (Magellan, TECAN) with optical density measurements at 405 nm, and corrected with 550 nm.

2.7.5 Plasma copeptin assay

Plasma copeptin concentration was quantified using a copeptin ELISA kit (Cloud-Clone Corp., TX, USA). Plasma samples and standards (50 µL) were prepared and assayed according to the manufacturer's instructions. The plate was read on a microplate reader (Magellan, TECAN) with optical density measurements at 450 nm.

2.7.6 Plasma electrolytes and osmolality

Electrolytes were measured in the plasma using an electrolyte analyser (SpotChem EL, Arkray). Osmolality was measured in undiluted samples, using an osmometer (Type 15 Löser Osmometer, Camlab, Cambridge, UK).

2.7.7 CBG binding capacity assay

CBG binding capacity assay is a saturation binding assay used to determine specific binding by CBG to glucocorticoids. CBG binding capacity was measured as previously described by Hammond and Lähteenmäki (1983) but modified by Delehanty et al (2015) to minimise plasma volume required^{151,152}. Terminal plasma samples were diluted (1:100) and stripped of endogenous steroids using dextran-coated charcoal. To calculate CBG specific binding, non-specific binding had to be subtracted from total binding. To measure total binding, samples were incubated with radioactively labelled corticosterone ([1,2,6,7-³H]corticosterone). To measure non-specific binding, samples were first incubated with high concentrations of unlabelled corticosterone in addition to radioactively labelled corticosterone. This meant that it was likely that the [1,2,6,7-³H]corticosterone would bind to unsaturated non-specific binding proteins. Free [1,2,6,7-³H]corticosterone was removed by further incubation with dextran-coated charcoal. The remaining radioactively labelled corticosterone bound to CBG was quantified by scintillation spectrophotometry. The maximal binding capacity (B_{max}), measured in disintegrations per minute, was estimated using non-linear regression.

2.8 Estimating abundance of messenger RNA transcript by reverse transcription quantitative real-time polymerase chain reaction

2.8.1 Tissue harvest

Mice were culled by decapitation prior to dissection. Right kidney cortex, right kidney medulla, right adrenal gland, liver, hypothalamus, hippocampus, anterior pituitary, renal artery and mesenteric artery were dissected. Renal and mesenteric arteries were pooled from 2 mice and dissected vessels were placed in 1 mL RNAlater (Thermo Fisher, MA, USA) at 4°C for 24 h before being removed from solution and stored at -80°C. The remaining tissue was placed on dry ice following dissection before storage at -80°C prior to RNA extraction.

2.8.2 RNA extraction

RNA was extracted from mouse tissue, using the RNeasy Mini Kit (Qiagen, Hilden, Germany), by adding 300 – 600 µL RLT buffer (from the RNeasy Mini Kit) and a 5 mm stainless steel bead (Qiagen) to a 2 mL tube containing specific mouse tissue for homogenization, carried out using TissueLyser II (Qiagen) at 30 Hz for 1 min. The volume of RLT buffer was dependent on tissue weight as per manufacturers' instructions. Samples were centrifuged for 3 min at 8000 x *g*, and supernatant mixed with 1 volume of 70% ethanol (50% ethanol for liver tissue) and transferred to a spin column. Various spin steps and a DNAase step (RNase-free DNase Set, Qiagen) were then carried out according to manufacturer's protocol before RNA was eluted in 30-50 µL RNase free water (Thermo Fisher).

For RNA extraction of vessels, pooled vessels (2 mesenteric arteries or 4 renal arteries) were ground into a fine powder on dry ice using a pestle (Thermo Fisher) before homogenisation. An optimised protocol for RNA extraction was carried out using the RNeasy Micro kit (Qiagen). RLT buffer (300 µL) was added to the ground vessels, along with a steel bead, for homogenisation in a TissueLyserII at 30 Hz for 1 min. Proteinase K (Thermo Fisher; diluted 1:60)

was added to each sample before being incubated at 55°C for 10 min. Samples were centrifuged for 3 min at 8000 x *g*, and supernatant mixed with 350 µL 70% ethanol and transferred to a Micro-Elute spin column. Various spin steps were then carried out according to manufacturer's protocol and RNA eluted in 14 µL RNase free water.

RNA concentrations, in ng/mL, were measured using a nanodrop 1000 spectrophotometer (Thermo Fisher). RNA integrity was assessed using automated electrophoresis (Figure 2.5; 2100 Bioanalyzer, Agilent, CA, USA). The software assigns an RNA integrity number (RIN) to a total RNA sample, which is an objective metric of RNA quality. RIN ranges from 1 (completely degraded RNA) to 10 (highly intact RNA). RNA samples of a RIN of 7 or above were taken forward for reverse transcription.

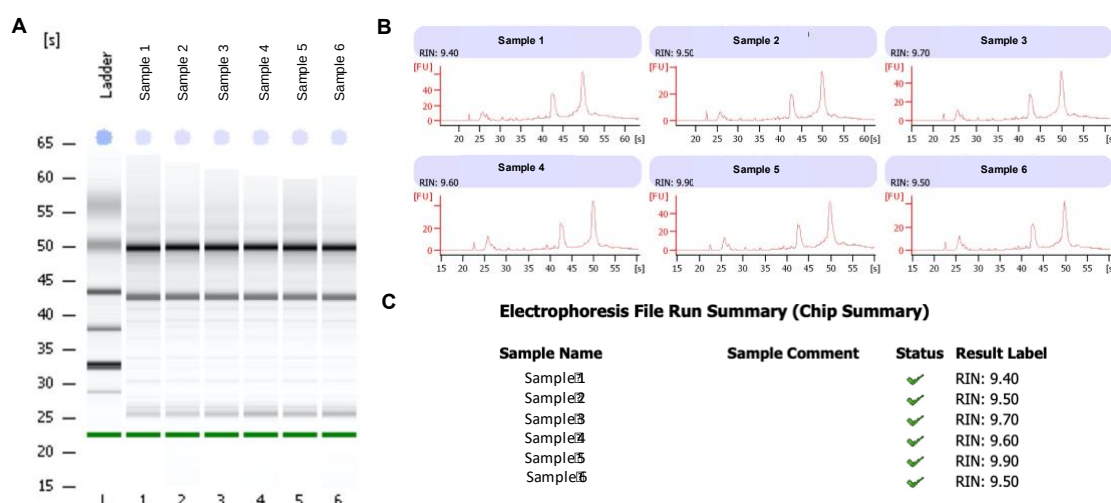


Figure 2.5: Schematic example of automated electrophoresis of RNA samples. (A) 500 ng of RNA was resolved on an automated electrophoresis gel. The intensity of the top band (28S) was approximately double of that of the bottom band (18S) of each RNA sample indicating highly intact RNA. (B) Electropherogram of each RNA sample (first peak 18S, second peak 28S). (C) RNA integrity number (RIN), an objective metric of total RNA integrity, of each sample ranging from 1 (completely degraded) and 10 (highly intact).

2.8.3 Reverse transcription quantitative real-time polymerase chain reaction

cDNA was synthesised from 500 ng of RNA, using the High Capacity cDNA Reverse Transcription (RT) kit (Applied Biosystems, CA, USA), where 10 μ L of 2x RT buffer and 1 μ L of 20x enzyme mix were added to the diluted RNA and made up to 20 μ L with nuclease free water. This was then reverse transcribed on a Veriti Thermal Cycler (Applied Biosystems) using cycling conditions described in Table 2.1. Negative controls were included, where the reverse transcriptase enzyme or RNA was replaced with an equal volume of water.

Table 2.1: Cycling conditions for reverse transcription.

Step	1	2	3
Temperature ($^{\circ}$ C)	37	95	4
Time (min)	60	5	∞

Primers for RT quantitative real-time polymerase chain reaction (RT-qPCR) were designed using the Universal Probe Library (UPL) Assay Design Centre

([URL:http://lifescience.roche.com/shop/CategoryDisplay?identifier=Universal+Probe+Library](http://lifescience.roche.com/shop/CategoryDisplay?identifier=Universal+Probe+Library)).

All designed UPL primers were synthesised by Integrated DNA technologies (IDT, IA, USA). Primers were designed where the amplicon spanned an intron, excluding the first or last intron. The resulting cDNA from the RT reaction was amplified and quantified using qPCR. Equal volumes of cDNA from samples were pooled together to construct an 8 point standard curve. The top standard of pooled samples were diluted 1:5 with nuclease-free water before serial dilutions (1:2) were carried out for standards 2-7. The final standard was used as a blank (nuclease-free water). The remaining cDNA was diluted 1:40 to fit in the middle of the standard curve. Sample/standard (2 μ L) was added to 8 μ L of the qPCR master mix (Table 2.2) in triplicate in a 384 well plate. Primers and dual hybridisation probes used in RT-qPCR reactions are illustrated in Table 2.3. Negative controls, as described above, were also included. The

Roche LightCycler 480 (Roche Diagnostics Ltd., Basel, Switzerland) was used to perform thermocycling and fluorescence detection, using the specific cycling conditions (Table 2.4). Threshold cycle/crossing point (C_q) values for each well were quantified, using the automated LightCycler 480 software (Figure 2.6). Each triplicate was analysed and excluded if the C_q SD was > 0.5 . For each gene of interest and reference gene, the standard curve was analysed and had to meet criteria of standard curve efficiency being within the range of 1.7-2.1 and error < 0.05 . Gene expression was analysed as relative expression as each gene was normalised against the mean expression of a minimum of 2 reference genes. A panel of reference genes were tested for each tissue (including *Actb*, *Gapdh*, *Hprt*, *Rn18s* and *Tbp*) and appropriate reference genes were selected for each tissue on the basis that they did not differ between treatment groups. Data were then log transformed to ensure symmetry of the data set and normality tests were conducted¹⁵³. RT-qPCR data were handled in accordance to the MIQE guidelines¹⁵⁴. Q values, adjusted p values controlling for the false discovery rate (FDR), were also reported to adjust for multiple comparisons in gene expression data for renal and mesenteric arteries. The two-stage set-up method of Benjamini, Krieger and Yekutieli was used, with desired FQR set to 5%. This method examines the distribution of p values to estimate the proportion of the null hypotheses that are actually true and whether the p value is low enough to be a discovery¹⁵⁵.

Table 2.2: qPCR master mix.

UPL master mix	Volume/Sample (μL)
PerfeCTa [®] FastMix [®] II (Quanta Biosciences Inc. [™] , MA, USA)	5.0
Primer pair	0.1
UPL Probe	0.1
Nuclease free H ₂ O	2.8
cDNA	2.0

Table 2.3: Primer sequences and UPL probes for qPCR.

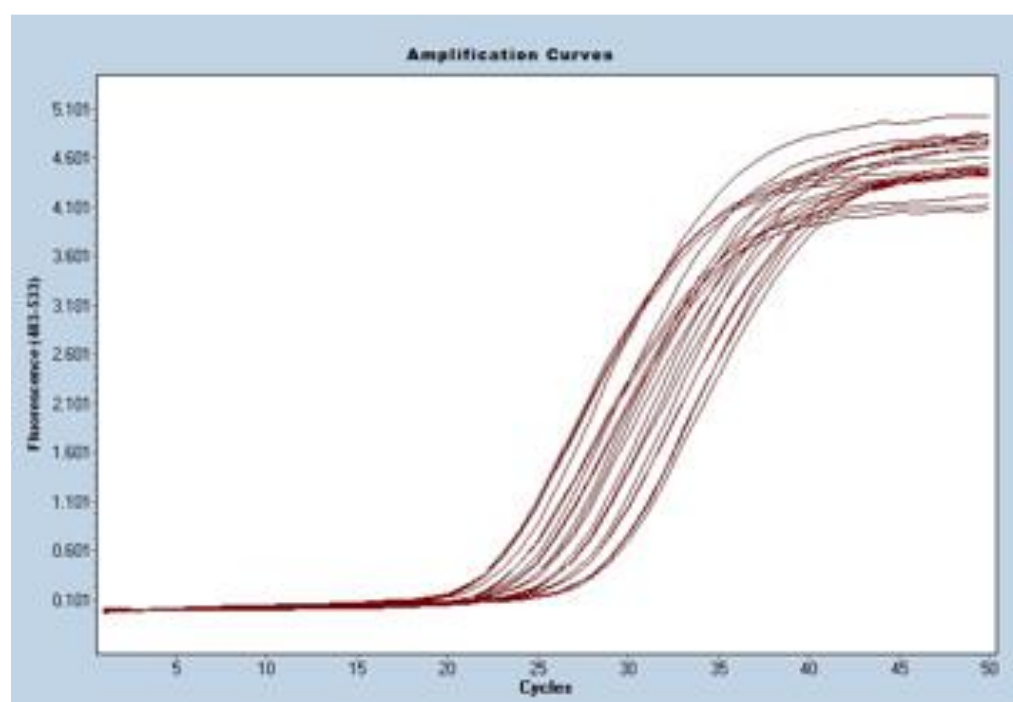
Efficiency and error values presented are from qPCR performed in Chapter 6. Efficiency and error values from all other assays were similar to the values presented here and met the criteria stated above.

Protein	Gene	Forward primer	Reverse Primer	UPL probe no.	Efficiency	Error
11βHSD1	<i>Hsd11b1</i>	tctacaaatgaagagttcagaccag	gccccagtgacaatcacttt	1	1.977	0.00373
11βHSD2	<i>Hsd11b2</i>	cactcgaggggacgtattgt	gcaggggtatggcatgtct	26	1.88	0.0122
18S	<i>Rn18S</i>	gccgtagaggtgaaattctt	cgtctcgaacctccgact	93	1.905	0.00938
5α-reductase 1	<i>Srd5a1</i>	gggaaactggatacaaaataccc	ccacgagctcccaaaata	41	1.992	0.00463
Adrenergic receptor alpha 1a	<i>Adra1a</i>	actgaaggtccgcttctct	ggaatatttgctgagaccgaag	1	1.861	0.0181
Agt	<i>Agt</i>	cggaggcaaactgaacaac	tcctcctcctgctttag	84	1.888	0.00497
Arginine vasopressin	<i>Avp</i>	gctgccaggaggagaactac	aaaaccgtcgtggcactc	88	1.871	0.00568
βactin	<i>Actb</i>	ctaaggccaaccgtgaaaag	accagaggcatacagggaaca	64	1.878	0.0199
Coricosteroid binding globulin	<i>Serpina6</i>	ccaccaaagacactcccttg	gggtgtacaggaggccatt	40	1.805	0.0227
Corticotropin releasing hormone	<i>Crh</i>	gaggcatcctgagagaagtcc	atgttagggcgctctctc	34	1.957	0.0219
Corticotropin releasing hormone receptor 1	<i>Crhr1</i>	gggccattgggaaacttta	atcagcaggaccaggatca	81	1.892	0.0163
Cytochrome P450, family 11, subfamily b, polypeptide 1	<i>Cyp11b1</i>	gccatccaggctaactcaat	cattaccaagggggtgatg	11	1.779	0.0261
Cytochrome P450, family 11, subfamily b, polypeptide 2	<i>Cyp11b2</i>	aagctcagacttggtgcttca	cggcccatggagtagagata	3	1.836	0.0148
Elastin	<i>Eln</i>	tgcagtactgtaaccccgctt	agggtggggtggtgagacat	1	2.038	0.00913
Elastin microfibril interfacier 1	<i>Emilin1</i>	cctgtctggctccagtgc	gctctagctgctgcacctt	46	2.059	0.00806
endothelial NOS	<i>Nos3</i>	atccagtgccctgcttca	gcagggcaagttaggatcag	12	1.962	0.0317
FK506 binding protein 51	<i>Fkbp5</i>	aaacgaaggagcaacggtaa	tcaaatgtcctccaccaca	97	1.95	0.00231
GAPDH	<i>Gapdh</i>	gggttcctataaatacggactgc	ccattttgtctacgggacga	52	2.036	0.0154
GR	<i>Nr3c1</i>	gacgtgtggaagctgtaaagt	catttctccagcacaaagg	56	1.971	0.0053
HPRT	<i>Hprt</i>	tcctcctcagaccgctttt	aacctgggtcatcatcgctaa	95	2.062	0.0212
Melanocortin 2 receptor	<i>Mc2r</i>	caccacaatcctctaccctca	cctctccttggttgcac	96	1.799	0.0204
MR	<i>Nr3c2</i>	ttcggagaaagaactgtcctg	cccagcttcttgactttcg	50	2.002	0.00707
Pro-opiomelanocortin-alpha	<i>Pomc</i>	agtgccaggacctcacca	cagcgagagggtcgagtttg	62	2.003	0.00375
Protein kinase cGMP-dependent type 1	<i>Prkg1</i>	acattccagagccttctga	tctccatttcatagtgggtctc	73	2.095	0.013
Steroidogenic acute regulatory protein	<i>StAR</i>	aaggctggaagaaggaaagc	ccacatctggcaccatctta	2	1.826	0.0174
TBP	<i>Tbp</i>	gggagaatcatggaccagaa	gatgggaattccaggagtca	97	1.999	0.005
TGF-β1	<i>Tgfb1</i>	ccctactctcccaagctgt	tgtgaagtgggtggtctccag	108	1.975	0.0205
Vasopressin receptor 1A	<i>Avpr1a</i>	gggataccaatttcgtttgg	aagccagtaacgccgtgat	31	1.846	0.0154
Vasopressin receptor 1B	<i>Avpr1b</i>	cattgtgctggcctacattg	tggtgaaagccacattggta	104	1.95	0.0392
Vasopressin receptor 2	<i>Avpr2</i>	cctggtgtctaccacgtctg	gcaccagactggcatgtatct	27	1.749	0.039

Table 2.4: qPCR cycling conditions for UPL assay.

Step	Temperature (°C)	Time	No. cycles
Pre-incubate	95	5 min	1
Program	95	10 sec	50
	60	30 sec	
Cool	40	30 sec	1

A



B

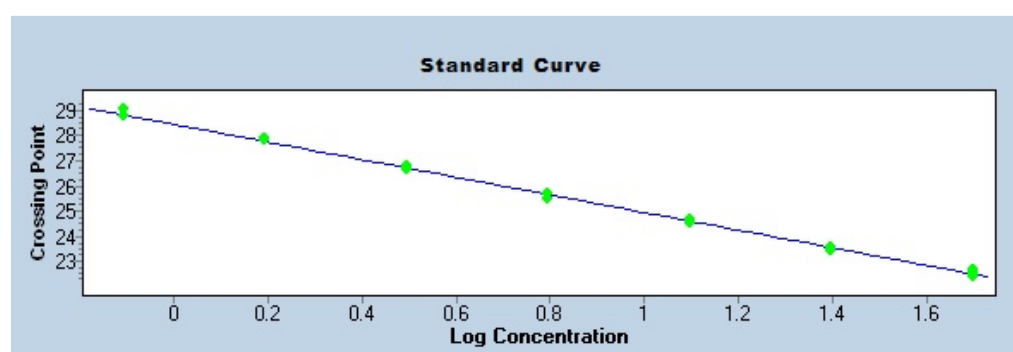


Figure 2.6: Representative amplification and standard curve for RT-qPCR.

(A) Amplification curves of fluorescent intensity over cycle number for serial dilutions (7 points) of pooled cDNA samples performed in triplicate. (B) A 7 point standard curve (each in triplicate) of crossing point (C_q) values derived from the amplification curve.

2.9 Histology

Dissected left adrenal glands were fixed in 4% paraformaldehyde for 24 h at room temperature before being transferred to 70% ethanol. Samples were dehydrated, using an ethanol series, followed by xylene and embedded into paraffin blocks. Fixed adrenal glands were sectioned (5 μ m), stained with haematoxylin and eosin (H&E) and examined by light microscopy and photographed by an AxioScan slide scanner (Zeiss, Cambridge, UK). Adrenal gland cross-sectional area was calculated using QuPath software¹⁵⁶. The adrenal glands were analysed in a randomised order by an observer blinded to the treatment groups.

2.10 Statistical analysis

Data are presented as mean $\pm$ SD. Prior to performing analysis on data, the distribution of data sets were assessed using the Shapiro-Wilk normality test. Data were defined as normally distributed when $p > 0.05$. Therefore, parametric analysis, including Student's t test or ANOVA with Holm-Šidák's *post hoc* test, was carried out, where appropriate, using GraphPad Prism 8.0 Software. Holm-Šidák's *post hoc* test was used for multiple comparisons as has increased power compared with other methods. A value of $p < 0.05$ was considered significant. For data that did not pass normality testing, non-parametric analysis was carried out using Wilcoxon test. Where possible, exact p values are reported from comparisons with control/baseline measurements, unless otherwise stated. The number of biological replicates (n) and statistical analysis used are stated in the figure legends in each result chapter.

Chapter 3: Glucocorticoid excess amplifies salt-sensitivity and induces non-dipping blood pressure

3.1 Introduction

Under the current *American Heart Association* (AHA) Scientific Statement on Animal Models of Hypertension, C57BL/6J mice are salt-resistant making this strain ideal for investigating the driving force for initiation of salt-sensitivity¹⁴⁷. The objective of this chapter was to measure the effect of high salt before and after chronic glucocorticoid excess on BP in C57BL/6JCrI mice. I hypothesise that glucocorticoid excess will result in the transition from a salt-resistant to a salt-sensitive phenotype in C57BL/6JCrI mice. To model glucocorticoid excess in mice, I used the ACTH-dependent Cushing syndrome mouse model.

The experiments were designed to investigate:

1. The impact of flattening ACTH, using an osmotic minipump, on diurnal plasma glucocorticoids.
2. The interaction between salt and glucocorticoid excess on BP homeostasis, using radio telemetry, in C57BL/6JCrI mice.
3. The impact of flattening ACTH on the circadian rhythm of BP.
4. The impact of salt on diurnal plasma glucocorticoids.

3.2. Methods

Details of each procedure used in this chapter are outlined in the main methods section (Chapter 2). All experiments in this chapter were carried out on adult male C57BL/6JCrI mice, aged 8-10 weeks, with implanted minipumps releasing 2.5 µg/day of ACTH or saline (0.9% NaCl) as a control over a minimum of 14 days (see Materials and methods 2.2). The design of each experiment is described below.

3.2.1 BP measurements in conscious mice

Single-housed adult male mice ($n = 8$) were implanted with radio telemetry devices (see Materials and methods 2.3). Following a recovery period, SBP and DBP were recorded over a 7-day period, where mice were given *ad libitum* access to control salt diet (0.3% Na⁺ by weight) and water. Mice were then placed on a high salt diet (3% Na⁺ by weight) for 7 days before returning to a control salt diet for 5 days. After this period, an Alzet 2004 minipump was implanted for continuous infusion of ACTH. BP was not recorded on the day of implantation as mice were allowed time to recovery from the anaesthesia. After 14 days of ACTH infusion, the mice were placed on high salt diet, as illustrated in Figure 3.1.

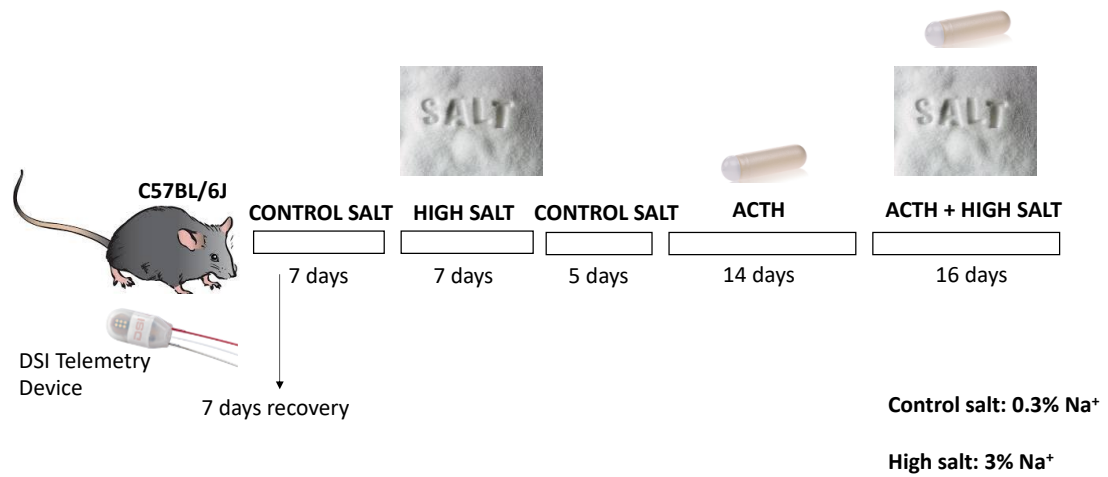


Figure 3.1: Timeline of the experimental design of ACTH and high salt telemetry study.

Male C57BL/6J mice ($n = 8$) were implanted with radio telemetry devices, with a 7-day recovery period. Blood pressure (BP) recordings were collected in these mice on a control salt diet (0.3% Na⁺ by weight) for 7 days, before being placed on a high salt diet (3% Na⁺ by weight) for 7 days. Mice were returned to a control salt diet for 5 days before implantation of minipumps releasing 2.5 µg/day ACTH over 30 days. Following 14 days of ACTH infusion, mice were placed back on a high salt diet for the remaining 16 days.

A second telemetry study was carried out where single-housed mice ($n = 8$) were implanted with radio telemetry devices and left to recover for 7 days. SBP, DBP and activity were recorded over a 3-day period, where mice were given *ad libitum* access to control salt diet and water. An Alzet model 2006 minipump was implanted into mice for a continuous infusion of ACTH (over 21 days), as illustrated in Figure 3.2.

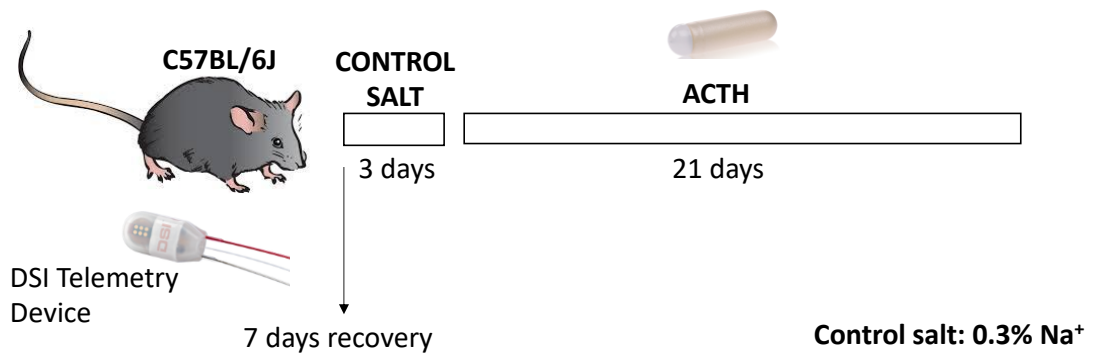


Figure 3.2: Timeline of the experimental design of ACTH telemetry study.

Male C57BL/6J mice ($n = 8$) were implanted with radio telemetry devices, with a 7-day recovery period. Blood pressure (BP) recordings were collected in these mice on a control salt diet (0.3% Na⁺ by weight) for 3 days before implantation of minipumps releasing 2.5 µg/day ACTH over 21 days.

3.2.2 Timed plasma corticosterone sampling

To measure the effect of high salt before and after chronic ACTH infusion on plasma corticosterone levels in mice ($n = 8$), blood samples were collected at 7 AM and 7 PM by tail venesection and quantified by an ELISA (see Material and methods 2.7). Samples were collected in the same mouse at the end of each treatment. Therefore, an AM and PM sample was taken at the end of 7 days control salt, 7 days high salt, 5 days control salt, 14 days ACTH and 14 days ACTH and high salt (Figure 3.3).

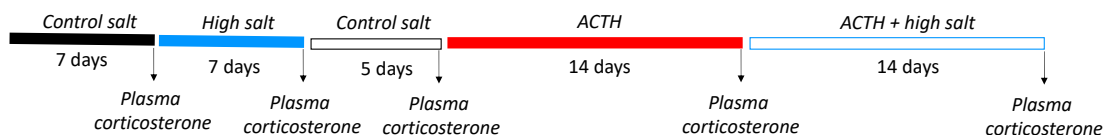


Figure 3.3. Plasma corticosterone sampling protocol.

Blood was collected from male C57BL/6J mice ($n = 8$), using tail venesection, to measure plasma corticosterone levels. Blood samples were taken at 7 AM and 7 PM at the end of each treatment in the same mice. Treatments included a control salt diet (0.3% Na⁺ by weight) for 7 days, a high salt diet (3% Na⁺ by weight) for 7 days, 5 days control salt, 14 days of 2.5 µg/day ACTH, infused via a minipump, and finally 28 days of ACTH infusion with 14 days of a high salt diet.

In different mice the effect of 28 day ACTH infusion on plasma corticosterone was measured, sampling at 7 AM and 7 PM, as before.

3.2.3 Timed plasma aldosterone sampling

Measurements of plasma aldosterone by ELISA necessitates large sample volume. Terminal blood samples were used, collected at 7 AM in mice treated with 7 days control diet ($n = 10$), 7 days high salt ($n = 9$), 14 days ACTH ($n = 8$) and 14 days ACTH and high salt ($n = 8$). Furthermore, terminal samples were collected at 7 AM in mice treated with 28 days ACTH. To measure the effect of chronic ACTH on the diurnal rhythm of plasma aldosterone, terminal samples were also collected at 7 PM in mice treated with 7 days control salt and 14 days ACTH only ($n = 8$).

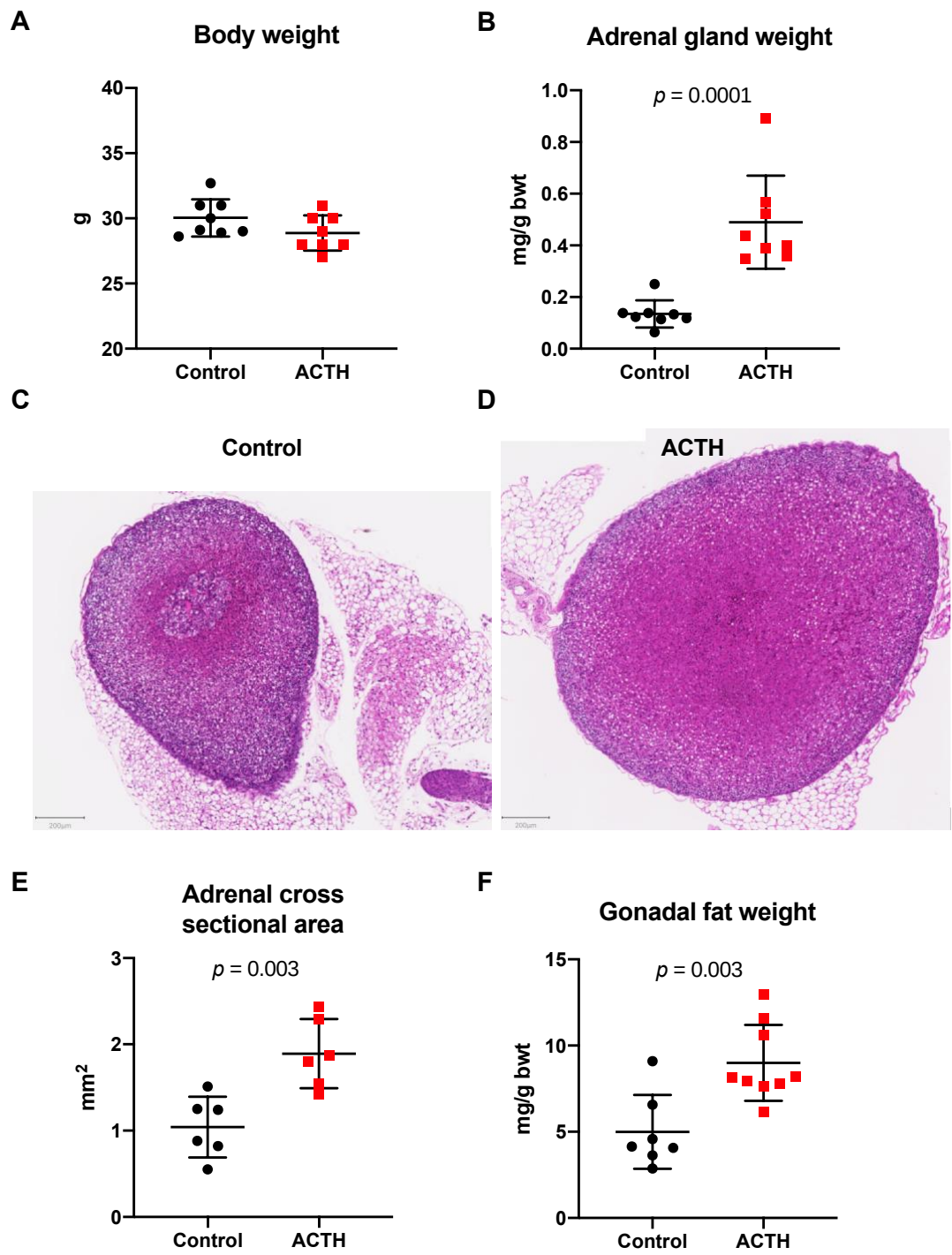
3.3 Results

3.3.1 The ACTH-dependent Cushing syndrome mouse model

ACTH-treated mice had similar body weight to controls, but adrenal glands were enlarged, with an increased weight and cross-sectional area and gonadal fat pad mass was increased (Figure 3.4). Diurnal rhythm of plasma corticosterone was measured in control and ACTH-treated mice to confirm glucocorticoid excess, which is covered further in Section 3.3.6.

Figure 3.4: Body and organ weights following treatment of ACTH.

Male C57BL/6J mice were treated with ACTH (red square; $n = 6-9$) or vehicle (black circle; $n = 6-8$) for 14 days. (A) Body weights at the end of the treatment. (B) Left adrenal gland weight normalised to body weight. Representative H&E-stained right adrenal gland sections (scale bar 200 μm magnification) for controls (C) and ACTH-treated mice (D), with adrenal gland cross-sectional area calculated (E). (F) Gonadal fat weight normalised to body weight. Measurements from individual mice and group mean $\pm$ SD are shown and were analysed by Student's unpaired t test.



3.3.2 Glucocorticoid excess amplifies salt-sensitive blood pressure

Radio telemetry was used to measure BP over a 49-day, multi-phase experiment. Figure 3.5 gives an overview of 24 h mean SBP and DBP across the entire experiment. On control diet, the 7-day average SBP was 122.7 ± 2.4 mmHg and DBP was 92.2 ± 2.7 mmHg. High salt increased BP to a peak of ~ 12 mmHg at 3 days. Thereafter, BP declined but did not return to baseline but stabilised after 7 days high salt, where SBP was 132.2 ± 1.9 mmHg and DBP was 98.8 ± 3.7 mmHg. Mice were returned to control diet for 5 days, by the end of which, SBP was 123.8 ± 4.2 mmHg, DBP at 96.2 ± 5.6 mmHg, similar to baseline measurements.

All mice were then implanted with a minipump containing ACTH. After 14 days ACTH infusion, SBP was 133.8 ± 1.7 mmHg and DBP was 101.4 ± 2.1 mmHg. Mice were transitioned to a high salt diet, with duration determined by stabilisation of BP. Following ACTH infusion (30 days) with the latter 16 days on a high salt diet, SBP was 154.4 ± 6.1 mmHg and DBP was 124.2 ± 8.1 mmHg.

The Δ SBP induced by high salt was 10.9 ± 3.5 mmHg compared with 17.6 ± 9.6 mmHg induced by high salt following ACTH infusion (paired t test: $p = 0.141$). Similarly, this was seen in the Δ DBP (Δ DBP high salt 8.7 ± 6.2 mmHg vs. Δ DBP ACTH + high salt 14.2 ± 8.9 mmHg; $p = 0.250$). This indicates a tendency for a greater BP change following ACTH infusion.

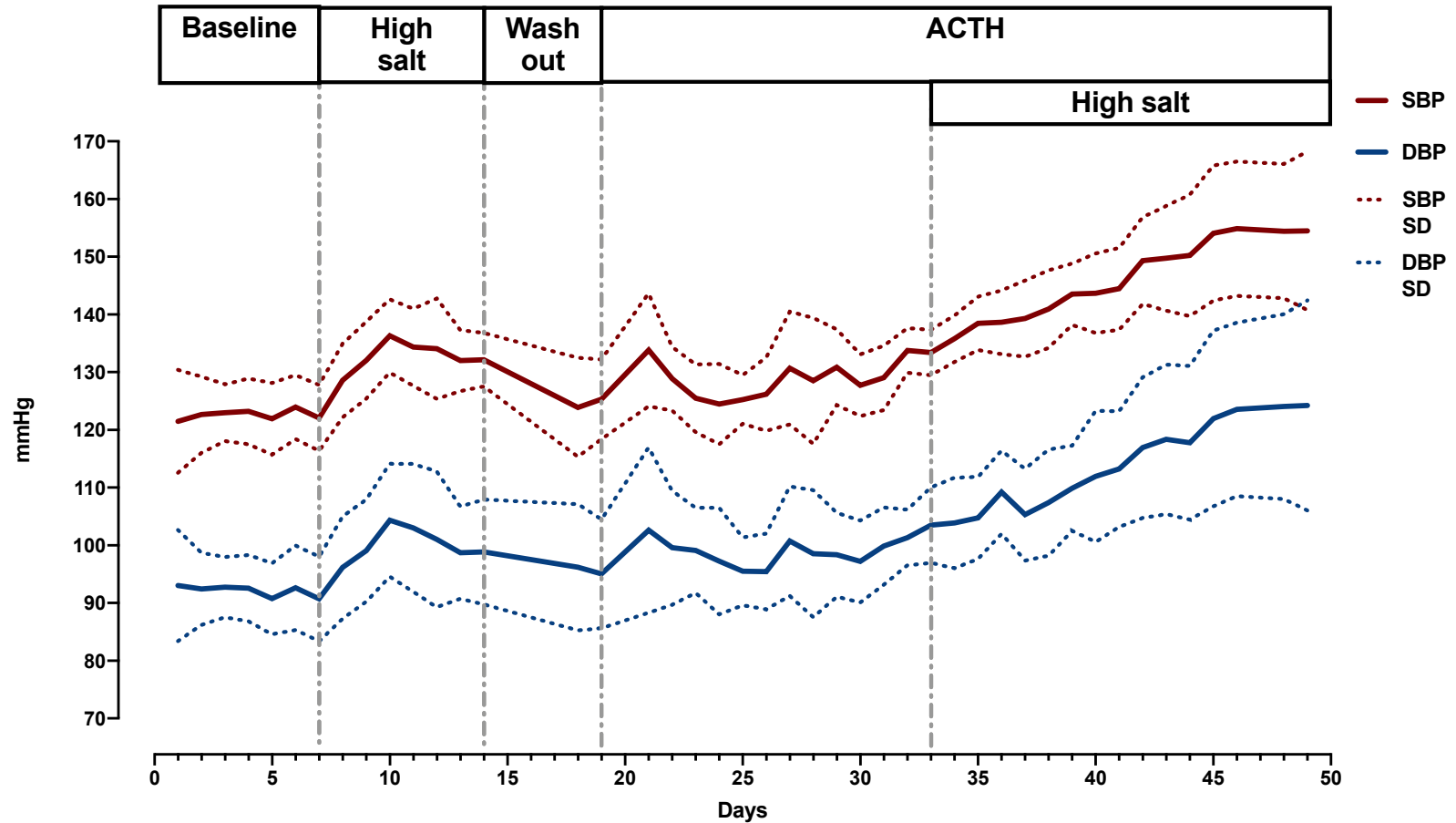


Figure 3.5: Systolic and diastolic blood pressure after treatment of high salt diet in the absence and presence of ACTH infusion. Averaged 24 h systolic blood pressure (SBP; red line) and diastolic blood pressure (DBP; blue line), measured by radio telemetry, of male C57BL/6J mice ($n = 6-7$). Data were acquired every 20 min over a longitudinal protocol (Figure 3.1) incorporating control diet (0.3% Na^+) baseline, high salt diet (3% Na^+) for 7 days, a washout period when mice returned to control of 5 days and treatment with ACTH for 30 days with the latter 16 days on high salt diet. Data are presented as group mean (solid line) $\pm$ SD (dashed lines).

3.3.3 ACTH flattens diurnal variation of systolic blood pressure

Salt-sensitivity is often accompanied by the development of non-dipping BP⁵⁶, and therefore 12 h day (7 AM – 7 PM) and night (7 PM – 7 AM) SBP were calculated, aligning with the light cycle. These values were used to calculate the night:day variation of SBP (Δ night and day SBP) throughout the protocol (Figure 3.6 A). During baseline, the change was 12.3 ± 2.9 mmHg, indicating a significant decline in SBP during the daytime, when the mice are inactive. During the high salt period, the change was 10.6 ± 5.6 mmHg and 11.8 ± 3.1 mmHg when returned to control diet. This change was reduced significantly to 4.9 ± 2.4 mmHg following 14 days ACTH infusion ($p = 0.013$ comparisons with wash out made by one-way ANOVA with Holm-Šidák's *post hoc* test). Following ACTH + high salt, the night:day variation was 3.7 ± 1.7 mmHg. This indicated the SBP dip between night and day is lost following ACTH infusion, also depicted in Table 3.1 which shows the %Dip (Δ night and day SBP expressed as a percentage of BP during active phase) on the last 24 h cycle in each experimental phase.

Table 3.1: Night:day systolic blood pressure (SBP) variation.

Data shown as mean $\pm$ SD ($n = 6-7$) and comparisons made by repeated measures one-way ANOVA with Holm-Šidák's *post hoc* test. *P* values stated are from comparisons made with previous treatment.

<i>SBP</i>	Baseline	High salt	Wash out	ACTH	ACTH + high salt
<i>Active or night (mmHg)</i>	127.4 $\pm$ 6.6	139.3 $\pm$ 8.5	131.7 $\pm$ 8.7	132.6 $\pm$ 3.3	147.3 $\pm$ 17.5
<i>Inactive or day (mmHg)</i>	116.8 $\pm$ 5.5	125.0 $\pm$ 4.4 ($p = 0.006$)	118.9 $\pm$ 5.5	134.9 $\pm$ 5.2 ($p = 0.001$)	145.5 $\pm$ 17.8
<i>Night-day variation (mmHg)</i>	10.6 $\pm$ 4.4	14.3 $\pm$ 9.8	12.8 $\pm$ 4.5	-2.3 $\pm$ 4.0 ($p = 0.01$)	1.8 $\pm$ 1.9
<i>% Dip</i>	8.3%	10.3%	9.7%	-1.7% ($p = 0.006$)	1.2%

To further explore the daily rhythms of SBP, data were smoothed using a 5 h rolling average and aligned into 3-day bins (Figure 3.6 B) for cosinor analysis (Table 3.2, Table 3.3).

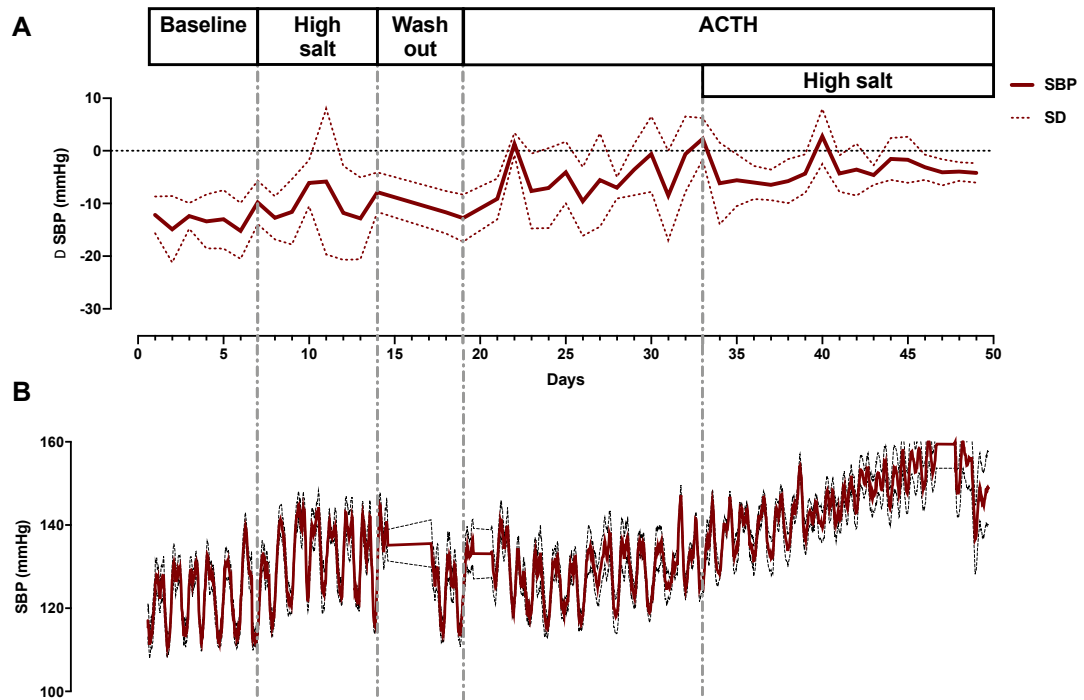


Figure 3.6: Night:day systolic blood pressure variation.

(A) Night:day systolic blood pressure (SBP) variation and (B) 5 h rolling averages of SBP from male C57BL/6J mice ($n = 6-7$) over a longitudinal protocol incorporating control diet (0.3% Na^+) baseline, high salt diet (3% Na^+) for 7 days, a washout period when mice returned to control of 5 days and treatment with ACTH for 30 days with the latter 16 days on high salt diet. Data are presented as group mean (solid line) $\pm$ SD (dashed lines).

Cosinor analysis was performed for each treatment. High salt diet significantly increased MESOR but did not change period, acrophase (time of the peak of the rhythm) and amplitude (difference between peak and MESOR). Robustness (strength of the rhythm) was significantly reduced in SBP with high salt diet (Table 3.2). This level of robustness is still indicative of rhythm as levels above 20% are accepted as a signal¹⁴⁹.

Table 3.2: Cosinor analysis of systolic blood pressure (SBP).

Data shown as mean $\pm$ SD ($n = 7$) and comparisons made by paired t-test.

<i>SBP</i>	Baseline	High salt
<i>Period (hours)</i>	24.0 $\pm$ 0.0	22.8 $\pm$ 0.7
<i>Acrophase (ZT)</i>	17h48 $\pm$ 00h09	16h13 $\pm$ 01h56
<i>Amplitude (mmHg)</i>	11.2 $\pm$ 1.1	9.5 $\pm$ 1.6
<i>MESOR (mmHg)</i>	123.1 $\pm$ 2.3	132.7 $\pm$ 2.5 ($p = 0.003$)
<i>Robustness (%)</i>	76.0 $\pm$ 5.1	52.9 $\pm$ 8.4 ($p = 0.006$)

ACTH did not influence period, acrophase or amplitude but increased SBP MESOR compared to the control diet. Robustness was reduced ~25%. Following transition to a high salt diet, the period reduced from ~24 h during ACTH infusion to ~12 h with ACTH + high salt. The acrophase was between ~15-17 ZT during normal salt but exhibited a shift with ACTH + high salt. ACTH + high salt did not influence amplitude or robustness compared to ACTH alone but increased MESOR (Table 3.3).

Table 3.3: Cosinor analysis of systolic blood pressure (SBP).

Data shown as mean $\pm$ SD ($n = 6$) and comparisons made by repeated measures one-way ANOVA with Holm-Šidák's *post hoc* test. *P* values stated are from comparisons made with previous treatment.

<i>SBP</i>	Wash out	ACTH	ACTH + high salt
<i>Period (hours)</i>	24.0 $\pm$ 0.0	24.0 $\pm$ 0.0	12.1 $\pm$ 0.1 ($p < 0.0001$)
<i>Acrophase (ZT)</i>	16h30 $\pm$ 00h16	16h50 $\pm$ 03h20	05h44 $\pm$ 00h57 ($p = 0.053$)
<i>Amplitude (mmHg)</i>	8.7 $\pm$ 1.0	6.4 $\pm$ 0.7	4.2 $\pm$ 0.3
<i>MESOR (mmHg)</i>	125.5 $\pm$ 3.5	130.6 $\pm$ 2.1 ($p = 0.0348$)	153.2 $\pm$ 5.1 ($p = 0.012$)
<i>Robustness (%)</i>	60.8 $\pm$ 6.8	36.2 $\pm$ 3.3 ($p = 0.062$)	38.3 $\pm$ 2.6

3.3.4 ACTH flattens diurnal variation of diastolic blood pressure

Similar to SBP, 12 h day (7 AM – 7 PM) and night (7 PM – 7 AM) DBP were calculated for the night:day variation of DBP throughout the protocol (Figure 3.7 A). During baseline, the DBP change was 12.8 $\pm$ 2.5 mmHg, again showing a BP dip. During the high salt period, the change was 9.5 $\pm$ 5.8 mmHg and 11.7 $\pm$ 3.2 mmHg when returned to control diet. This change was reduced to 6.6 $\pm$ 1.8 mmHg following 14 days ACTH infusion. Following ACTH + high salt, the night:day variation was 4.0 $\pm$ 1.4 mmHg. This showed ACTH infusion reduced the BP dip, also depicted in Table 3.4 on the last 24 h cycle in each experimental phase.

Table 3.4: Night:day diastolic blood pressure (DBP) variation.

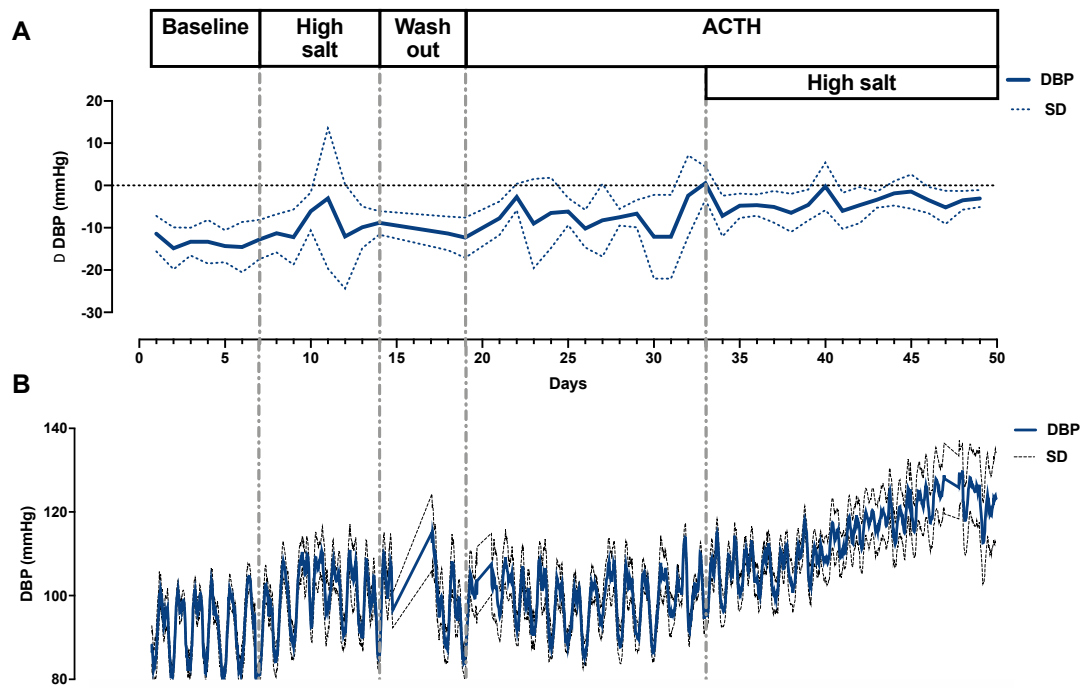
Data shown as mean $\pm$ SD ($n = 6-7$) and comparisons made by repeated measures one-way ANOVA with Holm-Šidák's *post hoc* test. *P* values stated are from comparisons made with previous treatment.

<i>DBP</i>	Baseline	High salt	Wash out	ACTH	ACTH + high salt
<i>Active or night (mmHg)</i>	97.3 $\pm$ 8.5	105.3 $\pm$ 12.3	101.8 $\pm$ 11.7	101.1 $\pm$ 3.7	121.9 $\pm$ 21.6
<i>Inactive or day (mmHg)</i>	84.1 $\pm$ 6.8	92.4 $\pm$ 7.5 (<i>p</i> = 0.004)	89.8 $\pm$ 8.5	101.6 $\pm$ 6.4 (<i>p</i> = 0.01)	119.1 $\pm$ 21.5
<i>Night-day variation (mmHg)</i>	13.2 $\pm$ 4.4	13.0 $\pm$ 9.1	12.0 $\pm$ 4.9	0.6 $\pm$ 3.9 (<i>p</i> = 0.021)	2.9 $\pm$ 3.3
<i>% Dip</i>	13.6%	12.3%	11.8%	0.6% (<i>p</i> = 0.014)	2.4%

To further explore the daily rhythms of DBP, data were smoothed using a 5 h rolling average and aligned into 3-day bins (Figure 3.7 B) for cosinor analysis (Table 3.5, Table 3.6).

Figure 3.7: Night:day diastolic blood pressure variation.

(A) Night:day diastolic blood pressure (DBP) variation and (B) 5 h rolling averages of DBP from male C57BL/6J mice ($n = 6-7$) over a longitudinal protocol incorporating control diet (0.3% Na⁺) baseline, high salt diet (3% Na⁺) for 7 days, a washout period when mice returned to control of 5 days and treatment with ACTH for 30 days with the latter 16 days on high salt diet. Data are presented as group mean (solid line) $\pm$ SD (dashed lines).



Cosinor analysis for DBP was performed for each treatment. High salt diet did not change period, acrophase, amplitude and MESOR. Robustness was significantly reduced in DBP with high salt diet (Table 3.5).

Table 3.5: Cosinor analysis of diastolic blood pressure (DBP).

Data shown as mean $\pm$ SD ($n = 7$) and comparisons made by paired t-test.

<i>DBP</i>	Baseline	High salt
<i>Period (hours)</i>	24.0 $\pm$ 0.0	22.7 $\pm$ 0.8
<i>Acrophase (ZT)</i>	17h04 $\pm$ 00h10	15h56 $\pm$ 01h43
<i>Amplitude (mmHg)</i>	10.9 $\pm$ 1.2	8.3 $\pm$ 1.7
<i>MESOR (mmHg)</i>	91.8 $\pm$ 2.7	100.8 $\pm$ 3.8
<i>Robustness (%)</i>	76.2 $\pm$ 5.0	44.2 $\pm$ 6.6 ($p = 0.0002$)

ACTH did not influence period, amplitude or MESOR but shifted acrophase to the right compared to the control diet. Robustness was reduced ~30%. Following transition to a high salt diet, the period reduced from ~24 h during ACTH infusion to ~14 h with ACTH + high salt. The acrophase exhibited a shift to the left with ACTH + high salt. ACTH + high salt did not influence amplitude or robustness compared to ACTH alone but increased MESOR (Table 3.6).

Table 3.6: Cosinor analysis of diastolic blood pressure (DBP).

Data shown as mean $\pm$ SD ($n = 6$) and comparisons made by repeated measures one-way ANOVA with Holm-Šidák's *post hoc* test. *P* values stated are from comparisons made with previous treatment.

<i>DBP</i>	Wash out	ACTH	ACTH + high salt
<i>Period (hours)</i>	23.8 $\pm$ 0.2	24.0 $\pm$ 0.0	13.9 $\pm$ 2.0 (<i>p</i> = 0.008)
<i>Acrophase (ZT)</i>	15h19 $\pm$ 00h11	19h46 $\pm$ 00h21 (<i>p</i> = 0.027)	08h08 $\pm$ 02h07 (<i>p</i> = 0.004)
<i>Amplitude (mmHg)</i>	8.9 $\pm$ 0.8	7.2 $\pm$ 1.1	4.0 $\pm$ 0.4
<i>MESOR (mmHg)</i>	96 $\pm$ 4.4	99.5 $\pm$ 3.0	121.2 $\pm$ 6.5 (<i>p</i> = 0.031)
<i>Robustness (%)</i>	63.6 $\pm$ 4.7	30.0 $\pm$ 4.6 (<i>p</i> = 0.003)	32.9 $\pm$ 3.1

3.3.5 Prolonged ACTH duration did not increase blood pressure

A question arising from glucocorticoid excess amplifying salt-sensitivity was – is the BP rise with salt following ACTH simply a delayed response to ACTH? Therefore, the impact of increased duration of glucocorticoid excess on BP homeostasis, using radio telemetry, was assessed. Figure 3.8 gives an overview of the entire experiment, plotting 24 h average SBP and DBP in conscious unrestrained mice. After baseline measurements, averaged over 3 days, SBP was 123.0 $\pm$ 4.3 mmHg and DBP was 87.1 $\pm$ 2.5 mmHg. Following 21 days of ACTH infusion, SBP was 130.7 $\pm$ 5.2 mmHg and DBP was 90.0 $\pm$ 4.9 mmHg.

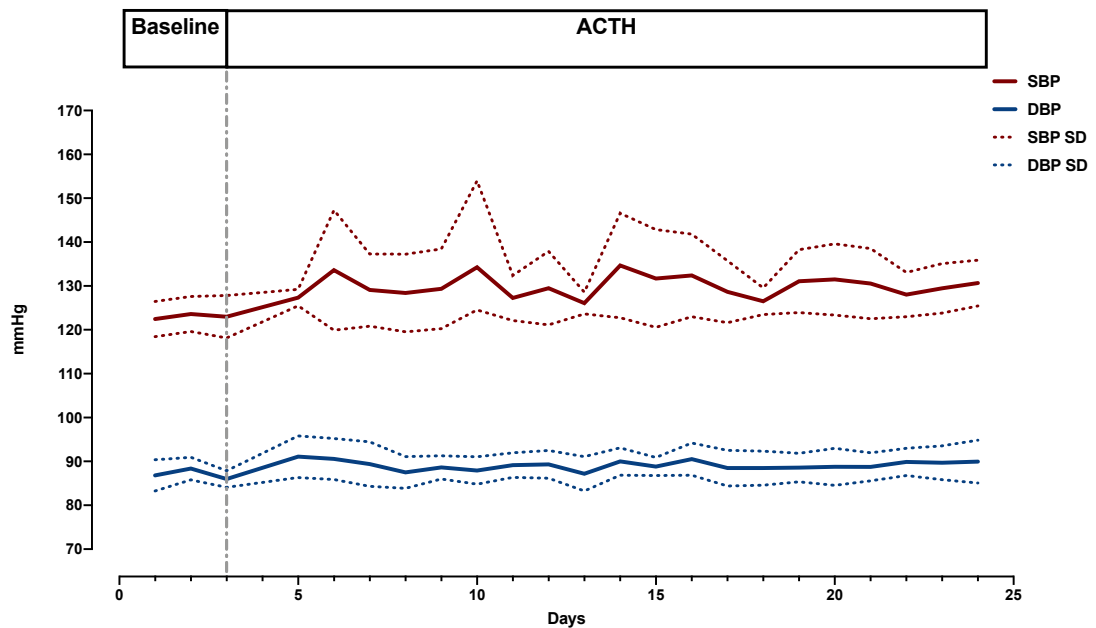


Figure 3.8: Systolic and diastolic blood pressure following 21 days of ACTH infusion.

Averaged 24 h systolic blood pressure (SBP; red line) and diastolic blood pressure (DBP; blue line), measured by radio telemetry, of male C57BL/6J mice ($n = 4$). Data were acquired every 20 min over a longitudinal protocol (Figure 3.2) incorporating control diet (0.3% Na⁺) baseline and treatment with ACTH for 21 days. Data are presented as group mean (solid line) $\pm$ SD (dashed lines).

During baseline, the SBP change was 8.6 ± 5.1 mmHg and remained similar following 21 days of ACTH treatment (6.1 ± 6.8 mmHg), suggesting no loss in BP dip (Figure 3.9 A). However, loss of BP dip is seen during the last 24 h cycle of ACTH treatment, depicted in Table 3.7.

Table 3.7: Night:day systolic blood pressure (SBP) variation for 21 days ACTH. Data shown as mean $\pm$ SD ($n = 4$) and comparisons made by paired t-test.

<i>SBP</i>	Baseline	ACTH (21 days)
<i>Active or night (mmHg)</i>	129.2 ± 5.5	131.8 ± 4.4
<i>Inactive or day (mmHg)</i>	116.8 ± 5.6	129.6 ± 6.4 ($p = 0.027$)
<i>Night-day variation (mmHg)</i>	12.3 ± 5.3	2.2 ± 3.3 ($p = 0.02$)
<i>% Dip</i>	9.5%	1.7% ($p = 0.015$)

To further explore the daily rhythms of SBP, data were smoothed using a 5 h rolling average and aligned into 3-day bins (Figure 3.9 B) for cosinor analysis (Table 3.8). ACTH infusion over 21 days did not influence period, acrophase, amplitude, MESOR or robustness.

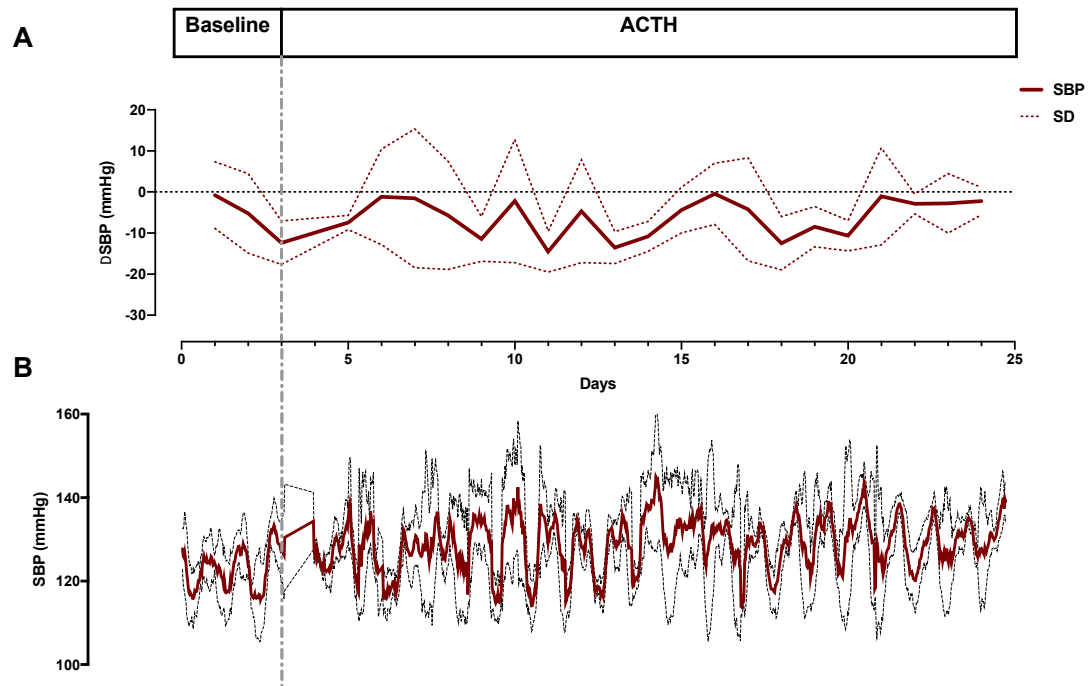


Figure 3.9: Night:day systolic blood pressure variation for 21 days ACTH.

(A) Night:day systolic blood pressure (SBP) variation and (B) 5 h rolling averages of SBP from male C57BL/6J mice ($n = 4$) over a longitudinal protocol incorporating control diet (0.3% Na⁺) baseline and treatment with ACTH for 21 days. Data are presented as group mean (solid line) $\pm$ SD (dashed lines).

Table 3.8: Cosinor analysis of systolic blood pressure (SBP) for 21 days ACTH.
Data shown as mean $\pm$ SD ($n = 4$) and comparisons made by paired t-test.

<i>SBP</i>	Baseline	ACTH (21 days)
<i>Period (hours)</i>	24.0 $\pm$ 0.0	23.8 $\pm$ 0.3
<i>Acrophase (ZT)</i>	18h03 $\pm$ 01h10	18h55 $\pm$ 01h49
<i>Amplitude (mmHg)</i>	9.5 $\pm$ 3.2	6.9 $\pm$ 1.9
<i>MESOR (mmHg)</i>	123.0 $\pm$ 4.3	129.4 $\pm$ 5.0
<i>Robustness (%)</i>	60.7 $\pm$ 16.7	49.3 $\pm$ 9.8

During baseline, the DBP change was 7.1 $\pm$ 4.2 mmHg and remained similar following 21 days of ACTH treatment (8.6 $\pm$ 3.7 mmHg), suggesting no loss in BP dip (Figure 3.10 A). However, a reduced BP dip was seen during the last 24 h cycle of ACTH treatment, depicted in Table 3.9.

Table 3.9: Night:day diastolic blood pressure (DBP) variation for 21 days ACTH.
Data shown as mean $\pm$ SD ($n = 4$) and comparisons made by paired t-test.

<i>DBP</i>	Baseline	ACTH (21 days)
<i>Active or night (mmHg)</i>	91.2 $\pm$ 3.4	92.2 $\pm$ 5.2
<i>Inactive or day (mmHg)</i>	80.7 $\pm$ 2.7	87.7 $\pm$ 4.9 ($p = 0.043$)
<i>Night-day variation (mmHg)</i>	10.5 $\pm$ 4.9	4.5 $\pm$ 2.4 ($p = 0.034$)
<i>% Dip</i>	11.3%	4.8% ($p = 0.026$)

DBP data were smoothed using a 5 h rolling average and aligned into 3-day bins (Figure 3.10 B) for cosinor analysis (Table 3.10). ACTH infusion over 21 days did not influence period, acrophase, amplitude, MESOR or robustness.

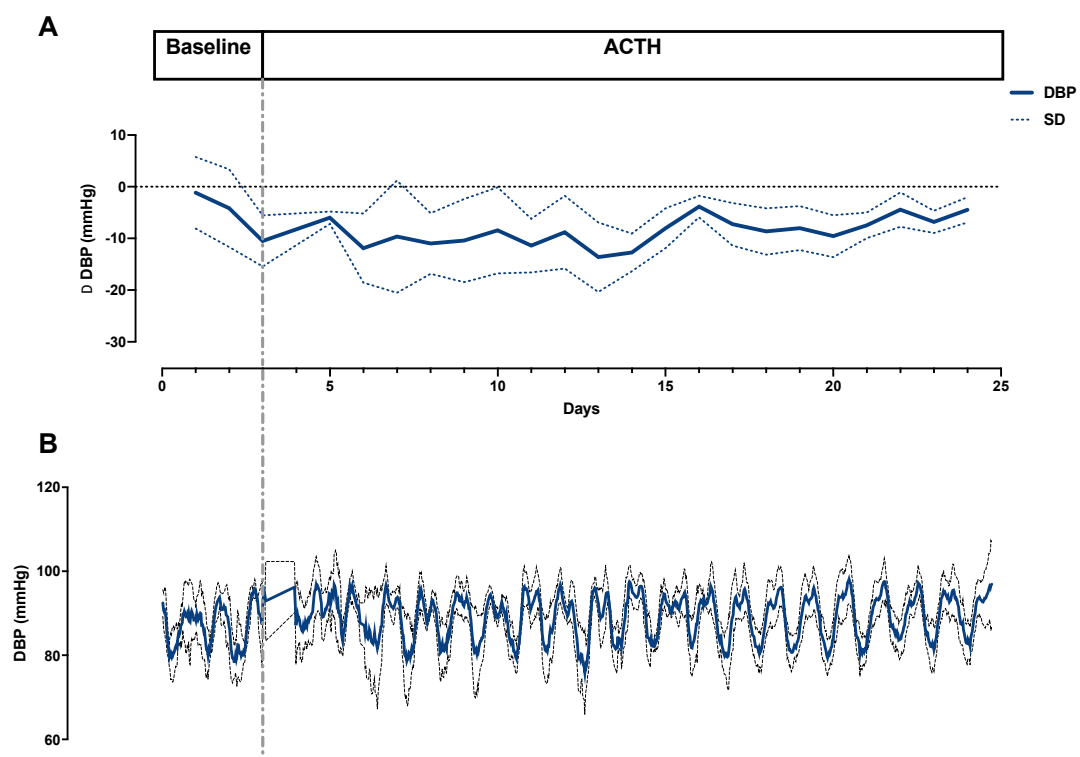


Figure 3.10: Night:day diastolic blood pressure variation for 21 days ACTH.
 (A) Night:day diastolic blood pressure (DBP) variation and (B) 5 h rolling averages of DBP from male C57BL/6J mice ($n = 4$) over a longitudinal protocol incorporating control diet (0.3% Na⁺) baseline and treatment with ACTH for 21 days. Data are presented as group mean (solid line) $\pm$ SD (dashed lines).

Table 3.10: Cosinor analysis of diastolic blood pressure (DBP) for 21 days ACTH.

Data shown as mean $\pm$ SD ($n = 4$) and comparisons made by paired t-test.

DBP	Control	ACTH (21 days)
<i>Period (hours)</i>	24.0 $\pm$ 0.0	23.5 $\pm$ 0.9
<i>Acrophase (ZT)</i>	18h05 $\pm$ 02h04	19h04 $\pm$ 00h55
<i>Amplitude (mmHg)</i>	7.3 $\pm$ 2.4	6.1 $\pm$ 1.8
<i>MESOR (mmHg)</i>	86.9 $\pm$ 2.6	89.8 $\pm$ 3.8
<i>Robustness (%)</i>	69.8 $\pm$ 10.3	51.5 $\pm$ 10.8

3.3.6 Activity levels were maintained following glucocorticoid excess

BP dipping occurs during the inactive phase, so when mice are sleeping. Disturbed sleep or poor sleep quality is associated with BP “non-dippers”¹⁵⁷. Therefore, the activity of mice was assessed during the inactive phase. To test the impact of glucocorticoid excess on activity, radio telemetry signal was used to calculate an activity count. Activity from the study above in Figure 3.8 was presented in Figure 3.11 A. Activity could not be measured during the study shown in Figure 3.4 due to a technical fault with the manufacturers. During baseline, the average day activity was 75.9 ± 28.9 counts per min and increased to 143.0 ± 23.3 counts per min during the night (activity change 67.2 ± 44.3 counts per min). ACTH treatment had no effect on activity as day activity was 49.4 ± 14.5 counts per min and increased to 138.7 ± 43.9 counts per min during the night (activity change 89.3 ± 33.6 counts per min). Table 3.11 illustrates the diurnal activity at the end of each period. There was a significant reduction in night:day activity variation at the end of treatment. However, this appears to be due to reduced activity of mice during their active phase, although not significant.

Table 3.11: Night:day activity variation for 21 days ACTH.

Data shown as mean $\pm$ SD ($n = 4$) and comparisons made by paired t-test.

<i>Activity</i>	Baseline	ACTH (21 days)
<i>Active or night (counts per min)</i>	183.9 ± 22.9	126.6 ± 51.2
<i>Inactive or day (counts per min)</i>	56.8 ± 22.0	60.9 ± 36.4
<i>Night-day variation (counts per min)</i>	127 ± 34.7	65.7 ± 34.2 ($p = 0.029$)

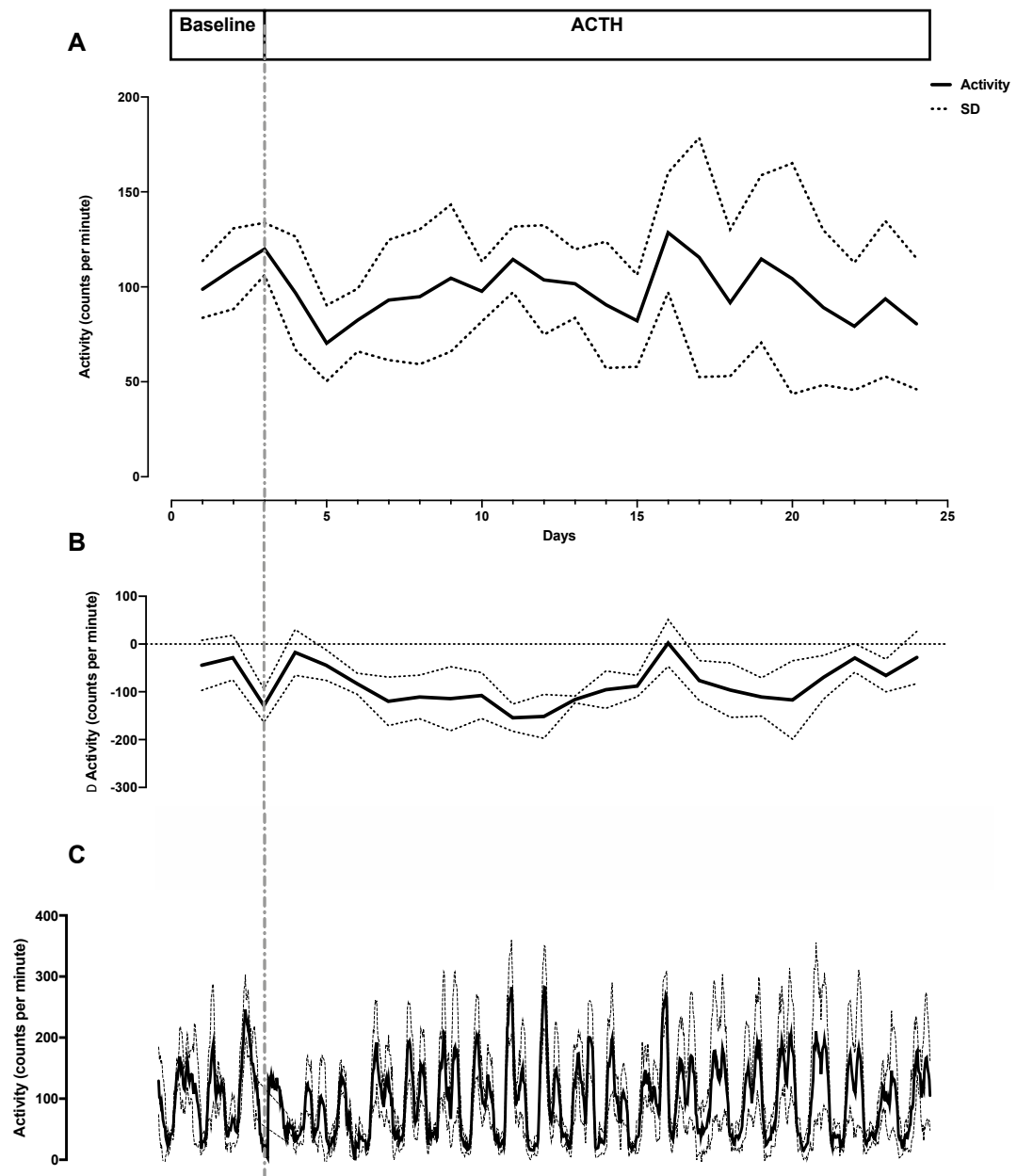


Figure 3.11: Night:day activity variation for 21 days ACTH.

(A) Average 24 h activity, (B) night:day activity variation and (C) 5 h rolling averages of activity from male C57BL/6J mice ($n = 4$) over a longitudinal protocol incorporating control diet (0.3% Na⁺) baseline and treatment with ACTH for 21 days. Data are presented as group mean (solid line) $\pm$ SD (dashed lines).

Activity data were smoothed using a 5 h rolling average and aligned into 3-day bins (Figure 3.11 B) for cosinor analysis (Table 3.12). ACTH infusion over 21 days did not influence period, acrophase, amplitude, MESOR or robustness.

Table 3.12: Cosinor analysis of activity for 21 days ACTH.

Data shown as mean $\pm$ SD ($n = 4$) and comparisons made by paired t-test.

<i>Activity</i>	Baseline	ACTH (21 days)
<i>Period (hours)</i>	23.1 $\pm$ 0.4	24.0 $\pm$ 0.0
<i>Acrophase (ZT)</i>	18h12 $\pm$ 00h31	18h02 $\pm$ 01h53
<i>Amplitude (counts per min)</i>	74.8 $\pm$ 18.0	59.2 $\pm$ 23.3
<i>MESOR (counts per min)</i>	108.1 $\pm$ 13.8	83.5 $\pm$ 33.3
<i>Robustness (%)</i>	62.3 $\pm$ 7.1	56.1 $\pm$ 5.4

3.3.7 ACTH disturbed diurnal plasma corticosterone rhythm

Mice initially had clear diurnal rhythm of plasma corticosterone, which was higher at 7 PM than at 7 AM. Diurnal variation was still evident after 7 days of high salt intake but 7 PM levels were lower than the control diet phase. Plasma corticosterone returned to previous levels when placed back on to control diet (ANOVA interaction of diurnal rhythm and salt treatment $p = 0.3675$; Figure 3.12 A).

In ACTH-treated mice, this diurnal variation was reversed, where 7 AM levels were higher than 7 PM (ANOVA interaction of diurnal rhythm and ACTH treatment $p = 0.0976$; Figure 3.12 B). Following ACTH infusion with high salt, the diurnal variation remained lost, with a significant increase in 7 PM plasma corticosterone compared with ACTH alone ($p < 0.0001$). Diurnal plasma corticosterone was also measured after 28 days of ACTH alone. Plasma corticosterone at 7 AM was similar to 14 days ACTH (293.5 $\pm$ 117.7 nmol/L). Plasma corticosterone at 7 PM was 352.5 $\pm$ 118.1 nmol/L ($p = 0.0667$; data not shown).

Diurnal rhythm of plasma aldosterone was maintained following ACTH infusion (ANOVA interaction of diurnal rhythm and ACTH treatment $p = 0.8013$; Figure 3.21 C). However, when comparing 7 AM plasma aldosterone, ACTH infusion increased plasma aldosterone and continued to do so following prolonged treatment. Following 28 days ACTH with the last 14 days high salt, plasma aldosterone was decreased but salt did not suppress plasma aldosterone to the levels seen prior to glucocorticoid excess (Figure 3.12 D).

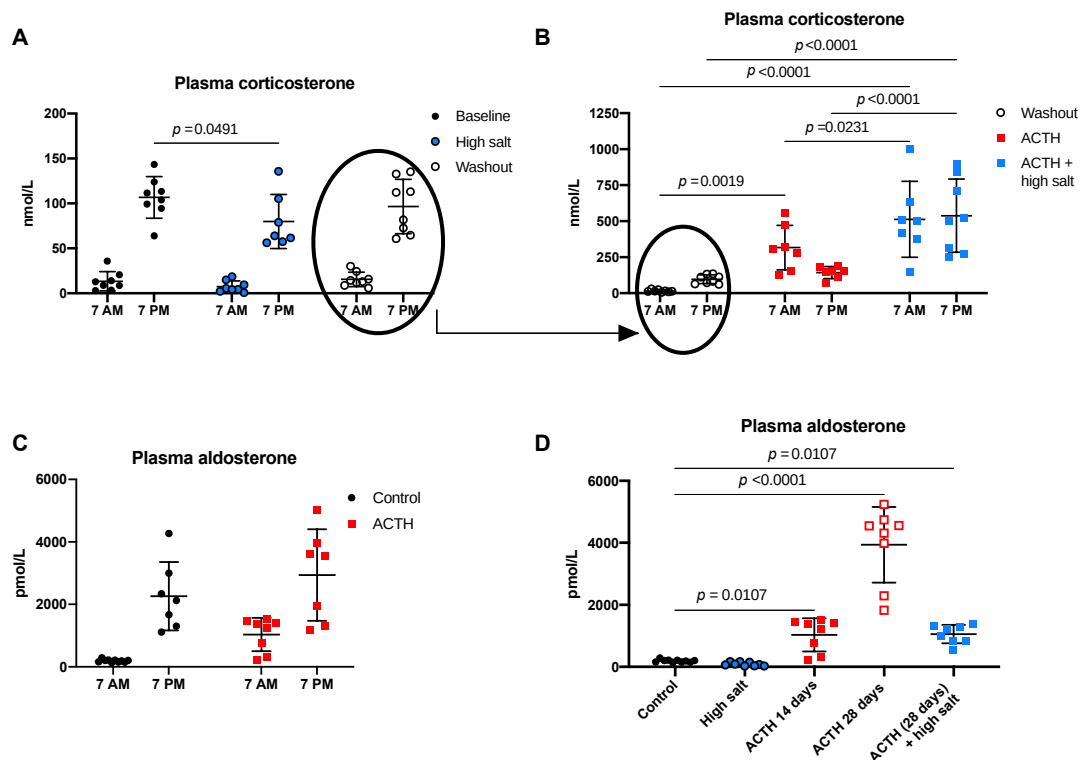


Figure 3.12: Plasma corticosterone and aldosterone at 7 AM and 7 PM.

Paired measurements of corticosterone in C57BL/6J mice ($n = 7-8$) on control diet ($0.3\% \text{ Na}^+$; black circles), after 7 days high salt diet ($3\% \text{ Na}^+$; blue circles), then after 5 days on control diet (open circles), (B) after 14 days of ACTH infusion (red squares) and then after 14 days of ACTH with high salt diet (blue squares). (C) Plasma aldosterone measurements in mice ($n = 7-10$) on either a control diet or after 14 days of ACTH infusion. Data analysed by two-way ANOVA with Holm-Šidák's *post hoc* test. (D) Plasma aldosterone measurements at 7 AM in mice ($n = 8-10$) either on control diet, after 7 days high salt diet, after 14 days ACTH infusion, after 28 days ACTH infusion (open red squares), or 28 day ACTH treatment with the last 14 days high salt. Data are mean $\pm$ SD and were analysed by one-way ANOVA with Holm-Šidák's *post hoc* test comparing with baseline.

3.4 Discussion

In this chapter, the interaction between salt and glucocorticoid excess on SBP and DBP homeostasis was assessed in C57BL/6JCrI mice. The main findings were that i) C57BL/6J mice showed unexpected salt-sensitivity; ii) salt-sensitivity was amplified by glucocorticoid excess; iii) glucocorticoid excess disrupted BP rhythmicity, particularly in combination with high salt.

In this model, ACTH infusion enlarged adrenal glands, which is consistent with previous studies^{127,158}. ACTH infusion increased adrenal cortex width, specifically the zona fasciculata was ~70% larger with a slight increase in width of the zona glomerulosa. There was no change in the adrenal medulla following ACTH treatment. Furthermore, gonadal fat mass was increased in ACTH-treated mice. This was measured in Cushing patients where it has been shown they experience a fat redistribution as there is a visceral adipose tissue (VAT) accumulation¹⁵⁹. Here, we measured one VAT, gonadal fat, as a read out for investigating VAT accumulation in ACTH-treated mice.

Growing evidence has suggested that glucocorticoid rhythmicity, as well as circulating glucocorticoid levels, have a significant impact on human health and disease¹⁶⁰. In our model, ACTH infusion flipped the circadian rhythm of plasma corticosterone. Human studies have shown disruption of glucocorticoid circadian rhythm in Cushing's syndrome, either flipping the circadian rhythm or flattening the rhythm. A study measuring salivary cortisol, which reflects the free active cortisol in plasma¹⁶⁰, in 39 Cushing's syndrome patients showed higher cortisol at 11 PM¹⁶¹, with a later study showing cortisol was higher at 10 PM in 120 patients¹⁶². However, studies measuring plasma cortisol in 7 Cushing's syndrome patients showed no diurnal rhythm¹⁶³, with a later study showing no difference in plasma cortisol in 103 patients between 2 time points (12 AM and 8 AM)¹⁶⁴. Unfortunately, ACTH could not be measured in this model due to lack of appropriate assays that could measure ACTH with limited sample volume and the required sensitivity. Nevertheless, disruption of the rhythmicity of glucocorticoids is a key feature in Cushing's syndrome and the ACTH-dependent Cushing's syndrome mouse model displays characteristic

hallmarks of glucocorticoid excess and was a useful model for investigating the interaction between salt and glucocorticoids and their impact on BP.

3.4.1 Salt-sensitivity in C57BL/6J mice

C57BL/6 substrains – primarily C57BL/6J and C57BL/6N – are widely used in biomedical research and are considered as salt-resistant under the current AHA Scientific Statement¹⁴⁷, with other studies demonstrating salt-resistance as high salt alone does not increase BP in either the parent strain or transgenic wild-types^{44,139,165}. The choice of using C57BL/6J mice was due to our lab previously observing salt-resistance in these animals⁴⁴. However, in our hands, male C57BL/6JCrI mice are salt-sensitive. This was supported by a study, using radio telemetry, demonstrating both C57BL/6J and C57BL/6N substrains were salt-sensitive, with this finding not seen when using tail-cuff plethysmography¹⁶⁶. Therefore, the BP response to salt cannot be assumed in strains and assessment of this response using radio telemetry must be an important consideration for study design. The mechanisms behind salt-sensitivity in these animals is not a focus of this thesis. However, I was involved in the work by Dr Ralph in the lab¹⁶⁷ which also demonstrated salt-sensitivity in male C57BL/6JCrI mice in an independent study. This study assessed the mechanisms behind this, indicating increased SNS activity.

3.4.2 Glucocorticoids excess induced an amplification of salt-sensitivity in C57BL/6JCrI mice

Studies have suggested that abnormal glucocorticoid signalling contributes to salt-sensitive BP^{58,76,77}. In this study, glucocorticoid excess amplified salt-sensitivity in C57BL/6JCrI mice.

The ACTH-dependent Cushing's syndrome model has been previously used by our group¹²⁸ using the same dose of ACTH (2.5 µg/day). However, the synthetic ACTH fragment 1-24 (Synacthen (Ciba-Geigy)) used was no longer available. ACTH fragment 1-24 was available from Sigma which had the same empirical formula and molecular weight as Synacthen, so this was used for our

model. Following 14 days of ACTH infusion, BP increased by ~ 8 mmHg. BP is reported to increase by ~20 mmHg in Cushing's patients¹⁶⁸. Previous mouse studies have shown ACTH infusion to increase BP by a similar magnitude using telemetry^{115,169–171}. However, these studies used doses considerably higher than our study, as illustrated in Table 3.13, which could explain the difference in BP response. Ivy et al measured SBP and DBP by telemetry in C57BL/6JCrI mice following 11 days of corticosterone infusion and showed a similar rise of BP to our study¹³⁴. Furthermore, with a similar infusion of ACTH, BP increase was similar in rats over 10 days of treatment although with no changes in diurnal rhythm¹⁷².

Table 3.13: ACTH dosing and blood pressure (BP) response.

Mouse strain (all male)	ACTH dose (for 25 g mouse)	BP response	Reference
Swiss Outbred	12.5 µg/day (Synacthen)	25 mmHg increase over 10 days	Schyvens et al 2001 ¹⁶⁹
Black Swiss	12.5 µg/day (unknown)	20 mmHg increase over 10 days	Whitworth 2001 ¹¹⁵
129sv/Black Swiss mixed background	37.5 µg/day (Sigma)	13 mmHg increase over 5 days	Lorenz et al 2008 ¹⁷¹
C57BL6/129sv mixed background	50 µg/day (Synacthen)	20 mmHg increase over 11 days	Zhang et al 2010 ¹⁷⁰

When ACTH-treated mice were placed on a high salt diet, BP again increased progressively and did not stabilise until ~ 14 days. Overall, the effect of salt on BP rise was approximately twice that observed with normal glucocorticoid status. My second telemetry experiment indicated that pronounced hypertension was not simply due to prolonged ACTH infusion: BP rose by ~7 mmHg when ACTH was given for 21 days. My data therefore suggests that pre-existing glucocorticoid excess amplifies salt-sensitivity in C57BL/6JCrI mice. Studies of abnormal glucocorticoid signalling, both glucocorticoid

excess^{46,128} and impaired glucocorticoid metabolism^{45,141}, have reported salt-sensitive hypertension but this is the first study to show salt-sensitivity in the ACTH-dependent Cushing's syndrome mouse model using radio telemetry.

ACTH-induced hypertension has been explored in other studies, albeit without the additional high salt diet challenge. A role of both GR and MR have been implicated. The effect of ACTH on circulating aldosterone has been reported to be transient, suggesting GR-mediated pathways are involved in ACTH-dependent hypertension^{127,128}. This has been supported by a study showing patients with ACTH-dependent Cushing's syndrome were more responsive to GR antagonism than MR antagonism¹⁷³. Although, activation of MR is considered a major factor in hypertension of glucocorticoid excess, as it can be activated by glucocorticoids when in excess to breach the 11 β -HSD2 barrier, as suggested in Cushing's syndrome¹⁷⁴. Furthermore, deficiency in 11 β -HSD2 activity has been shown to predict salt-sensitivity¹⁷⁵. MR can also be activated by 11-deoxycorticosterone (an intermediate in the corticosteroid biosynthetic pathway) if elevated¹⁷⁶.

In mice with ACTH excess, hypertension was partially prevented by pharmacological blockade of both GR and MR: combinatorial treatment was required to completely rescue the hypertensive phenotype¹²⁸. Here, ACTH infusion caused increased plasma corticosterone and aldosterone. Therefore, whether the increase in BP in response to salt was GR- or MR-mediated remains in question.

A potential limitation of this study was the lack of vehicle controls to ensure BP changes were due to ACTH and to test the effect of repeated high salt exposure on BP. Future studies using telemetry would incorporate vehicle controls, where mice would be infused with saline over the same duration, to test this.

3.4.3 Glucocorticoid excess induces non-dipping blood pressure

Non-dipping is associated with a plethora of diseases including CVD¹⁷⁷, diabetes¹⁷⁸ and metabolic syndrome¹⁷⁹. Circadian BP was assessed in patients with active Cushing's disease and 50% of patients had no nocturnal BP dip¹⁰⁸. Similarly, ACTH-treated mice had non-dipping BP due to BP increased during the inactive phase. Activity is a contributor to BP and corticosterone has been shown to increase sensitivity to sleep disturbances¹⁸⁰. Therefore, are these mice active when they should be asleep? Activity displayed a diurnal rhythm and similar counts per min in glucocorticoid excess, comparable to prior to minipump implantation. Therefore, non-dipping BP was not due to increased activity during the inactive phase. A role for renal salt handling has been suggested as corticosterone excess induced non-dipping BP by activating NCC¹³⁴. This non-dipping phenotype remained following high salt in ACTH-treated mice.

Following the end of ACTH + high salt period, the mice displayed poor health with symptoms including fuzzy facial fur, due to piloerection, with hunched posture. When mice were terminated, several had enlarged hearts. Individuals with essential hypertension who exhibit non-dipping BP have been associated with increased risk of target organ damage in the heart, kidney and vasculature¹⁸¹. We hypothesised that hypertension in these mice may have been about to enter a malignant phase. For welfare reasons, animals were culled, and this was not evaluated further.

3.4.4 ACTH infusion and salt loading alters plasma corticosteroid levels

Salt decreased 7 PM plasma glucocorticoids but diurnal rhythm was maintained over a 7-day period. This contradicts reports that have shown high salt increases glucocorticoids^{146,182}. Understanding this finding will be explored further in Chapter 6.

ACTH infusion reversed glucocorticoid diurnal rhythm. This is surprising as minipumps infused ACTH at a constant rate. Glucocorticoid rhythm is under

the control of the SCN⁸³, as well as the circadian clock in the adrenal gland. Clock control of glucocorticoid release is mediated via the HPA axis and also the sympathetic input to the adrenal gland which influence adrenal sensitivity of ACTH¹⁸³. This raises the question – is the clock control of glucocorticoid release dysregulated with ACTH infusion? This was not evaluated further in this thesis but would be of interest in future work.

Plasma glucocorticoids was increased further following a high salt diet. This suggests that salt alters glucocorticoids and is exaggerated in salt-sensitive hypertension. Our data are consistent with a growing literature in both experimental and clinical salt-sensitivity studies that the HPA axis is activated by increased dietary salt^{44,76,77,141,143}.

Diurnal plasma aldosterone was increased, particularly during the day, and circadian rhythm was not altered in ACTH excess. Day plasma aldosterone was significantly increased following 28 days ACTH infusion. This contradicts studies that have reported that plasma aldosterone transiently increases with prolonged ACTH administration¹²⁸. Day plasma aldosterone has only been measured in ACTH + high salt mice and was reduced with high salt, although to similar concentrations to that of 14 days ACTH infusion and not to levels pre-existing glucocorticoid excess. This non-modulation of aldosterone following salt-loading might contribute to amplification of salt-sensitivity.

3.4.5 Potential mechanisms behind amplification of salt-sensitivity

The underlying mechanisms behind glucocorticoid-induced hypertension are thought to be centred around direct and indirect effects on the vasculature and the kidney^{114,115}. However, the mechanisms behind the relationship between abnormal glucocorticoid signalling and salt-sensitive BP are still not fully understood. The suggested molecular mechanisms whereby glucocorticoids contribute to salt-sensitive hypertension focus around stimulation of renal sodium transport with a potential role of both MR and GR in some capacity^{46,141}. However, there are limited studies exploring the potential

contribution of the vasculature in glucocorticoid-induced salt-sensitive hypertension. The mechanism behind salt-sensitivity in our model is explored further in Chapter 4.

3.4.6 Summary of chapter findings

- C57BL/6J mice are salt-sensitive.
- Chronic glucocorticoid excess amplifies salt-sensitivity.
- Chronic glucocorticoid excess induces non-dipping BP.
- ACTH infusion flips the circadian rhythm of plasma corticosterone.
- High salt challenge alters plasma glucocorticoids in the absence and presence of glucocorticoid excess.
- ACTH infusion causes non-modulation of aldosterone following salt-loading, which might be contributory to amplification of salt-sensitivity.

**Chapter 4: Glucocorticoid excess
induces renal vascular dysfunction,
with no major impairment of
pressure natriuresis**

4.1 Introduction

Impaired renal salt excretion, suggesting compromised acute PN relationship, and vascular (endothelial) dysfunction are thought to be fundamental mechanisms of salt-sensitivity^{63,64}. The process that dominates remains contentious. Some authorities indicate a primary role for natriuretic dysfunction with vasoconstriction of nonrenal blood vessels being a secondary, sustaining event³⁴. Others propose a primary “vasodysfunction” theory, characterised as an attenuated reduction in TPR in response to increased salt intake as necessary and sufficient to induce salt-sensitive hypertension⁷⁰.

The primary objective of this chapter was to explore potential mechanisms underlying the amplification of salt-sensitivity in ACTH-treated mice.

The experiments were designed to investigate:

1. The impact of glucocorticoid excess on diurnal renal sodium and water handling.
2. The impact of glucocorticoid excess $\pm$ high salt on renal natriuresis and haemodynamics on the acute PN response.
3. The impact of glucocorticoid excess on vascular reactivity.
4. The impact of the interaction between salt and glucocorticoid excess on vascular reactivity.
5. The molecular mechanisms underlying the renal/vascular response to glucocorticoid excess.

4.2 Methods

Details of each procedure used in this chapter are outlined in the main methods section (Chapter 2). All experiments in this chapter were carried out on adult male C57BL/6JCrI mice, aged 8-10 weeks, with implanted minipumps releasing 2.5 µg/day of ACTH or saline as a control over a minimum of 14 days and given *ad libitum* access to control salt diet (0.3% Na⁺ and 0.7% K⁺ by weight) or high salt diet (3% Na⁺ and 0.6% K⁺ by weight) and water (see Materials and methods 2.2). The design of each experiment is described below.

4.2.1 Sodium and water excretion in conscious mice

Single housed mice ($n = 8$) before and after 14 days ACTH infusion were placed in metabolic cages for 24 h, with 2x 12 h urine collections between 7 AM and 7 PM, and 7 PM and 7 AM. Food and water intake was measured and urine collected for electrolyte analysis, details outlined in the Materials and methods section 2.4.

4.2.2 Pressure natriuresis and renal haemodynamics

The following experimental groups were used: 14 days ACTH and 14 days saline control ($n = 10$ each group); 28 days ACTH infusion and 28 days ACTH infusion with the final 14 days of high salt diet ($n = 10$ each group). Experiments were performed under non-recovery anaesthesia, using the protocol described in Materials and methods 2.5. Surgery was performed by Mr Kevin Stewart (*Ex vivo* Laboratory Manager). For each mouse, BP and RBF was measured and used to calculate RVR. GFR was measured by inulin clearance and sequential urine collections were used to calculate sodium and water excretion (see Materials and methods 2.5.4).

4.2.3 Vascular reactivity

To assess the effect of glucocorticoid excess on vascular reactivity, separate cohorts of mice were implanted with minipumps either infusing saline or ACTH for 14 days before being culled at 7 AM ($n = 8$ each group). Terminal blood

was collected for nitric oxide ELISA (see below) and renal and mesenteric arteries were dissected and mounted on a wire myograph as described in the Materials and methods section 2.8. KPSS was used as a depolarising contraction. Vascular reactivity was tested by generating cumulative concentration (1nM to 30µM) to the vasoconstrictor PE and vasodilators ACh and SNP.

To assess the interaction between salt and glucocorticoids, wire myography was performed on renal and second order mesenteric arteries in mice following 28 days ACTH or 28 days ACTH infusion with the latter 14 days fed a high salt diet ($n = 8$ each group).

4.2.3.1 Nitric oxide ELISA

Plasma nitric oxide (NO) concentration was quantified using a NO ELISA kit (Enzo Life Sciences). Plasma samples were diluted 1:10 with Reaction Buffer and ultrafiltration was carried out through a 10,000 molecular weight cut-off filter (Amicon® Ultra Centrifugal Filter, Sigma). Samples and standards were prepared and assayed according to the manufacturer's instructions. The plate was read on a microplate reader (Magellan, TECAN) with optical density measurements at 550 nm.

4.2.4 Timed artery dissections in saline and ACTH-treated mice

A separate cohort of saline-treated ($n = 12$) and ACTH-treated mice ($n = 16$) were culled between 7-8 AM, via decapitation, 14 days after treatment. Dissected renal and second order mesenteric arteries from 2 mice were pooled together and placed in RNAlater at 4°C for 24 h before storage at -80°C prior to RNA extraction (see Materials and methods 2.8).

4.3 Results

4.3.1. ACTH induces an abnormality in diurnal sodium excretion

Twenty four h food and water intake did not change following 14 days ACTH infusion. ACTH-treated mice had increased urine output and were able to excrete their dietary sodium load within a 24 h period however, their urinary potassium excretion was increased (Figure 4.1).

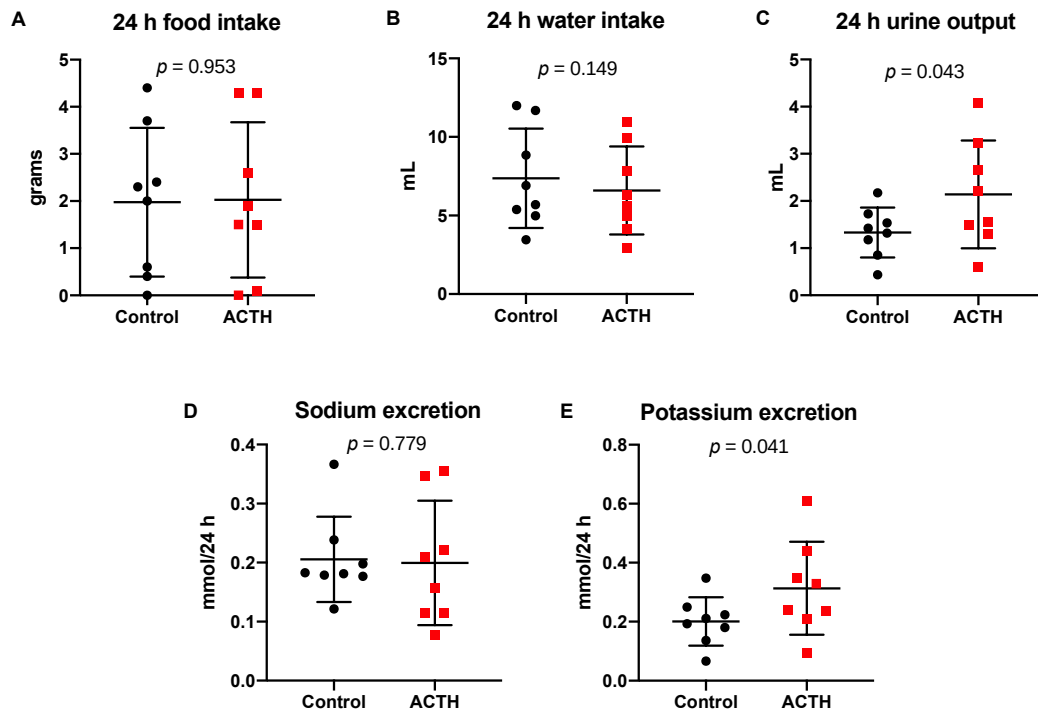


Figure 4.1: 24 hour urinary parameters following ACTH infusion.

Paired measurements of (A) food intake (0.3% Na⁺ and 0.7% K⁺ diet), (B) water intake, (C) urine flow rate, (D) sodium excretion and (E) potassium excretion over 24 h in the same mice ($n = 8$) before (control, black circles) and after 14 days ACTH infusion (red squares). Data are group means $\pm$ SD and were analysed by paired t test.

To assess these changes in excretion further, the diurnal excretion pattern was assessed. In ACTH-treated mice, the normal diurnal patterning of sodium excretion was lost (ANOVA interaction of diurnal variation and ACTH $p = 0.067$). However, urine output and potassium excretion retained rhythmicity (ANOVA interaction of diurnal variation $p = 0.493$ and $p = 0.994$, respectively; Figure 4.2).

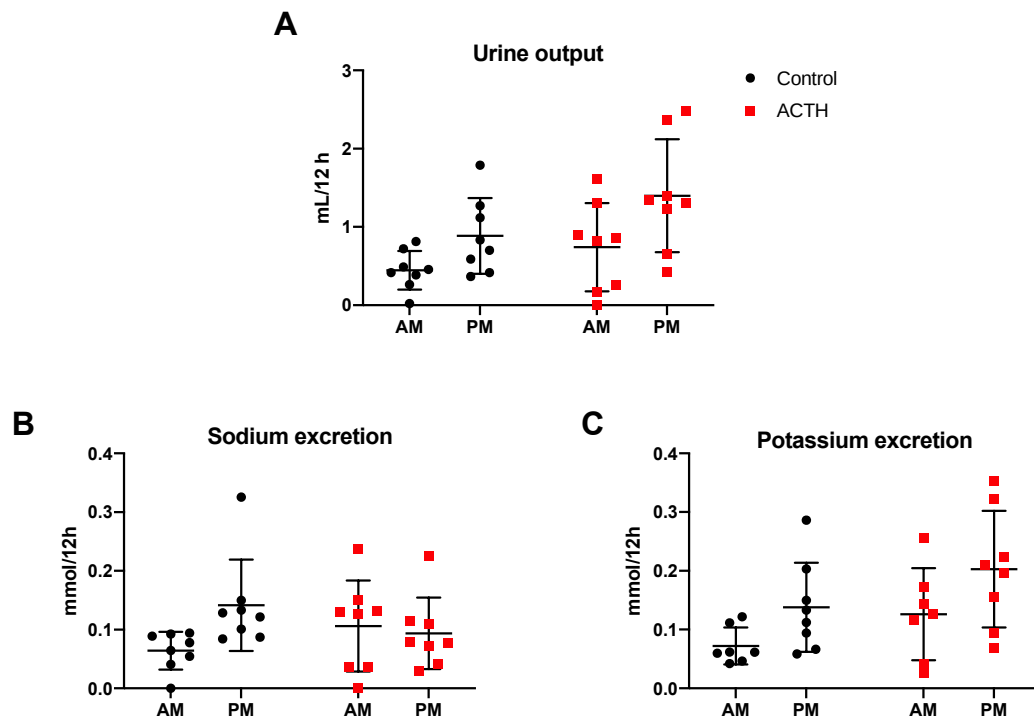


Figure 4.2: Diurnal urinary parameters following ACTH infusion.

Paired measurements of (A) urine flow rate, (B) sodium excretion and (C) potassium excretion over 2x12 h (7 AM-7 PM, AM; 7 PM-7 AM, PM) in the same mice ($n = 7-8$) before (control, black circles) and after 14 days ACTH infusion (red squares). Data analysed by two-way ANOVA with Holm-Šidák's *post hoc* test.

4.3.2 No impairment of acute pressure natriuresis in glucocorticoid excess

As ACTH induced an abnormality in diurnal sodium excretion, the acute PN response was measured in control and ACTH-treated mice. Figure 4.4 demonstrates that the procedure induced significant rises in BP following sequential ligations (ANOVA interaction of ligations $p < 0.0001$) and the effect was similar between both groups (ANOVA interaction of ligations and ACTH treatment $p = 0.319$).

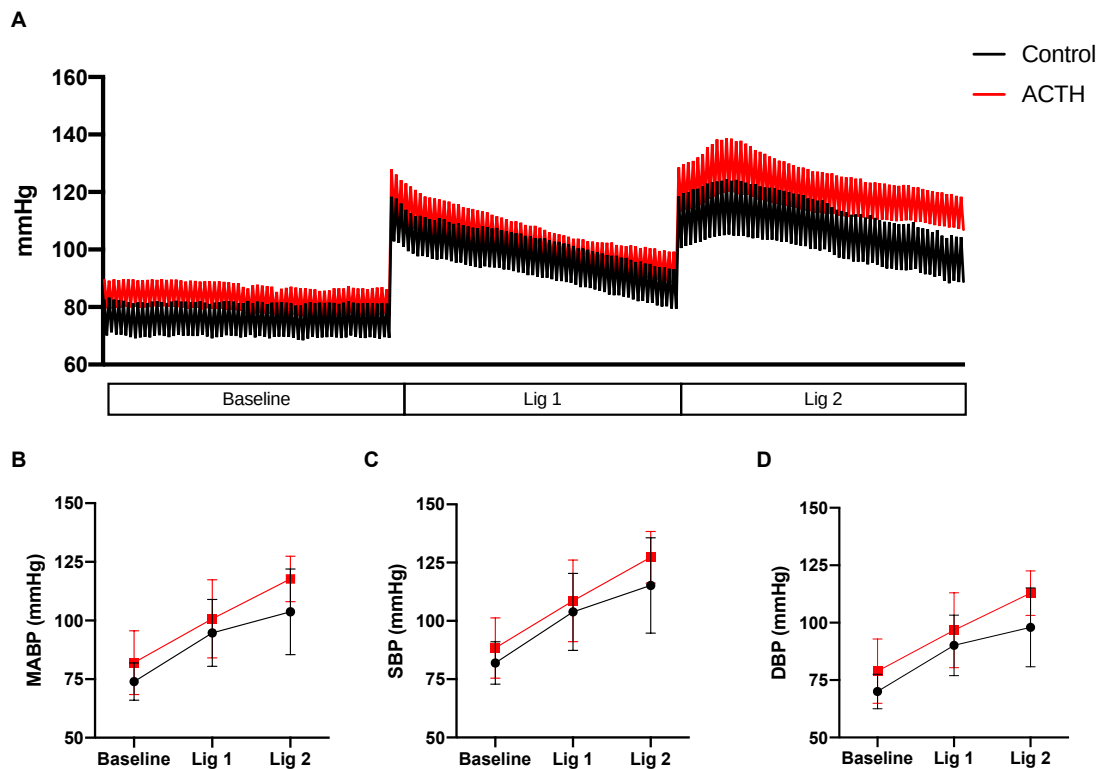


Figure 4.3: Blood pressure measurements during surgical assessment of ligation-induced acute pressure natriuresis following ACTH infusion.

Blood pressure (BP) measurements were taken following acute pressure natriuresis (PN) surgery in adult male C57BL/6J mice treated with ACTH (red square; $n = 9$) or vehicle (black circle; $n = 6$) for 14 days. (A) Averaged BP trace taken at 20 sec intervals for each mouse during baseline, mesenteric and coeliac artery ligation (Lig 1) and distal aorta artery ligation (Lig 2). (B) Average mean arterial BP (MABP), (C) systolic BP (SBP) and (D) diastolic BP (DBP) of each period (Baseline, Lig 1 and Lig 2). Data are group mean $\pm$ SD and analysed by two-way ANOVA with repeated measures and Holm-Šidák's *post hoc* test.

Renal haemodynamics: Pulsatile RBF measurements were collected during this procedure. At baseline, mean RBF was significantly increased in ACTH-treated mice (Figure 4.4 A). By dividing MABP by mean RBF, this gives you mean RVR which was significantly reduced following ACTH infusion (Figure 4.4 B). At baseline, GFR was not different between saline- and ACTH-treated mice (Figure 4.4 C). With increased RBF and stable GFR following ACTH infusion, this suggests the filtration fraction, i.e. the fraction of renal plasma flow filtered across the glomerulus, is changing.

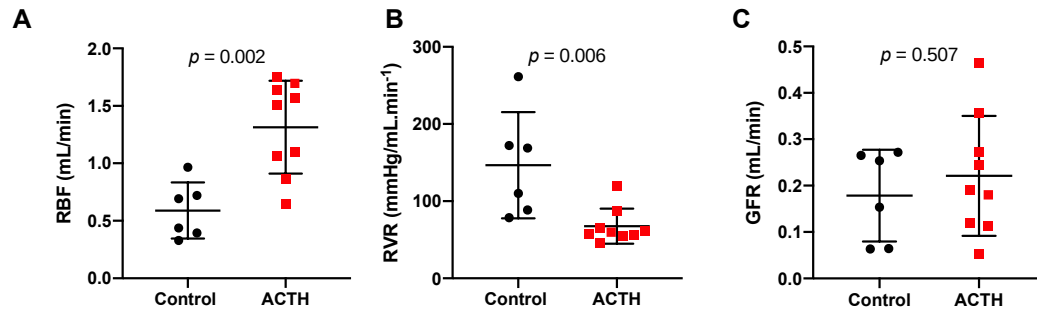


Figure 4.4: Baseline renal haemodynamics following ACTH infusion.

(A) Mean renal blood flow (RBF), (B) mean renal vascular resistance (RVR), (C) glomerular filtration rate (GFR) measurements were taken at baseline following acute pressure natriuresis (PN) surgery in adult male C57BL/6J mice treated with ACTH (red square; $n = 9$) or vehicle (black circle; $n = 6$) for 14 days. Measurements from individual mice and group mean $\pm$ SD are shown and were analysed by Student's unpaired t test.

With changes in baseline haemodynamic measurements with ACTH, was the response to acute BP increases different? Figure 4.5 A demonstrates averaged RBF traces during sequential ligation of arteries to induce pressure ramps in control and ACTH-treated mice. The relationship between RBF and SBP is shown in Figure 4.5 B. There was a significant increase in mean RBF in chronic ACTH from baseline (1.32 ± 0.40 mL/min) to Lig 1 (1.58 ± 0.45 mL/min) and Lig 2 (1.87 ± 0.60 mL/min), unlike control mice (Baseline 0.59 ± 0.24 mL/min, Lig 1 0.71 ± 0.34 mL/min, Lig 2 0.80 ± 0.42 mL/min). Therefore, there was a significant increase of RBF following Lig 1 and Lig 2 (both $p = 0.003$) in ACTH-treated mice compared with controls. The relationship between RVR and SBP is shown in Figure 4.5 C. There was no change in mean RVR in chronic ACTH from baseline (67.54 ± 22.79 mmHg/mL.min⁻¹) to Lig 1 (67.69 ± 18.88 mmHg/mL.min⁻¹) and Lig 2 (69.19 ± 22.46 mmHg/mL.min⁻¹), unlike control mice (Baseline 146.6 ± 68.77 mmHg/mL.min⁻¹; Lig 1 156.7 ± 76.09 mmHg/mL.min⁻¹; Lig 2 173.7 ± 94.95 mmHg/mL.min⁻¹). There was a treatment effect in RVR following ligations ($p = 0.003$). However, this was not reflected in the *post hoc* analysis. The relationship between GFR and SBP is shown in Figure 4.5 D. GFR was not different between groups following ligations.

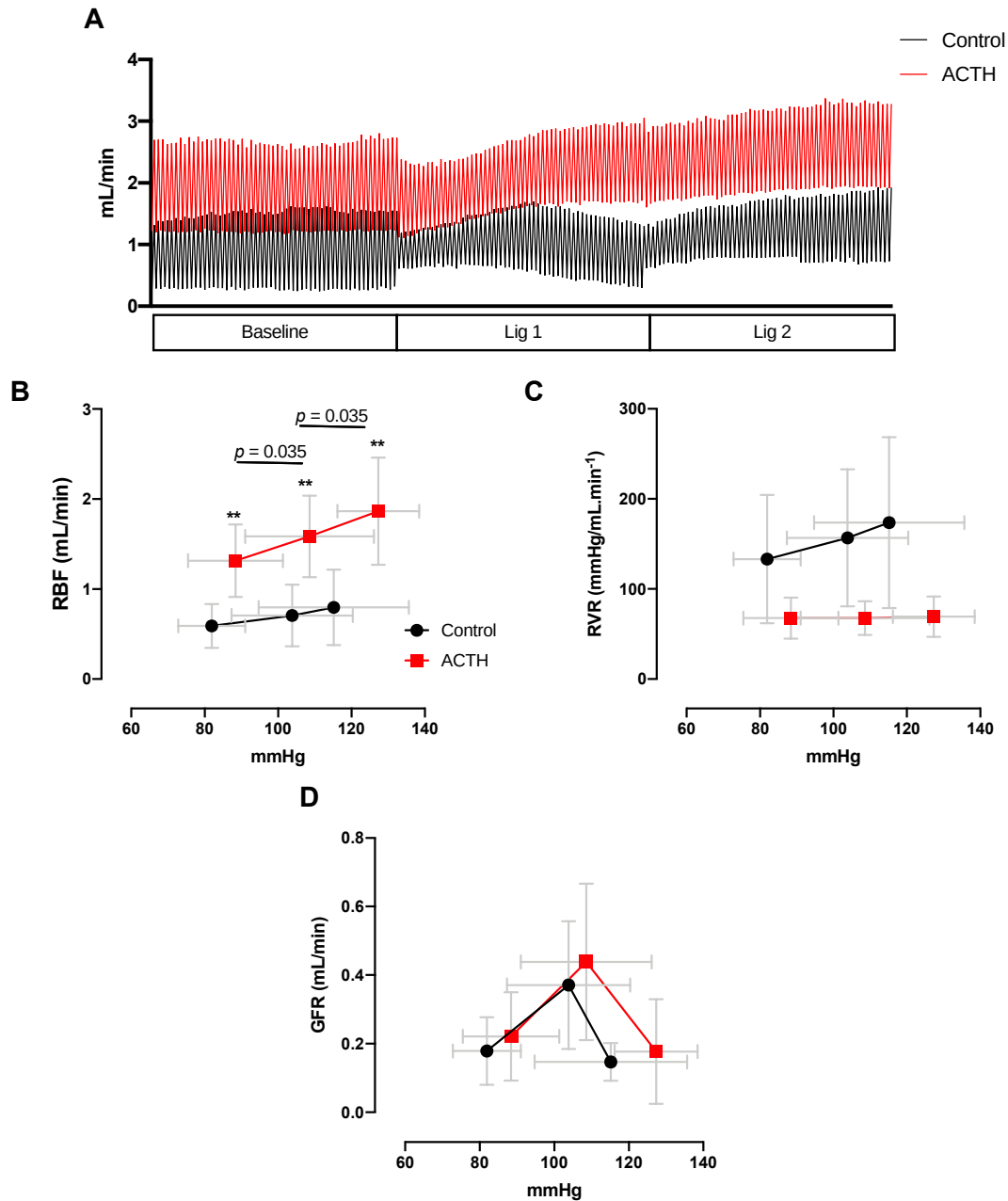


Figure 4.5: Renal haemodynamics during sequential ligation of arteries.

(A) Representative renal blood flow (RBF) trace calculated following sequential ligation of mesenteric and coeliac arteries (Lig 1) and distal aorta (Lig 2) in adult male mice treated with ACTH (red line) or vehicle (black line). The relationship between (B) mean RBF, (C) mean renal vascular resistance (RVR), (D) glomerular filtration rate (GFR) and systolic blood pressure (SBP) in adult male C57BL/6J mice treated with ACTH (red square; $n = 9$) or vehicle (black circle; $n = 6$) for 14 days. Each point for each group represents baseline, Lig 1 and Lig 2, respectively. Data are group mean $\pm$ SD and were analysed by two-way ANOVA with repeated measures and Holm-Sidák's *post hoc* test. $** p < 0.01$.

Water and sodium excretion: Urine flow, along with electrolyte excretion, were measured in anaesthetised control and ACTH-treated mice. Urine flow rate, sodium and potassium excretion, and fractional sodium and potassium excretion were not different between groups (Figure 4.6). Due to limited urine volume in baseline, electrolytes could not be measured in some samples.

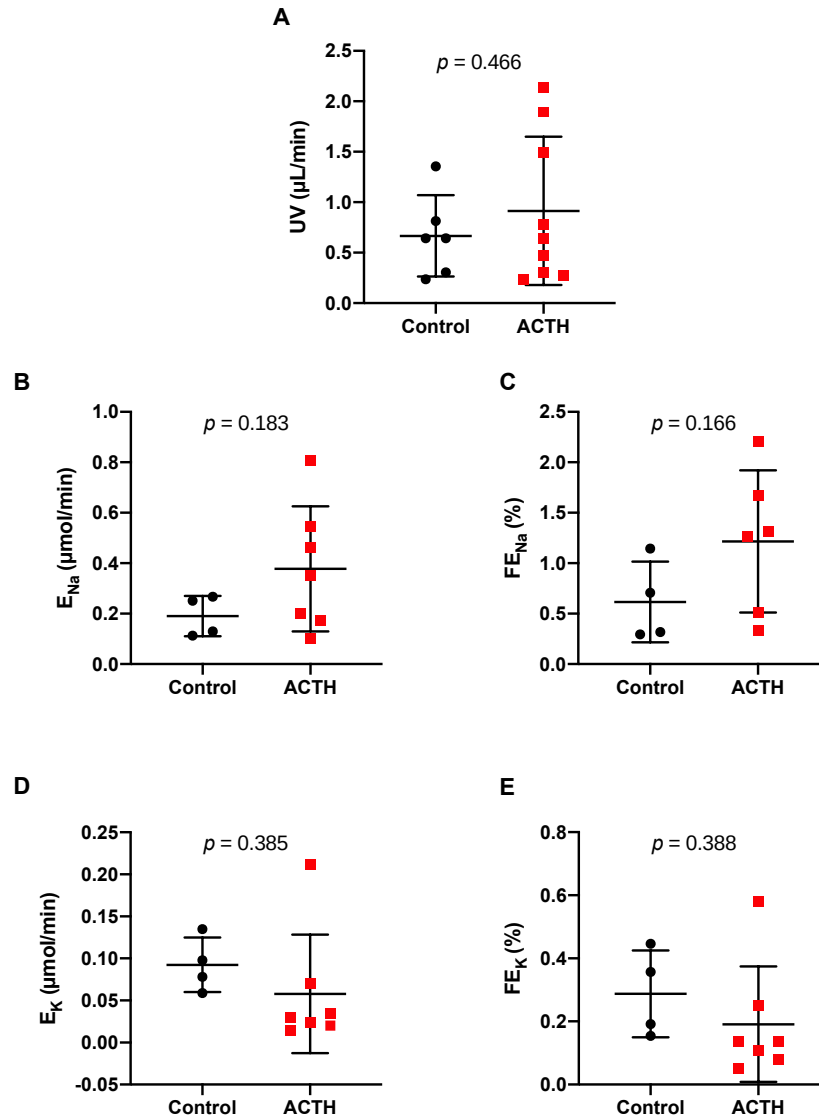


Figure 4.6: Baseline water and electrolyte excretion following ACTH infusion. (A) Urine flow rate (UV), (B) sodium excretion (E_{Na}), (C) fractional E_{Na} (FE_{Na}), (D) potassium excretion (E_{K}) and (E) FE_{K} measurements at baseline following acute pressure natriuresis (PN) surgery in adult male C57BL/6J mice treated with ACTH (red square; $n = 7-9$) or vehicle (black circle; $n = 4-6$) for 14 days. Measurements from individual mice and group mean $\pm$ SD are shown and were analysed by Student's unpaired t test.

The response of urine flow rate to acute BP rises, plotted as the relationship between urine flow rate and SBP, was significantly increased ($p = 0.0002$) with no difference between control and ACTH-treated mice (Figure 4.7 A). This was also reflected in the relationship between sodium excretion and SBP (Figure 4.7 B), fractional sodium, potassium and fractional potassium excretion (Figure 4.7 C-E).

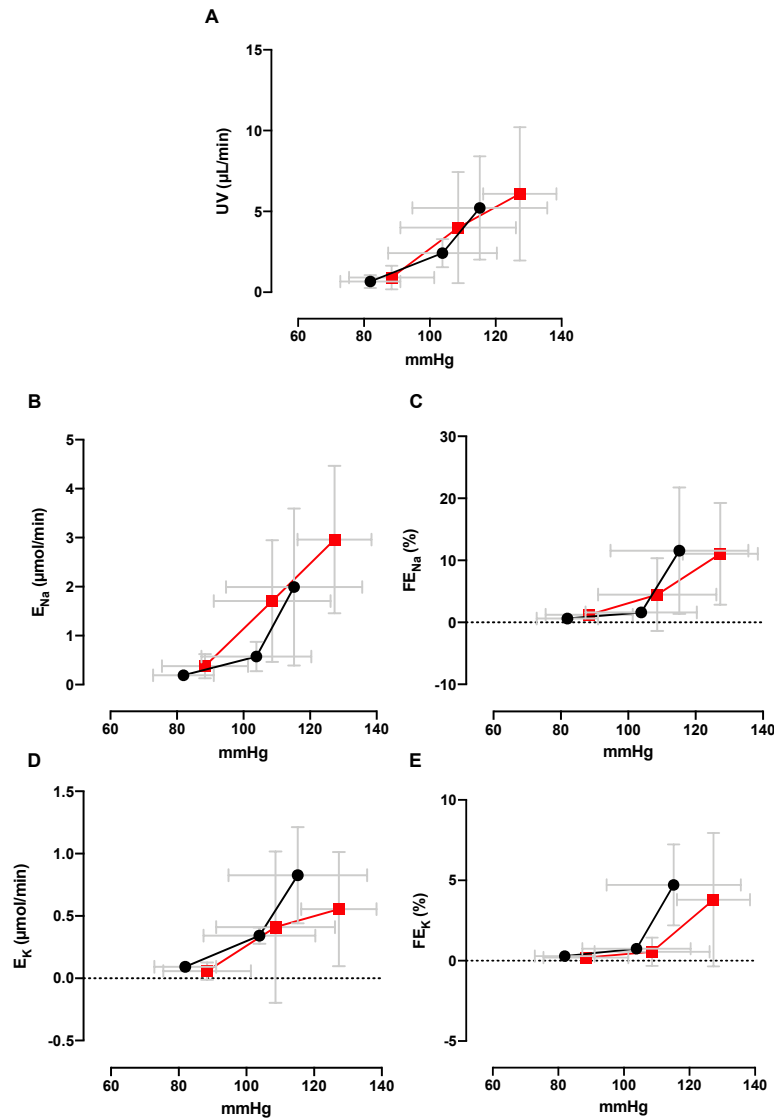


Figure 4.7: Water and electrolyte excretion during sequential ligation of arteries. (A) Urine flow rate (UV), (B) sodium excretion (E_{Na}), (C) fractional E_{Na} (FE_{Na}), (D) potassium excretion (E_{K}) and (E) FE_{K} measurements following acute pressure natriuresis (PN) surgery in adult male mice treated with ACTH (red square; $n = 7-9$) or vehicle (black circle; $n = 4-6$) for 14 days. Each point for each group represents baseline, mesenteric and coeliac ligation (Lig 1) and distal aorta ligation (Lig 2), respectively. Data are group mean $\pm$ SD and were analysed by two-way ANOVA with repeated measures and Holm-Šidák's post hoc test.

Plasma volume and electrolytes: There was a small, but not significant, increase in plasma sodium, along with hypokalaemia, following ACTH treatment compared to the control group. Plasma osmolality was increased in ACTH-treated mice, with increased 7 AM copeptin, a surrogate marker for AVP (Table 4.1). Volume status was assessed by measuring haematocrit (Hct) at the start and the end of the experiment. Starting values were similar (control, $39.2 \pm 3.5\%$; ACTH, $39.3 \pm 6.0\%$; $p = 0.9761$) and neither group showed significant changes in plasma volume during the experiment (data not shown).

Table 4.1: Plasma electrolytes and osmolality following ACTH infusion.
Data shown as mean $\pm$ SD ($n = 6-8$) and comparisons made by paired t-test.

	Control	ACTH
P_{Na} (mmol/L)	145.7 $\pm$ 2.0	149.5 $\pm$ 4.4 ($p = 0.08$)
P_K (mmol/L)	4.2 $\pm$ 0.3	3.5 $\pm$ 0.5 ($p = 0.01$)
P_{Osm} (mOsm/kg)	354.3 $\pm$ 6.8	373.2 $\pm$ 9.2 ($p = 0.002$)
P_{Copeptin} (pg/mL)	15.8 $\pm$ 10.6	92.3 $\pm$ 30.6 ($p < 0.0001$)

4.3.3 No impairment of acute pressure natriuresis in glucocorticoid excess $\pm$ high salt

ACTH had no effect on the acute PN response but as the significant BP rise occurs following ACTH + high salt, this leads to questioning the impact of ACTH + high salt on the acute PN response.

Figure 4.8 demonstrates that the procedure induced significant rises in BP following sequential ligations (ANOVA interaction of ligations $p < 0.0001$) and the effect was similar between ACTH + high salt and 28-day ACTH groups (ANOVA interaction of ligations and ACTH treatment $p = 0.494$).

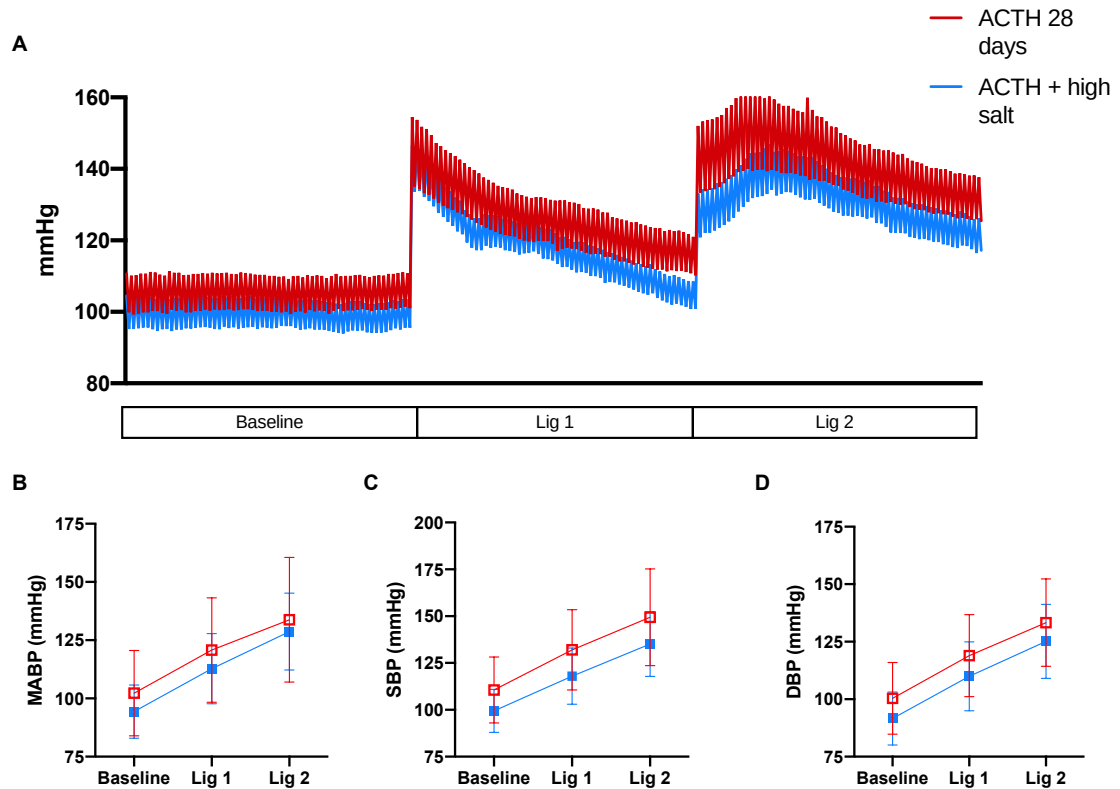


Figure 4.8: Blood pressure measurements during surgical assessment of ligation-induced acute pressure natriuresis following ACTH ± high salt.

Blood pressure (BP) measurements were taken following acute pressure natriuresis (PN) surgery in adult male mice treated with ACTH for 28 days on control diet (0.3% Na⁺; open red square; $n = 7$) or ACTH for 28 days on high salt diet for the latter 14 days (3% Na⁺; blue squares; $n = 7$). (A) Averaged BP trace taken at 20 sec intervals for each mouse during baseline, mesenteric and coeliac artery ligation (Lig 1) and distal aorta artery ligation (Lig 2). (B) Average mean arterial BP (MABP), (C) systolic BP (SBP) and (D) diastolic BP (DBP) of each period (Baseline, Lig 1 and Lig 2). Data are group mean $\pm$ SD and analysed by two-way ANOVA with repeated measures and Holm-Šidák's *post hoc* test.

Renal haemodynamics: At baseline, mean RBF was not different in ACTH + high salt mice compared with 28 day ACTH-treated mice. Similarly, there was no changes in RVR and GFR between groups (Figure 4.9).

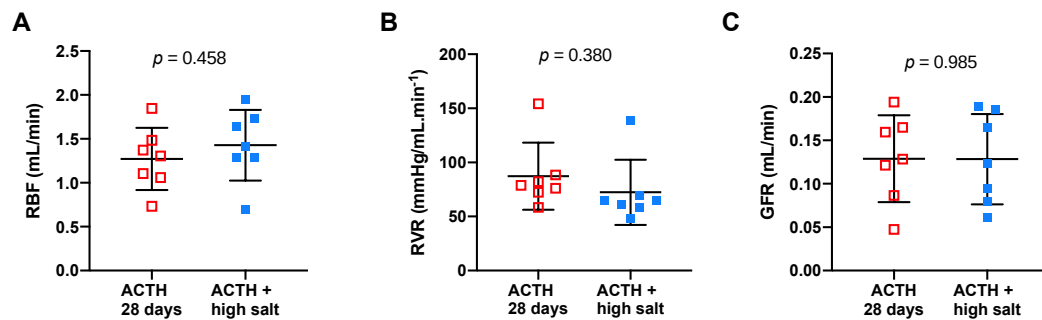


Figure 4.9: Baseline renal haemodynamics following ACTH ± high salt.

(A) Mean renal blood flow (RBF), (B) mean renal vascular resistance (RVR) and (C) glomerular filtration rate (GFR) measurements were taken at baseline following acute pressure natriuresis (PN) surgery in adult male mice treated with ACTH for 28 days on control diet (0.3% Na⁺; open red square; $n = 7$) or ACTH for 28 days on high salt diet for the latter 14 days (3% Na⁺; blue squares; $n = 7$). Measurements from individual mice and group mean $\pm$ SD are shown and were analysed by Student's unpaired t test.

Figure 4.10 A demonstrates averaged RBF traces during sequential ligation of arteries to induce pressure ramps in 28 day ACTH-treated and ACTH + high salt mice. Mean RBF remained stable as BP rose in both groups (Figure 4.10 B). The relationship between RVR and SBP was similar between groups (Figure 4.11 C). There was a small but significant increase in RVR in ACTH + high salt from baseline to Lig 1, with no change in Lig 2. The relationship between GFR and SBP is shown in Figure 4.10 D. GFR was not different between groups and following ligations.

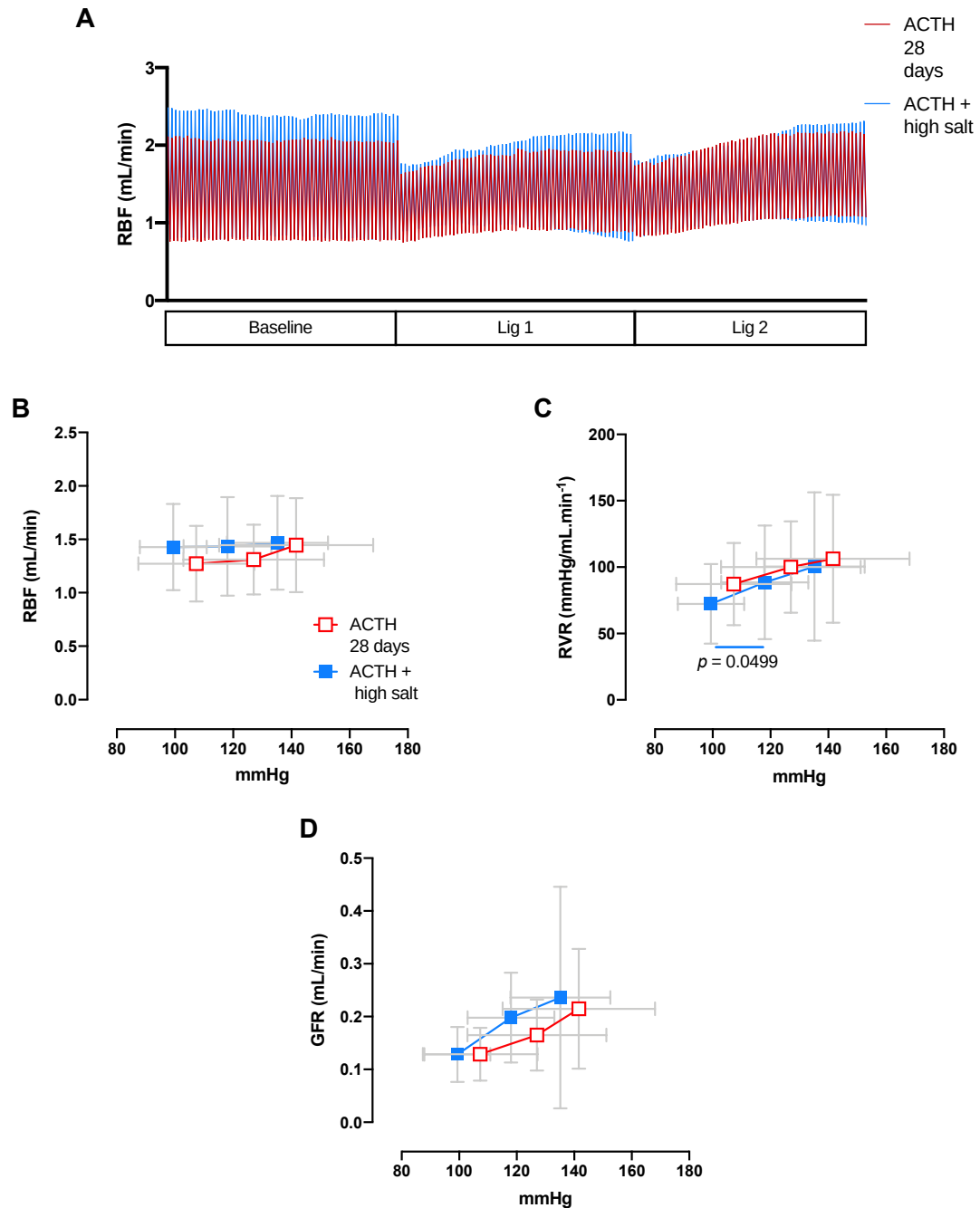


Figure 4.10: Renal haemodynamics during sequential ligation of arteries.

(A) Representative renal blood flow (RBF) trace calculated following sequential ligation of mesenteric and coeliac arteries (Lig 1) and distal aorta (Lig 2) in adult male mice treated with ACTH (red line) or vehicle (black line). The relationship between (B) mean RBF, (C) mean renal vascular resistance (RVR), (D) glomerular filtration rate (GFR) and systolic blood pressure (SBP) in adult male treated with ACTH for 28 days on control diet (0.3% Na⁺; open red square; $n = 7$) or ACTH for 28 days on high salt diet for the latter 14 days (3% Na⁺; blue squares; $n = 7$). Each point for each group represents baseline, Lig 1 and Lig 2, respectively. Data are group mean $\pm$ SD and were analysed by two-way ANOVA with repeated measures and Holm-Šidák's *post hoc* test.

Water and sodium excretion: Urine flow, along with electrolyte excretion, were measured in anaesthetised control and ACTH-treated mice. Urine flow rate, sodium excretion and fractional sodium excretion were not different between groups (Figure 4.11). Potassium in urine samples collected exceeded maximum range of electrolyte analyser (>80 mmol/L) so was not analysed further.

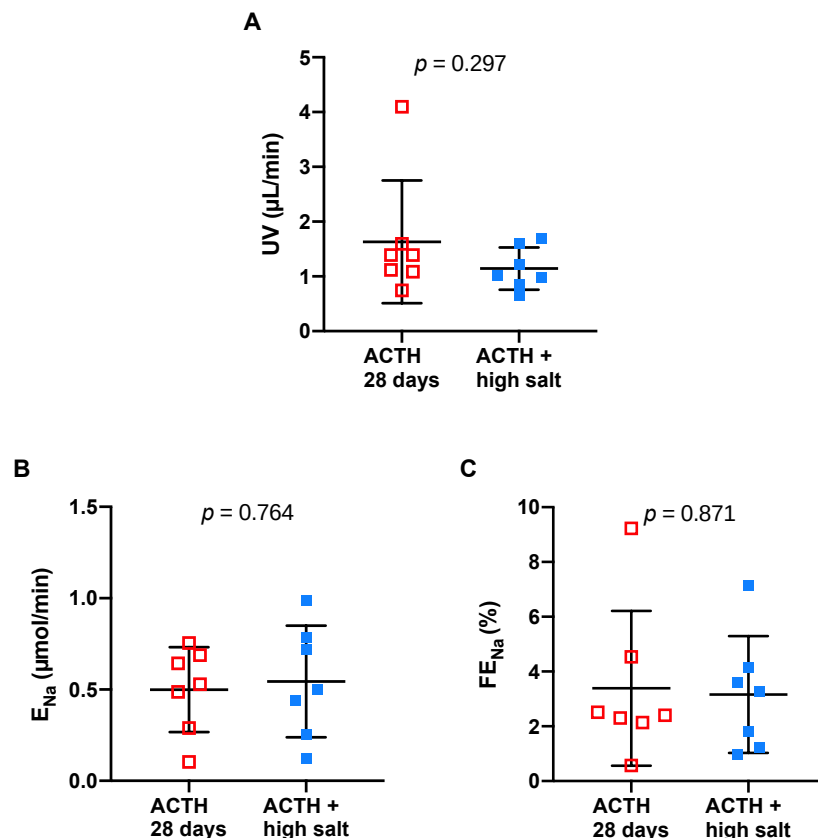


Figure 4.11: Baseline water and electrolyte excretion following ACTH ± high salt. (A) Urine flow rate (UV), (B) sodium excretion (E_{Na}) and (C) fractional E_{Na} (FE_{Na}) measurements were taken at baseline following acute pressure natriuresis (PN) surgery in adult mice treated with ACTH for 28 days on control diet (0.3% Na^+ ; open red square; $n = 7$) or ACTH for 28 days on high salt diet for the latter 14 days (3% Na^+ ; blue squares; $n = 7$). Measurements from individual mice and group mean $\pm$ SD are shown and were analysed by Student's unpaired t test.

The response of urine flow rate to acute BP rises, plotted as the relationship between urine flow rate and SBP, was significantly increased ($p < 0.0001$) with no difference between 28-day ACTH-treated mice and ACTH + high salt (Figure 4.12 A). This was also reflected in the relationship between sodium

excretion and SBP (Figure 4.7 B) and fractional sodium excretion and SBP (Figure 4.7 C).

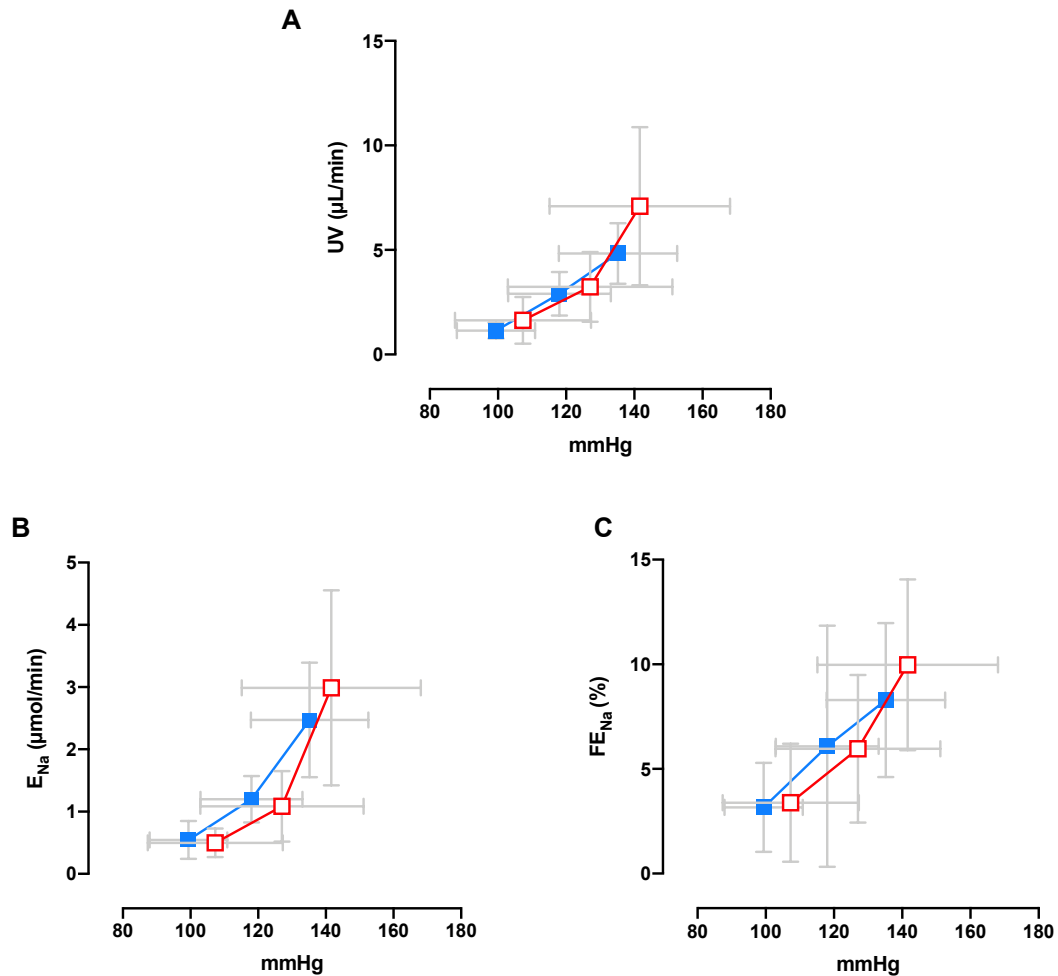


Figure 4.12: Water and electrolyte excretion during sequential ligation of arteries.

(A) Urine flow rate (UV), (B) sodium excretion (E_{Na}) and (C) fractional E_{Na} (FE_{Na}) measurements following acute pressure natriuresis (PN) surgery in adult mice treated with ACTH for 28 days on control diet (0.3% Na^+ ; open red square; $n = 7$) or ACTH for 28 days on high salt diet for the latter 14 days (3% Na^+ ; blue squares; $n = 7$). Each point for each group represents baseline, mesenteric and coeliac ligation (Lig 1) and distal aorta ligation (Lig 2), respectively. Data are group mean $\pm$ SD and were analysed by two-way ANOVA with repeated measures and Holm-Šidák's *post hoc* test.

Plasma volume and electrolytes: Plasma sodium was not affected by ACTH treatment as was similar to previous control measurements, but ACTH + high salt diet induced significant hypernatremia. ACTH (28 days) and ACTH + high salt induced hypokalaemia, similar to ACTH 14 days (Table 4.2).

Volume status was assessed by measuring Hct at the start and the end of the experiment. Starting values were similar (ACTH 28 days, $47.0 \pm 3.1\%$; ACTH, $50.9 \pm 3.9\%$; $p = 0.8276$) and neither group showed significant changes in plasma volume during the experiment (data not shown).

Table 4.2: Plasma electrolytes following ACTH $\pm$ high salt.

Data shown as mean $\pm$ SD ($n = 6-8$) and comparisons made by paired t-test.

	ACTH (28 days)	ACTH + high salt
P_{Na} (mmol/L)	148.2 ± 1.0	154.2 ± 3.4 ($p = 0.002$)
P_K (mmol/L)	3.6 ± 0.5	3.6 ± 0.4

4.3.4 Renal, but not mesenteric, vascular dysfunction in glucocorticoid excess

ACTH infusion had no effect on the acute PN response but there was evidence of abnormal renal haemodynamics *in vivo*, suggesting vascular dysfunction. To investigate if ACTH induces vascular abnormalities, renal and mesenteric artery reactivity to a known vasoconstrictor (PE), an endothelium-dependent vasodilator (ACh) and an endothelium-independent vasodilator (SNP) was assessed by wire myography.

Concentration response curves for PE in renal arteries of ACTH-treated mice, with saline-treated mice as control, is shown in Figure 4.13 A. The contractile response to depolarisation with KPSS was not significantly different between control and ACTH groups and did not deteriorate over the time course of the protocol. Normalised to the KPSS response, renal arteries from ACTH-treated mice showed significant reduction in both the sensitivity to PE (logEC₅₀) and maximal contractile response to PE (%E_{max}) (Figure 4.13 B). In contrast, the response of mesenteric arteries to PE was not different in ACTH-treated mice compared with controls (Figure 4.13 C, D).

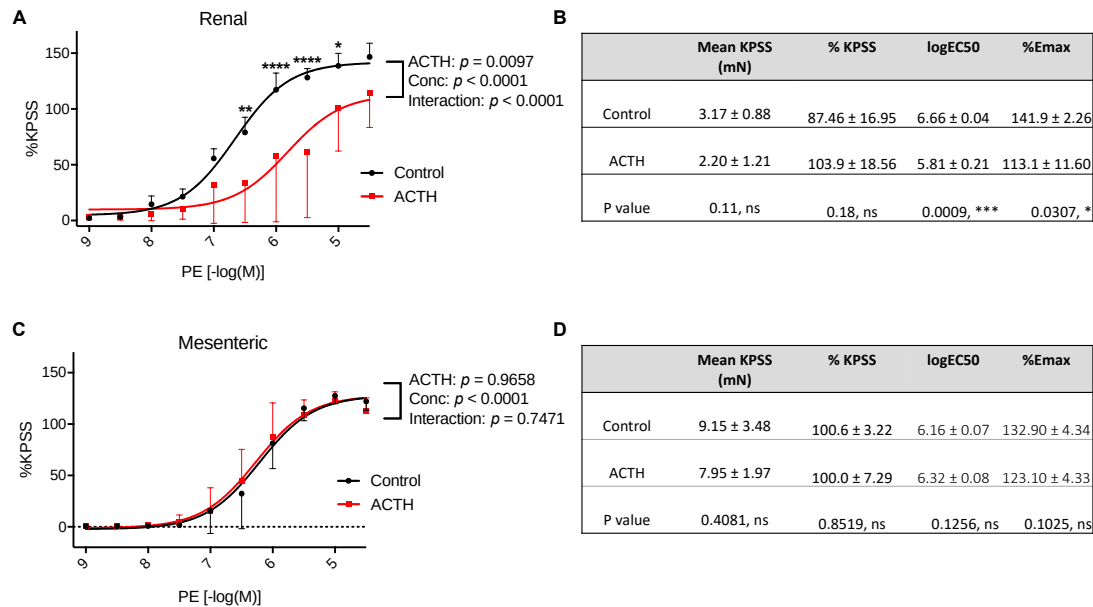


Figure 4.13: Contractile response to phenylephrine following ACTH infusion.

Arteries were isolated at 7 AM from adult male C57BL/6J mice treated for 14 days with either ACTH (red square; $n = 8$) or vehicle (black circle; $n = 8$). Concentration response curves to phenylephrine (PE) were obtained from (A) renal and (C) second order mesenteric arteries. Constriction was normalised to the maximum response induced by a high potassium physiological salt solution (KPSS) and concentration response curves generated by non-linear regression. Mean KPSS, %KPSS, logEC50 and %EMax were calculated for each concentration response curve for renal (B) and mesenteric arteries (D). Data are group mean $\pm$ SD and were analysed by two-way ANOVA with repeated measures and Holm-Šidák's *post hoc* test. Conc, concentration. **** $p < 0.0001$; ** $p < 0.01$; * $p < 0.05$.

In the renal artery, the response to ACh was different in ACTH-treated mice (ANOVA interaction between ACTH and concentration of ACh, $p = 0.002$). The sensitivity (logIC50) and the maximum vasodilation (%Preconstriction) were both decreased (Figure 4.14 A, B).

In mesenteric arteries, there was a small but significant reduction in sensitivity in ACTH-treated mice (Figure 4.14 C, D). The maximum vasodilation was not different between groups and overall there was no strong evidence for impaired endothelium-dependent relaxation in ACTH-treated mice.

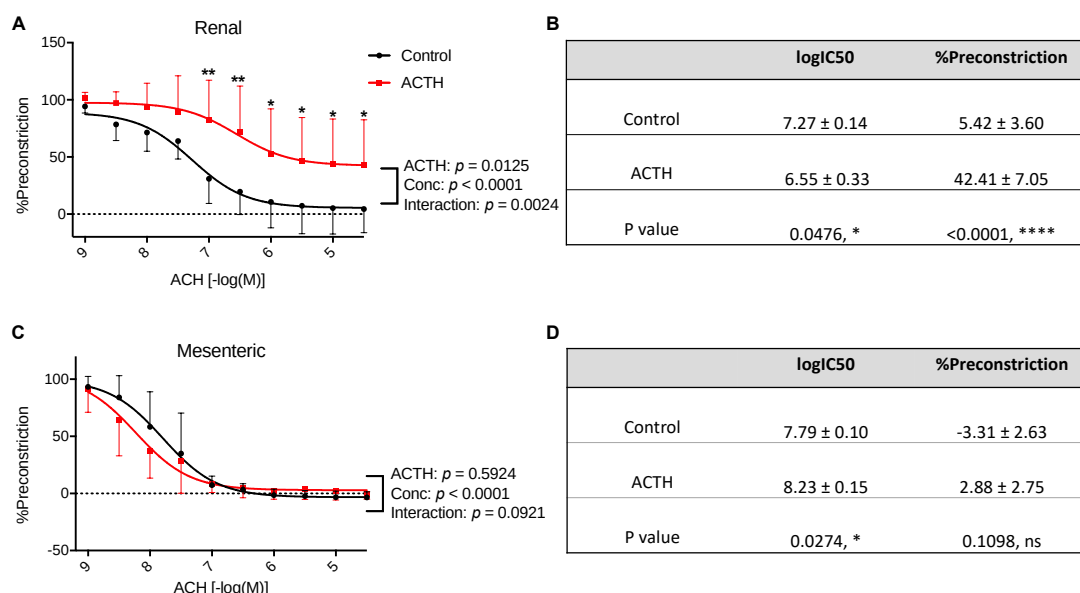


Figure 4.14: Dilatory response to acetylcholine following ACTH infusion.

Arteries were isolated at 7 AM from adult male C57BL/6J mice treated for 14 days with either ACTH (red square; $n = 8$) or vehicle (black circle; $n = 8$). Concentration response curves to acetylcholine (ACh) were obtained from (A) renal and (C) second order mesenteric arteries. Dilation was normalised to a percentage of each vessels response to a half maximal concentration of phenylephrine (%Precontraction) and concentration response curves shown as non-linear regression. LogIC50 and %Precontraction were calculated for each concentration response curve for renal (B) and mesenteric arteries (D). Data are group mean $\pm$ SD and were analysed by two-way ANOVA with repeated measures and Holm-Šidák's *post hoc* test. Conc, concentration. ** $p < 0.01$; * $p < 0.05$.

In the renal artery, the response to SNP was different in ACTH-treated mice (ANOVA interaction between ACTH and concentration of SNP, $p = 0.0002$). The sensitivity and the maximum vasodilation were both significantly decreased (Figure 4.15 A, B).

In mesenteric arteries, there was a small but significant reduction in sensitivity in ACTH-treated mice. The maximum vasodilation was not different between groups (Figure 4.15 C, D).

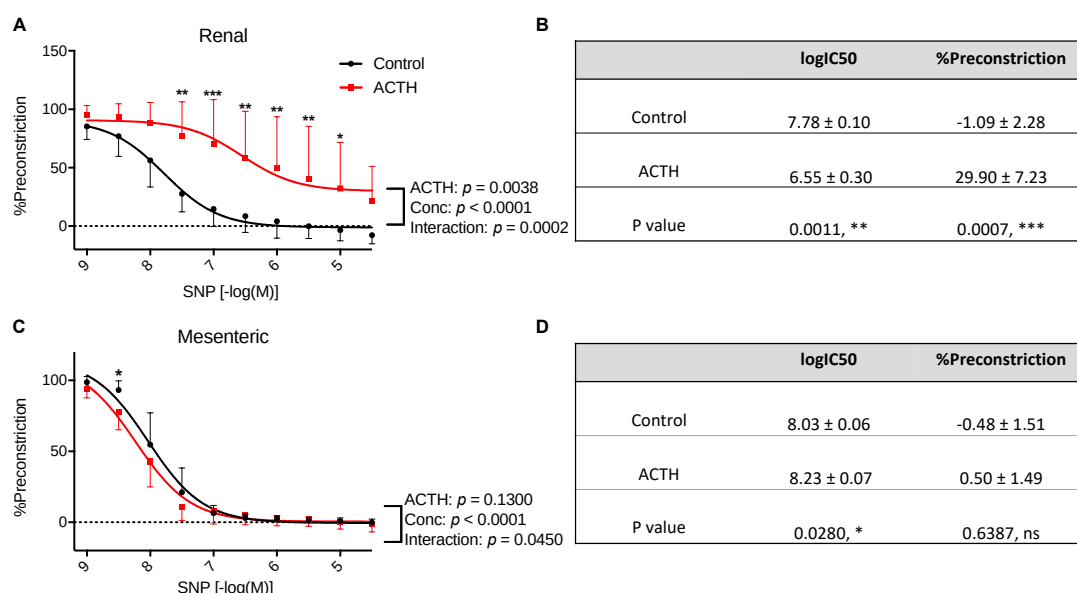


Figure 4.15: Dilatory response to sodium nitroprusside following ACTH infusion.

Arteries were isolated at 7 AM from adult male C57BL/6J mice treated for 14 days with either ACTH (red square; $n = 8$) or vehicle (black circle; $n = 8$). Concentration response curves to sodium nitroprusside (SNP) were obtained from (A) renal and (C) second order mesenteric arteries. Dilation was normalised to a percentage of each vessels response to a half maximal concentration of phenylephrine (%Preconstriction) and concentration response curves shown as non-linear regression. LogIC50 and %Preconstriction were calculated for each concentration response curve for renal (B) and mesenteric arteries (D). Data are group mean $\pm$ SD and were analysed by two-way ANOVA with repeated measures and Holm-Šidák's *post hoc* test. Conc, concentration. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

4.3.5 Mesenteric vascular dysfunction in prolonged glucocorticoid excess

The effect of different durations of glucocorticoid excess (14 and 28 days) on renal and mesenteric artery reactivity was assessed. Concentration response curves for PE in renal and mesenteric arteries of mice treated with either 14 or 28 days ACTH are shown in Figure 4.16 A and C. The %KPSS was not significantly different between groups and did not deteriorate over the time course of the protocol. The response of renal arteries to PE from 14 and 28 day ACTH-treated mice was not different (Figure 4.16 B). In mesenteric arteries, there was a small but significant increase in sensitivity to PE following 28 days ACTH compared to 14 days. The maximum contractile response was not different between groups and overall there was no strong evidence for impaired PE-induced constriction comparing durations of ACTH treatment (Figure 4.16 D).

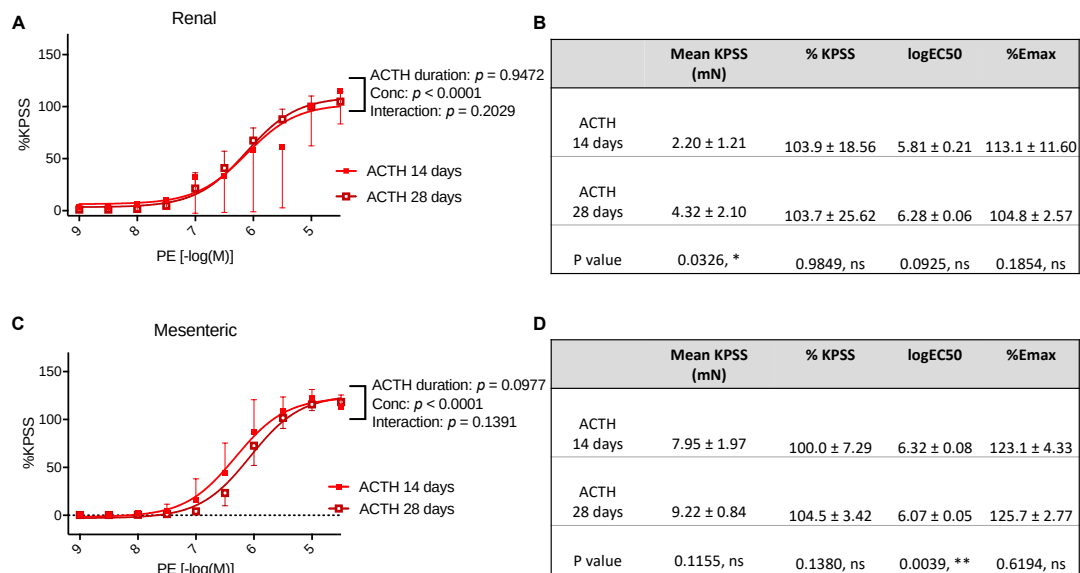


Figure 4.16: Contractile response to phenylephrine following prolonged ACTH. Arteries were isolated at 7 AM from adult male C57BL/6J mice treated with ACTH for either 14 days (red square; $n = 7-8$) or 28 days (open red squares; $n = 7-8$). Concentration response curves to phenylephrine (PE) were obtained from (A) renal and (C) second order mesenteric arteries. Constriction was normalised to the maximum response induced by a high potassium physiological salt solution (KPSS) and concentration response curves generated by non-linear regression. Mean KPSS, %KPSS, logEC50 and %EMax were calculated for each concentration response curve for renal (B) and mesenteric arteries (D). Data are group mean $\pm$ SD and were analysed by two-way ANOVA with repeated measures and Holm-Šidák's *post hoc* test. Conc, concentration.

In the renal artery, the maximum vasodilation was significantly increased following 28 days ACTH compared with 14 days, with no change in sensitivity to ACh (Figure 4.17 A, B).

In mesenteric arteries, the sensitivity to ACh was different in 28-day ACTH-treated mice (ANOVA interaction between ACTH and concentration of ACh, $p < 0.0001$). The maximum vasodilation was not different between groups (Figure 4.17 C, D).

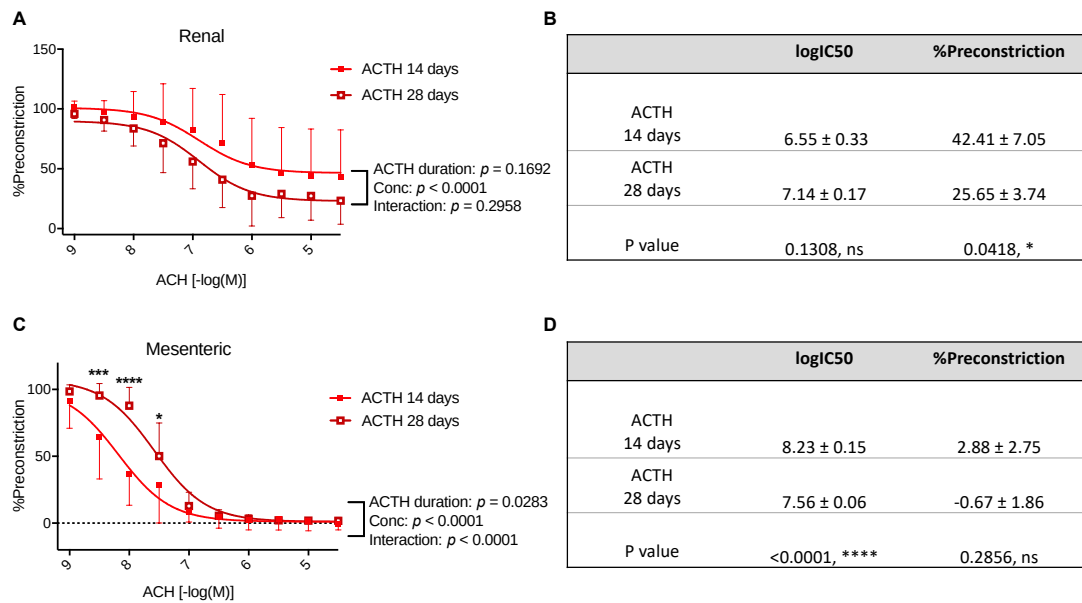


Figure 4.17: Dilatory response to acetylcholine following prolonged ACTH.

Arteries were isolated at 7 AM from adult male C57BL/6J mice treated with ACTH for either 14 days (red square; $n = 7-8$) or 28 days (open red squares; $n = 7-8$). Concentration response curves to acetylcholine (ACh) were obtained from (A) renal and (C) second order mesenteric arteries. Dilation was normalised to a percentage of each vessels response to a half maximal concentration of phenylephrine (%Precontraction) and concentration response curves shown as non-linear regression. LogIC50 and %Precontraction were calculated for each concentration response curve for renal (B) and mesenteric arteries (D). Data are group mean $\pm$ SD and were analysed by two-way ANOVA with repeated measures and Holm-Šidák's *post hoc* test. **** $p < 0.0001$; *** $p < 0.001$; * $p < 0.05$.

In the renal artery, the response to SNP was not different between 14 and 28 days ACTH infusion (Figure 4.18 A, B).

In contrast, in mesenteric arteries, the response to SNP was different in 28-day ACTH-treated mice compared with 14 days ACTH (ANOVA interaction between ACTH and concentration of ACh, $p < 0.0001$). The sensitivity was significantly decreased, with no change in the maximal dilatory response (Figure 4.18 C, D).

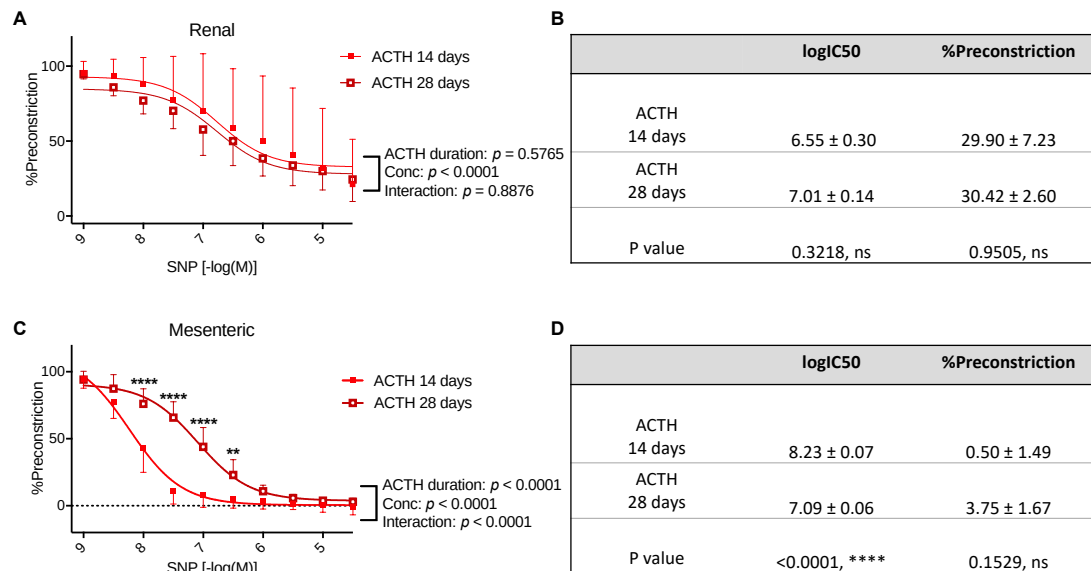


Figure 4.18: Dilatory response to sodium nitroprusside following prolonged ACTH.

Arteries were isolated at 7 AM from adult male C57BL/6J mice treated with ACTH for either 14 days (red square; $n = 7-8$) or 28 days (open red squares; $n = 7-8$). Concentration response curves to sodium nitroprusside (SNP) were obtained from (A) renal and (C) second order mesenteric arteries. Dilation was normalised to a percentage of each vessels response to a half maximal concentration of phenylephrine (%Precontraction) and concentration response curves shown as non-linear regression. LogIC50 and %Precontraction were calculated for each concentration response curve for renal (B) and mesenteric arteries (D). Data are group mean $\pm$ SD and were analysed by two-way ANOVA with repeated measures and Holm-Šidák's *post hoc* test. Conc, concentration. **** $p < 0.0001$; ** $p < 0.01$.

4.3.6 High salt does not alter renal and mesenteric reactivity in glucocorticoid excess

As the BP began to rise significantly with ACTH + high salt, does the interaction between salt and glucocorticoid excess worsen vascular reactivity? To answer this, the effect of a high salt challenge on glucocorticoid excess on renal and mesenteric artery reactivity was assessed.

Concentration response curves for PE in renal and mesenteric arteries of mice following 28 days ACTH or 28 days ACTH infusion with the latter 14 days fed a high salt diet (Figure 4.19 A, C). The %KPSS was not significantly different between groups and did not deteriorate over the time course of the protocol. The response of renal arteries to PE from 28-day ACTH-treated and ACTH + high salt mice was not different (Figure 4.19 B). In mesenteric arteries, there

was a small but significant increase in sensitivity to PE following ACTH + high salt compared to 28 days ACTH. The maximum contractile response was not different between groups and overall there was no strong evidence for impaired PE-induced constriction comparing ACTH $\pm$ high salt treatment (Figure 4.19 D).

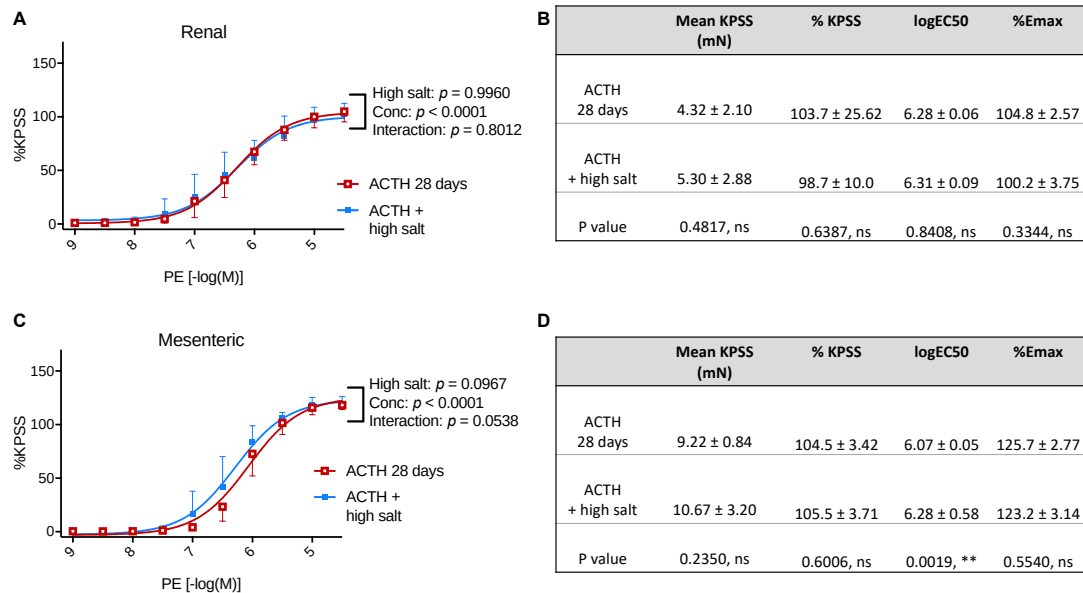


Figure 4.19: Contractile response to phenylephrine following ACTH $\pm$ high salt. Arteries were isolated at 7 AM from adult male C57BL/6J mice treated with ACTH for 28 days on control diet (0.3% Na⁺; open red square; $n = 8$) or 28 days on high salt diet for the latter 14 days (3% Na⁺; blue squares; $n = 8$). Concentration response curves to phenylephrine (PE) were obtained from (A) renal and (C) second order mesenteric arteries. Constriction was normalised to the maximum response induced by a high potassium physiological salt solution (KPSS) and concentration response curves generated by non-linear regression. Mean KPSS, %KPSS, logEC50 and %EMax were calculated for each concentration response curve for renal (B) and mesenteric arteries (D). Data are group mean $\pm$ SD and were analysed by two-way ANOVA with repeated measures and Holm-Šidák's *post hoc* test. Conc, concentration.

Concentration response curves to ACh in renal and mesenteric arteries in mice treated with 28 days ACTH or ACTH + high salt mice (Figure 4.20). There was no change in sensitivity and maximal dilatory response to ACh of the renal and mesenteric arteries between groups.

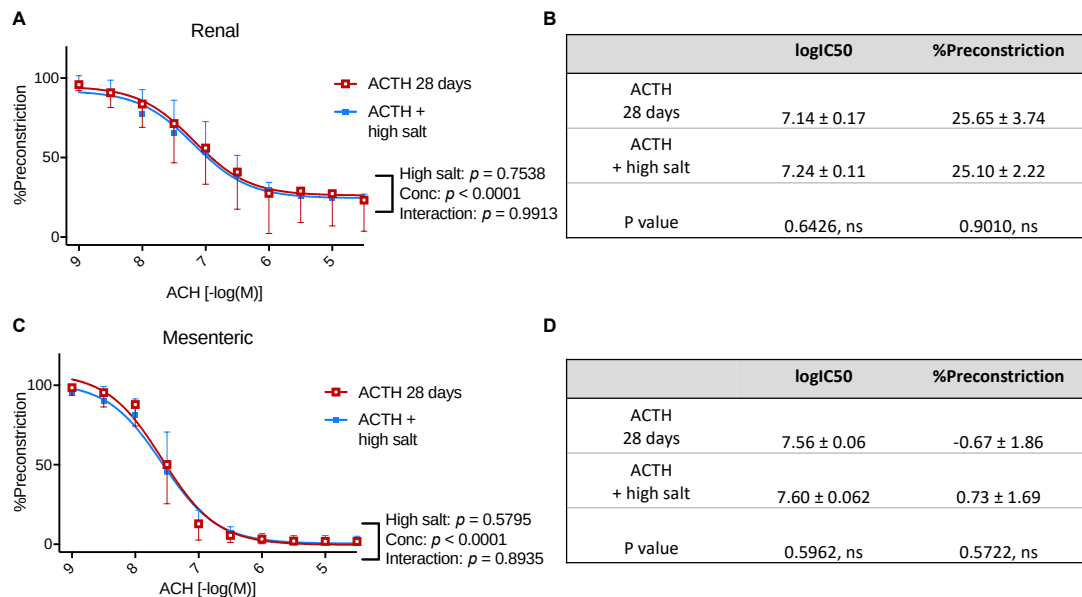


Figure 4.20: Dilatory response to acetylcholine following ACTH ± high salt.

Arteries were isolated at 7 AM from adult male C57BL/6J mice treated with ACTH for 28 days on control diet (0.3% Na⁺; open red square; $n = 8$) or 28 days on high salt diet for the latter 14 days (3% Na⁺; blue squares; $n = 8$). Concentration response curves to acetylcholine (ACh) were obtained from (A) renal and (C) second order mesenteric arteries. Dilation was normalised to a percentage of each vessels response to a half maximal concentration of phenylephrine (%Precontraction) and concentration response curves shown as non-linear regression. LogIC50 and %Precontraction were calculated for each concentration response curve for renal (B) and mesenteric arteries (D). Data are group mean ± SD and were analysed by two-way ANOVA with repeated measures and Holm-Šidák's *post hoc* test. Conc, concentration.

Concentration response curves to SNP in renal and mesenteric arteries in mice treated with 28 days ACTH or ACTH + high salt mice (Figure 4.21). There was no change in sensitivity and maximal dilatory response to ACh of the renal and mesenteric arteries between groups.

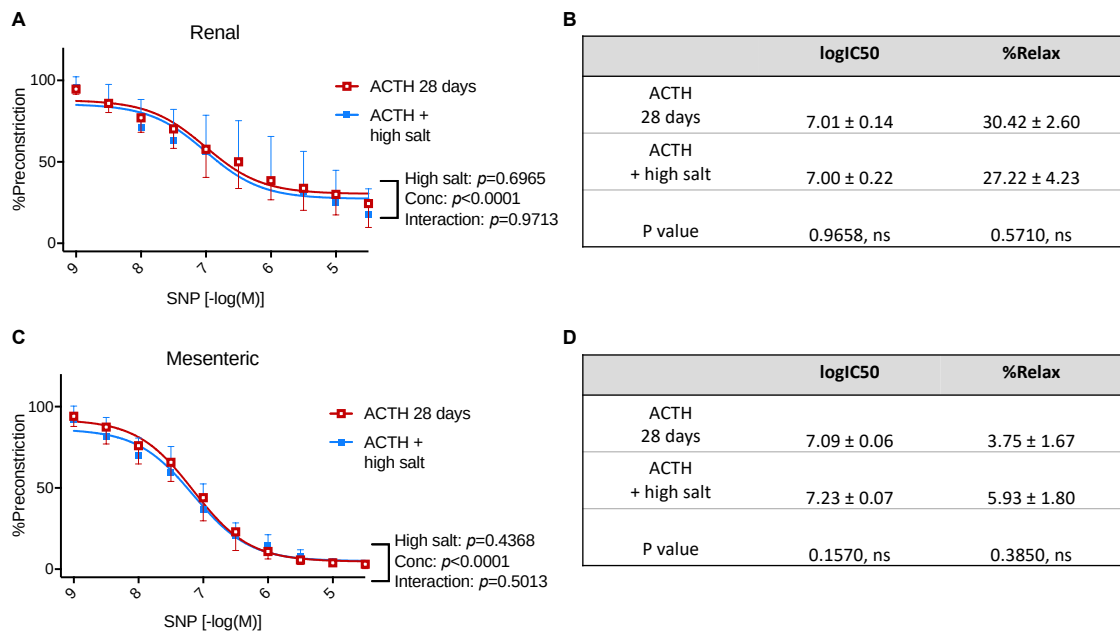


Figure 4.21: Dilatory response to sodium nitroprusside following ACTH ± high salt.

Arteries were isolated at 7 AM from adult male C57BL/6J mice treated with ACTH for 28 days on control diet (0.3% Na⁺; open red square; *n* = 8) or 28 days on high salt diet for the latter 14 days (3% Na⁺; blue squares; *n* = 8). Concentration response curves to sodium nitroprusside (SNP) were obtained from (A) renal and (C) second order mesenteric arteries. Dilation was normalised to a percentage of each vessels response to a half maximal concentration of phenylephrine (%Preconstriction) and concentration response curves shown as non-linear regression. LogIC₅₀ and %Preconstriction were calculated for each concentration response curve for renal (B) and mesenteric arteries (D). Data are group mean ± SD and were analysed by two-way ANOVA with repeated measures and Holm-Šidák's *post hoc* test. Conc, concentration.

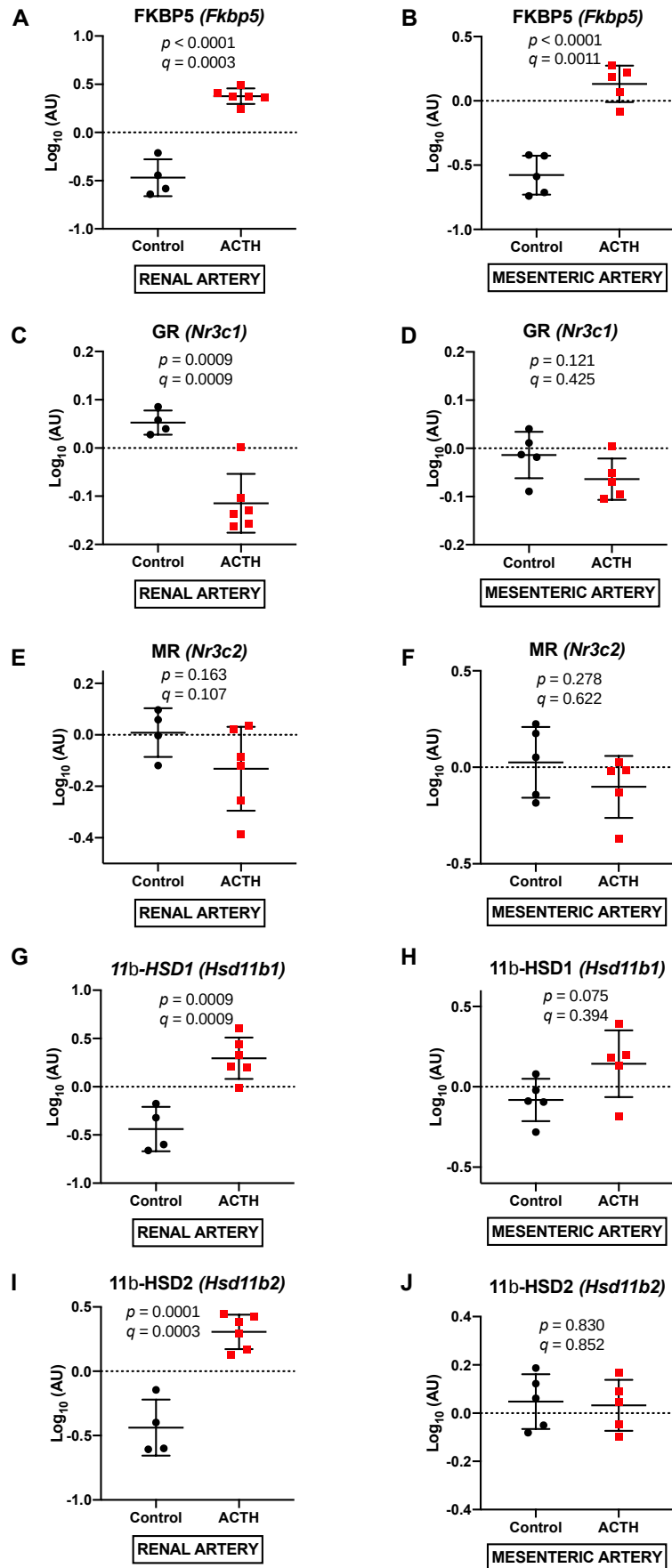
4.3.7 Glucocorticoid-induced alterations in the renal and mesenteric artery

A large panel of genes were selected to unpick the mechanisms behind renal vascular abnormalities seen in ACTH-treated mice. Firstly, expression of glucocorticoid-related genes were investigated in both renal and mesenteric arteries in mice treated with 14 days ACTH or saline as controls (Figure 4.22). For assessing whether there were changes in cellular glucocorticoids in renal and mesenteric arteries, the gene expression of FK506 binding protein 51 (*Fkbp5*) was measured. Renal and mesenteric artery *Fkbp* expression was significantly increased following chronic ACTH infusion. The gene encoding GR (*Nr3c1*) was significantly downregulated in renal arteries in ACTH-treated mice. However, unaltered in mesenteric arteries. The gene encoding MR

(*Nr3c2*) was unchanged in renal and mesenteric arteries in ACTH-treated mice compared with controls. Renal artery *Hsd11b1* and *Hsd11b2* was significantly increased in ACTH-treated mice, with no changes in mesenteric arteries of the same mice.

Figure 4.22: Expression of glucocorticoid-related genes in renal and mesenteric arteries following ACTH infusion.

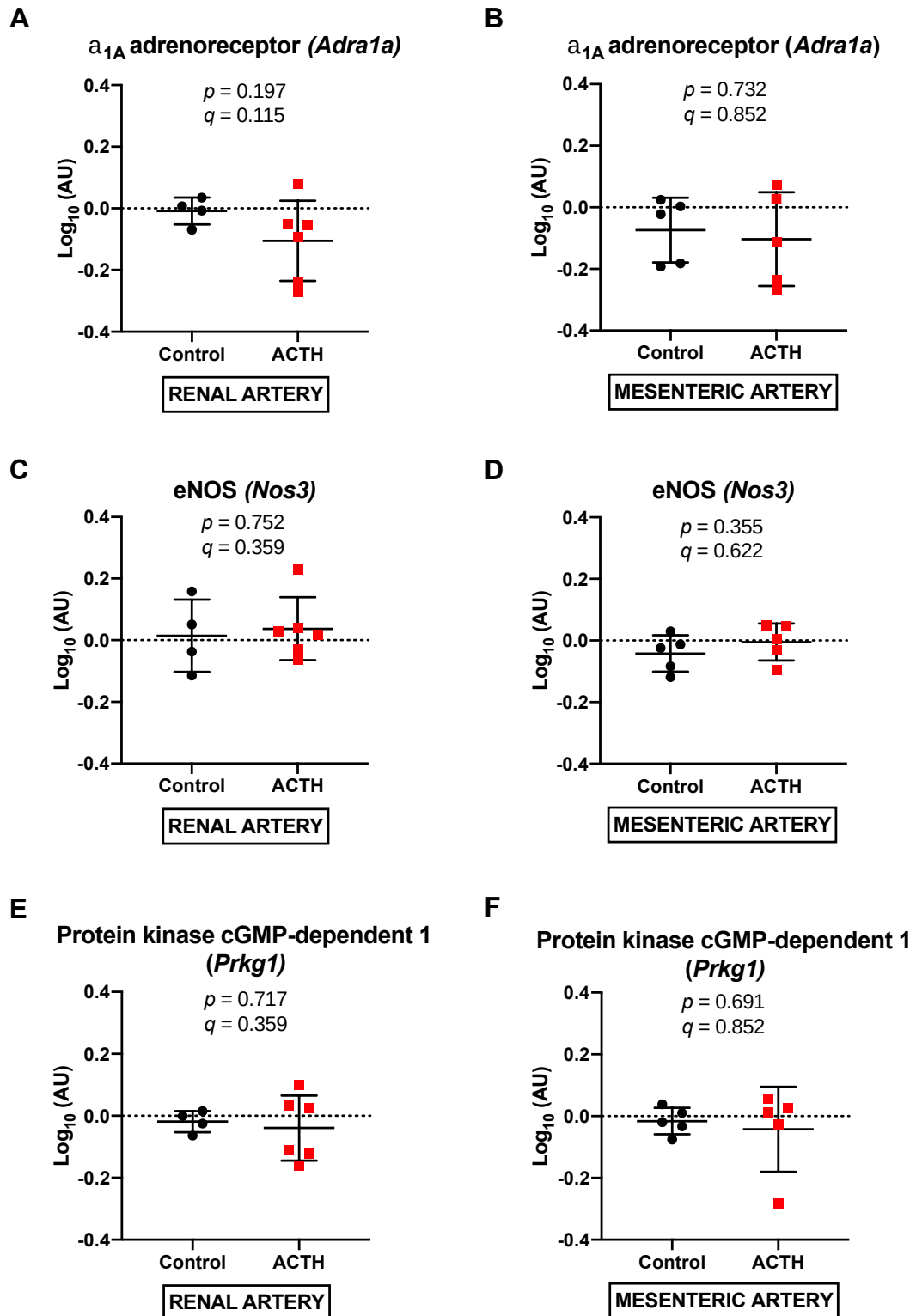
Glucocorticoid-related gene expression in renal and mesenteric arteries of 14-day saline-treated mice (black circles; $n = 4-5$) and ACTH-treated mice (red squares; $n = 5-6$). The names of proteins are presented alongside the genes-of-interest within brackets and normalised to the average expression of reference genes *Hprt*, *Rn18s*, *Actb* and *Tbp*. Data are mean $\pm$ SD and were analysed by Student's unpaired t test, adjusting for multiple comparisons using FDR. AU, arbitrary units.



Expression of the gene encoding α_{1a} receptor (*Adra1a*), involved in PE-induced vasoconstriction, was unaltered in both renal and mesenteric arteries of ACTH-treated mice, compared with controls (Figure 4.23 A, B). Expression of the gene encoding endothelial nitric oxide synthase (*Nos3*), involved in endothelium-dependent vasodilation, was unaltered in both renal and mesenteric arteries of ACTH-treated mice, compared with controls (Figure 4.23 C, D). Similarly, the gene encoding cGMP-dependent protein kinase 1 (*Prkg1*), involved in both endothelium-dependent and -independent vasodilation was similar between groups (Figure 4.23 E, F).

Figure 4.23: Expression of genes involved in vascular function following ACTH infusion.

Expression of genes involved in vascular function in renal and mesenteric arteries of 14-day saline-treated mice (black circles; $n = 4-5$) and ACTH-treated mice (red squares; $n = 5-6$). The names of proteins are presented alongside the genes-of-interest within brackets and normalised to the average expression of reference genes *Hprt*, *Rn18s*, *Actb* and *Tbp*. Data are mean $\pm$ SD and were analysed by Student's unpaired t test, adjusting for multiple comparisons using FDR. AU, arbitrary units.



Plasma NO concentration was assessed in terminal samples of saline- and ACTH-treated mice (Figure 4.24). Plasma NO was not significantly different between control and ACTH-treated mice.

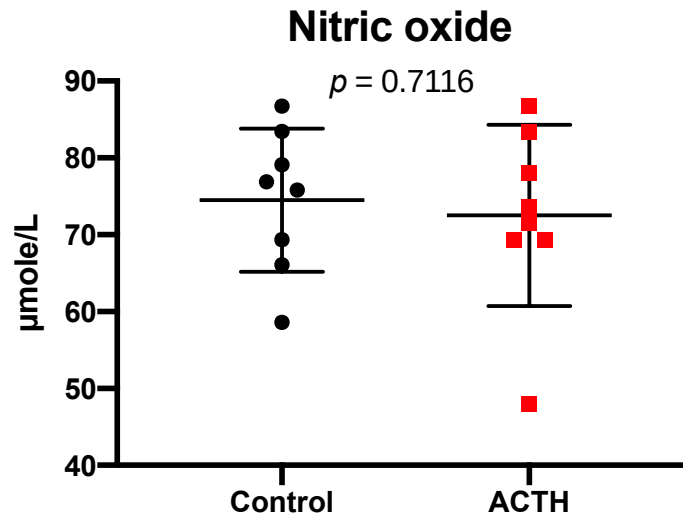


Figure 4.24: Plasma nitric oxide levels following ACTH infusion.

Plasma nitric oxide in mice of 14-day saline-treated mice (black circles; $n = 8$) and ACTH-treated mice (red squares; $n = 8$). Data shown as mean $\pm$ SD and were analysed by Student's unpaired t test.

Genes involved in the structural composition of the vasculature was assessed in the renal and mesenteric arteries of control and ACTH-treated mice (Figure 4.25). There was significant reduction in *Eln* (encoding elastin) and *Emilin1* in renal arteries of ACTH-treated mice compared with controls (Figure 4.25 A, B). However, *Tgfb1* was significantly increased following chronic ACTH infusion (Figure 4.25 C). There were no changes in *Eln*, *Emilin-1* or *Tgf- β* in mesenteric arteries in ACTH-treated mice compared with controls (Figure 4.25 D-F).

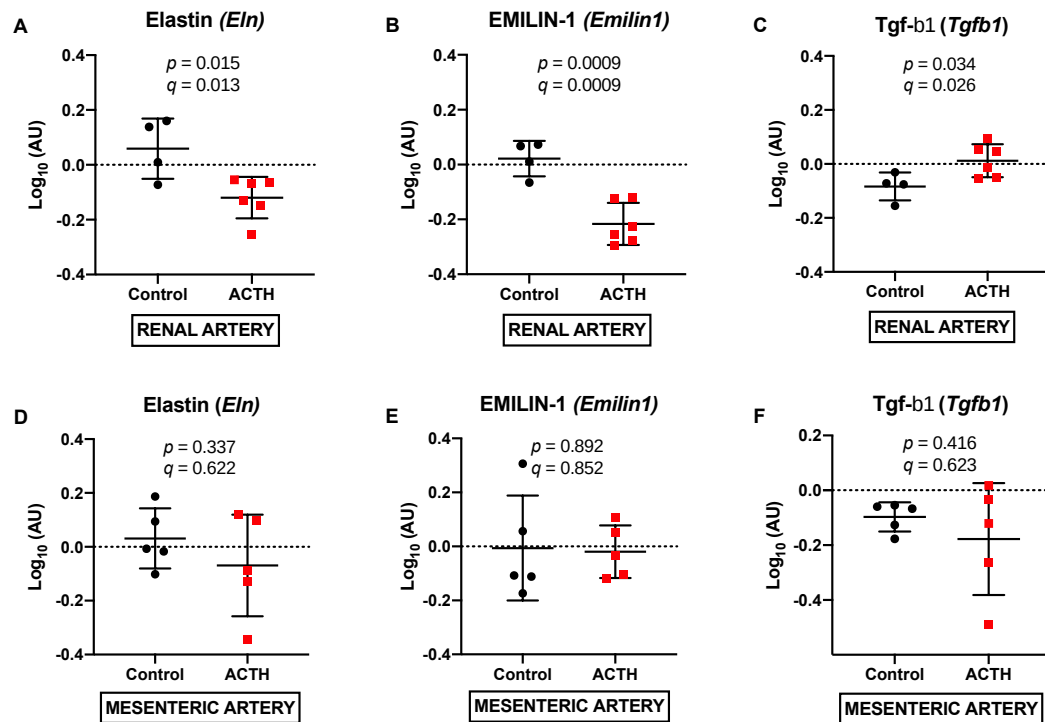


Figure 4.25: Expression of genes involved in regulating structural components of renal and mesenteric arteries following ACTH infusion.

Expression of genes involved in regulating structural components in renal and mesenteric arteries of 14-day saline-treated mice (black circles; $n = 4-5$) and ACTH-treated mice (red squares; $n = 5-6$). The names of proteins are presented alongside the genes-of-interest within brackets and normalised to the average expression of reference genes *Hprt*, *Rn18s*, *Actb* and *Tbp*. Data are mean $\pm$ SD and were analysed by Student's unpaired t test, adjusting for multiple comparisons using FDR. AU, arbitrary units.

4.4 Discussion

The main findings of this chapter are that ACTH does not induce major impairment of the acute natriuretic response to rising BP either alone or in combination with high salt diet. Instead, *in vivo* haemodynamic measurements and *ex vivo* myography point to a renal vascular phenotype that may contribute salt-sensitivity in ACTH-treated mice.

4.4.1 Does a natriuretic defect cause salt-sensitivity?

The use of metabolic cages allow for the measurement of water and electrolyte input and output in conscious, unrestrained mice. Metabolic cages also allow for the measurement of sodium and water balance but require acclimatation to the cages over a period of several days which is regarded as stressful for the animal. Therefore, to minimise stress, only 24 h collections were made. Food (control diet, 0.3% Na⁺ and 0.7% K⁺) and water intake was not different with glucocorticoid excess. Glucocorticoid excess has been reported to induce hyperphagia and obesity over a 28 day period of exogenous corticosterone treatment in mice, where food intake increased after 3 days of treatment and remained elevated with body weight significantly increased after 14 days¹⁸⁴. This was not seen in our model of glucocorticoid excess. ACTH-treated mice excreted more water over 24 h however, with no change in drinking. Potassium excretion was also increased, suggesting a persistent renal leak which might contribute to hypokalaemia. The increased water loss and no change to 24 h sodium excretion could explain hypernatremia with increased osmolality and plasma copeptin in ACTH-treated mice. It is well documented that Cushing's syndrome patients¹⁸⁵ and animal models of ACTH-induced hypertension¹²⁸ have hypernatremia, inducing activation of AVP in an aim to restore equilibrium. Overall, this suggests volume contraction, previously reported during ACTH excess¹²⁸, which may underestimate potassium depletion. Potassium depletion is a hallmark of mineralocorticoid excess, thought to be caused by MR activation in the distal nephron. With glucocorticoid and mineralocorticoid excess in ACTH-treated mice, MR overactivation in the distal nephron could behind hypokalaemia.

Rhythmicity of urine and potassium excretion was maintained. However, rhythmicity of diurnal sodium excretion was lost. It has been suggested that hypertensive individuals have blunted diurnal patterns of sodium and water excretion compared with normotensive subjects¹⁸⁶. Similarly, this is seen in non-dippers but diurnal sodium excretion was normalised following sodium restriction in these patients¹⁸⁷. Overall, this suggests ACTH excess induces a natriuretic defect. Therefore, I hypothesised impaired PN might be contributory to the BP abnormalities seen in ACTH-treated mice.

Chronic glucocorticoid excess had no effect on the relationship between RAP, a surrogate marker of BP, and urinary sodium excretion (the acute PN curve), with no change in pressure diuresis. Although, the acute PN response was measured in anaesthetised animals during their inactive phase. Rats anaesthetised, using inactin, during their inactive period showed significant increases in GFR and proximal tubular reabsorption compared with conscious animals. However, these parameters remained comparable to conscious animals when anaesthetised during their active period¹⁸⁸. Therefore, potential changes may be missed. Time of day for assessment of renal function under anaesthesia needs to be considered in future studies. The acute PN response was unaltered following ACTH treatment with high salt, previously seen to cause significant rises in BP. Furthermore, BP was not risen in these animals compared with ACTH alone. With inactin reported to decrease BP¹⁸⁹, the observations seen in conscious telemetry measurements are more likely to be reliable. Overall, this points to impaired PN not being contributory to salt-sensitivity.

4.4.1.1 Optimisation of experimental induction of the acute pressure natriuresis response in mice

The approach that was used to investigate the acute PN response involved experimentally inducing pressure ramps via arterial ligation to increase BP and, in turn, renal perfusion pressure. The ability of the kidneys to modify excretion of sodium and water in response to this short term rise in BP was

then investigated by measuring urinary sodium excretion. Alternative pharmacological approaches have been used to investigate acute PN response, including the use of aldosterone¹⁹⁰, vasopressin¹⁹¹ and angiotensin II¹⁹². However, inducing pressure ramps via arterial ligation is the current “gold standard” for inducing acute PN. This technique was first developed in dogs by Dresser et al¹⁹³, and modified to be performed in rats by Roman and Cowley¹⁹⁴. Roman and Cowley’s protocol involved simultaneous ligation of the coeliac and mesenteric arteries, followed by ligation of the distal aorta. Ligation-induced acute PN also allows the demonstration of GFR and RBF autoregulation. Studies have performed ligation-induced acute PN method in mice^{195,196}. However, methodology was limited. Therefore, I assisted Kevin Stewart, who performed the surgery, in optimising this protocol for mice, based on the Roman and Cowley method. Potential problems we faced were length of this invasive surgery, hypothermia and fluid loss due to extravasation. The length of the surgery was kept at roughly under 75 min and core body temperature was maintained at 37°C using a homoeothermic blanket system. However, extravasation could have still been an issue in our optimised protocol. Our optimised protocol induced appropriate pressure ramps, as demonstrated in Figure 4.3 A and 4.8 A. The GFR measurements in this chapter were in line with renal clearance measurements previously carried out on C57BL/6JCrI mice¹²⁸. However, both urine flow rate and urinary sodium excretion have been reported higher in renal clearance¹²⁸ and PN studies^{195,196}. This could be due to extravasation in our mice. To investigate this, we carried out a study to include 2% bovine serum albumin (BSA) in the infusate. Furthermore, increasing the infusion rate to 0.35 mL/10g bw/h. This is to ensure the replacement of fluid loss while, itself, inducing natriuresis. This was completed by Mr Kevin Stewart in a small cohort of C57BL/6JCrI mice (Figure 4.26). Preliminary data indicated that my approach to assessing PN was not fully optimised. Unfortunately, due to COVID-19, the experimental groups were unable to be completed within the timeframe of my PhD.

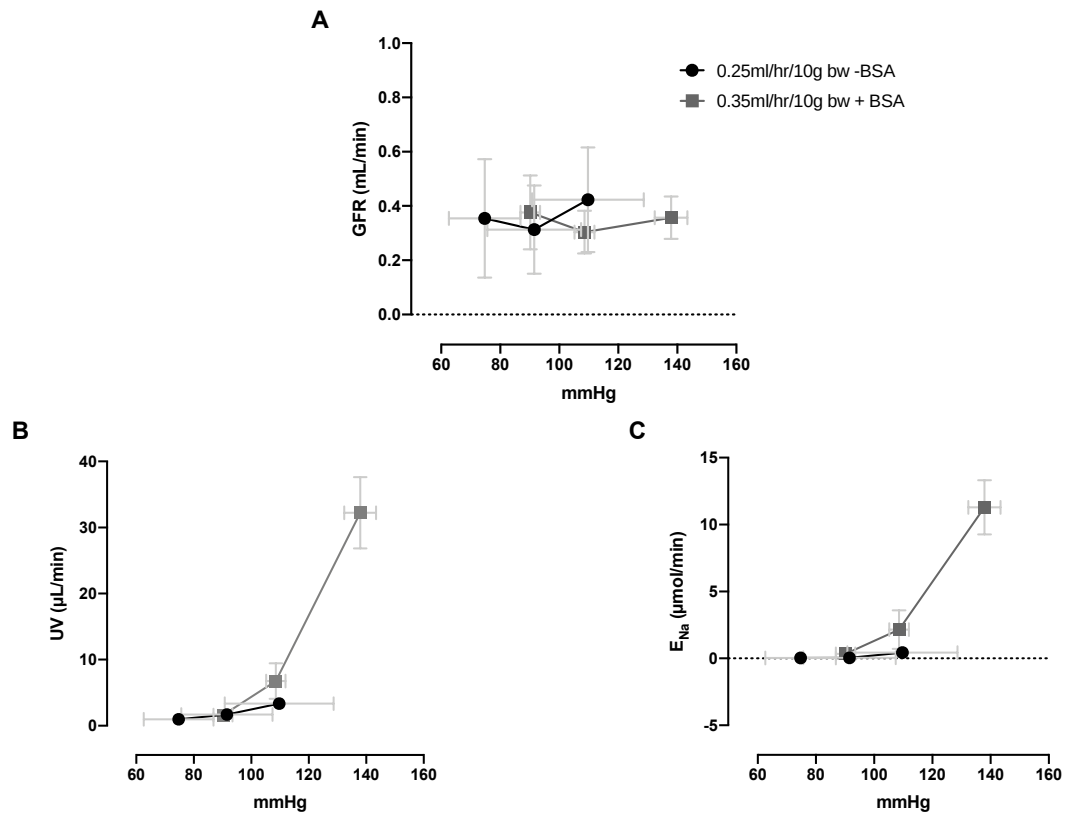


Figure 4.26: Comparison of pressure natriuresis approaches.

Preliminary data from Mr Kevin Stewart of the relationship between (A) glomerular filtration rate (GFR), (B) urine flow rate (UV) and (C) sodium excretion (E_{Na}) and systolic blood pressure following acute pressure natriuresis (PN) surgery in adult male C57BL/6J mice with infusion rate of 0.25 mL/10g bw/h without 2% bovine serum albumin (BSA) (black circles, $n = 4$) or with infusion rate of 0.35 mL/10g bw/h with 2% BSA (grey squares, $n = 4$). Each point for each group represents baseline, mesenteric and coeliac ligation (Lig 1) and distal aorta ligation (Lig 2), respectively. Data are group mean $\pm$ SD.

4.4.2 Renal vascular dysfunction in chronic glucocorticoid excess

4.4.2.1 Compromised renal autoregulation

Variable results have been reported for the effect of chronic glucocorticoid excess on both RBF and RVR^{75,125,126,128,197}. In our model, RBF was significantly increased, with decreased RVR.

Renal autoregulation is a key component for maintenance of renal function as it acts to stabilise RBF and GFR during changes in renal perfusion pressure. Changes in renal perfusion pressure often results in no change in steady-state whole kidney RBF, GFR and efferent arteriolar blood flow^{198,199}. However,

renal autoregulation requires RVR to change in proportion to renal perfusion pressure. Renal autoregulation is maintained by myogenic responses and the macula densa tubuloglomerular feedback response to regulate preglomerular VSM tone. Attenuated renal autoregulation contributes to renal damage in various renal and hypertensive models. Although, the mechanisms behind this are poorly understood¹⁹⁹.

Renal autoregulation is compromised in glucocorticoid excess as changes in renal perfusion pressure caused increases in RBF, with no changes in RVR. However, GFR remained stable. The tone of afferent arterioles are predominately responsible for pressure-induced autoregulation of RBF and GFR. Whereas the efferent arterioles are not involved in RBF autoregulation. Although, the efferent arterioles have been proposed to contribute to GFR autoregulation in conditions of low renal perfusion pressure, which in our model is not the case¹⁹⁹. A potential explanation of this phenomenon is intra-renal shunting, which diverts blood away from the glomerulus at low RVR (Figure 4.27)²⁰⁰. This could be a protective mechanism due to the increase in RBF. More work will need to be done to understand the impact of increased RBF, with no change in GFR, to BP. However, this is a challenging concept to investigate further.

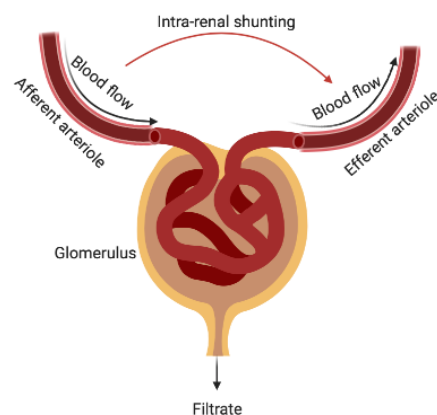


Figure 4.27: Schematic representation of proposed intra-renal shunting.
Diagram created with BioRender.com.

The “vasodysfunction” model, proposed by Morris et al supports the role of the vasculature in the induction of salt-induced hypertension. They proposed that

initiation of salt-induced rises in BP is due to subnormal decreases in TPR, involving subnormal decreases/failure to lower RVR. This was based on studies showing that in salt-sensitive individuals, they predominately were unable to reduce vascular resistance to the same extent as in salt-resistant individuals following salt loading. Interestingly in these patients, salt loading did not induce increases in sodium retention greater than that of salt-resistant subjects⁷⁰. Following chronic glucocorticoid excess, RVR was significantly decreased at baseline compared with controls. When ACTH-treated mice were faced with a high salt challenge, RVR remained stable. Therefore, reflecting a failure to lower RVR proposed by Morris et al. However, whether this contributes to salt-induced rises in BP is unknown. Overall, these findings suggest a dysregulation of renal haemodynamics in chronic glucocorticoid excess. However, whether that contributes to the amplification of salt-sensitivity remains elusive.

4.4.2.2 Reduced renal vascular reactivity

Studies have supported that glucocorticoids regulate vascular reactivity by acting on both endothelial and VSM cells, with both of its receptors (GR and MR) being present^{116,117,201}. There is a vast literature supporting the potentiation of vasoconstrictor action and loss of endothelium-mediated relaxation by chronic glucocorticoid excess^{118,119}. Although, there is limited studies that have investigated the vascular reactivity of the renal artery. Vascular reactivity was assessed in renal arteries to investigate the mechanisms behind the changes in renal autoregulation. Furthermore, mesenteric arteries were also investigated as mesenteric arteries contribute in peripheral vascular resistance and BP regulation²⁰².

PE acts on α_1 receptors on the VSM to cause vasoconstriction²⁰³ (Figure 4.28). The responsiveness of the renal artery to the vasoconstrictor PE was significantly reduced in ACTH-treated mice, with no change in the responsiveness of the mesenteric artery to PE. The response by the mesenteric artery is in line with a study that showed aortic rings and carotid

arteries in dexamethasone-treated rats responded similarly to control rats²⁰⁴. Although, there appears to be limited studies investigating renal arteries. This reduction in responsiveness is conflicting with many studies that suggest glucocorticoids potentiate the action of vasoconstrictors. However, most studies have investigated this using norepinephrine and angiotensin II in mesenteric arteries²⁰⁵, but not renal arteries.

This reduced PE response is suggested to be receptor-mediated in renal arteries as the vessels depolarised to a similar extent as control. The mRNA expression of α_{1a} receptor, which is present on the VSM, was similar between groups. However, binding affinity has not been measured and there are more subtypes (α_{1b} and α_{1d})²⁰⁶ which could be investigated. With increases in plasma copeptin, indicative of AVP, in ACTH-treated mice, the response of vessels to AVP (a vasoconstrictor) was assessed by a MSc student in the lab under my supervision. No changes in the response to AVP was seen in renal and mesenteric arteries and there were no changes in its receptor ($V_{1a}R$) gene expression in these arteries (data not shown). Further studies involving different vasoconstrictors (endothelin-1, serotonin, norepinephrine and angiotensin II) would be necessary to investigate the effect of glucocorticoid excess on vasoconstrictor action in both the renal and mesenteric artery of ACTH-treated mice.

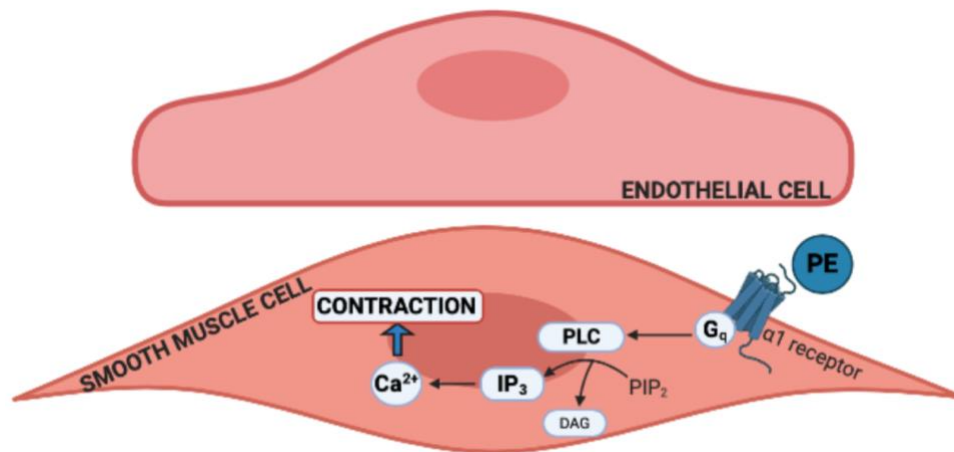


Figure 4.28: Schematic depicting the mechanisms of vasoconstriction within the vasculature in response to phenylephrine.

Signalling pathway of the endothelium-independent vasoconstrictor phenylephrine (PE) which acts through the alpha-1 (α_1) receptor on the vascular smooth muscle (VSM) cell, triggering a signalling cascade to increase intracellular calcium causing contraction. PLC, phospholipase C; PIP_2 , phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP_3 , inositol trisphosphate. Diagram created with BioRender.com.

To investigate vasodilation, the use of endothelium-dependent and -independent vasodilators in wire myography has allowed for distinguishing between endothelial and/or VSM impairment. Dysfunction in vasodilation is usually characterised by reduced NO bioavailability²⁰⁷. NO is synthesised by the endothelium, by eNOS, and acts on the VSM to cause relaxation (Figure 4.29). Therefore, if impaired, responses to endothelium-dependent vasodilators, such as ACh, are observed, and endothelium-independent vasodilator (SNP) can be used to determine if the impairment is localised to the endothelium or not.

There is a plethora of data to support that glucocorticoids reduce endothelium-dependent vasodilation, via inhibitory effects on eNOS synthesis^{120,121,208}. Specifically, studies have shown that administration of oral cortisol over 5 days in healthy normotensive men showed impairment of ACh-induced vasodilation, with no change in SNP response, in the forearm²⁰⁹. Similar findings were seen in dexamethasone-induced hypertensive rats where responses to ACh were impaired in aortic rings and resistance arterioles (using intravital microscopy

of the skin muscle), with no alteration of the SNP response, suggesting abnormal eNOS synthesis and no effects downstream of NO²¹⁰. It is thought that the VSM NO signalling pathway may compensate the glucocorticoid-induced low NOS activity in the endothelium¹¹⁸. To the best of my knowledge, the effect of glucocorticoid excess on mesenteric and renal vascular reactivity is limited. In our model of chronic glucocorticoid excess, there was a small reduction in responsiveness to endothelium-dependent ACh in renal arteries, but not in mesenteric arteries, with no differences in artery eNOS expression and plasma NO following ACTH treatment. However, eNOS in PVAT has been shown to play an important role in vasodilation²¹¹. PVAT was removed for wire myography measurements and for mRNA quantification. This could be an area to investigate in future work. Overall, there was no strong evidence for impaired endothelium-dependent relaxation in ACTH-treated mice.

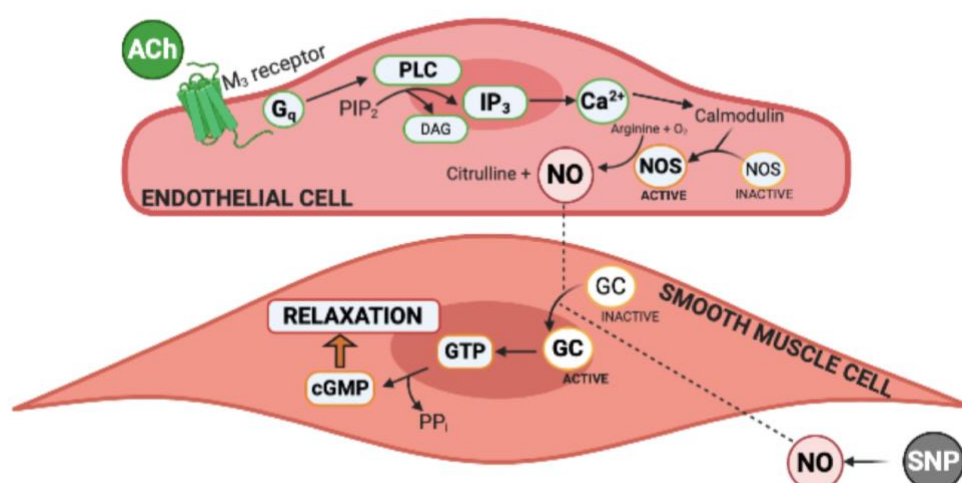


Figure 4.29: Schematic depicting the mechanisms of vasodilation within the vasculature in response to acetylcholine and sodium nitroprusside.

Signalling pathway of the endothelium-dependent vasodilator acetylcholine (ACh) which acts through the muscarinic 3 (M₃) receptor on the endothelial cell, triggering a signalling cascade to increase intracellular calcium causing nitric oxide (NO) synthesis. NO acts on the vascular smooth muscle (VSM) cell to cause relaxation. Signalling pathway of the endothelium-independent vasodilator sodium nitroprusside (SNP) which acts as a NO donor to cause relaxation. PLC, phospholipase C; PIP₂, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP₃, inositol trisphosphate; NOS, nitric oxide synthase; GC, guanylyl cyclase; GTP, guanosine-5'-triphosphate; cGMP, cyclic guanosine monophosphate. Diagram created with BioRender.com.

There was a significant reduction in maximal relaxation and sensitivity to endothelium-independent vasodilator SNP in renal arteries of ACTH-treated mice. This was only seen in the renal artery and not in the mesenteric artery of ACTH-treated mice. Although, following longer duration of ACTH (28 days), the sensitivity to SNP was significantly reduced, similar to the response to ACh. This suggests a VSM phenotype, especially since the response of PE-induced vasoconstriction was also reduced. NO induces generation of cGMP which activates cGMP-dependent protein kinase (PKG-I), which mediates vascular tone by inducing relaxation of VSM cells (Figure 4.29). Suppression of PKG-I (protein and/or mRNA) has been linked with many CVDs. Although, the mechanisms behind this decrease are not fully understood²¹². This was the rationale behind assessing the mRNA expression of PKG-I (*Prkg1*), although there was no changes seen between ACTH and saline-treated mice. Future work is necessary for understanding the mechanisms of this VSM phenotype in chronic glucocorticoid excess in different vascular beds.

4.4.2.4 Measurement of myogenic tone

The assessment of vascular reactivity by wire myography is important for exploring intrinsic responses to pharmacological stimuli. However, exploring intrinsic responses to physiological stimuli can only be measured using pressure myography. Pressure myography is an alternative method to wire myography which has improved sensitivity. Furthermore, this technique is reliable for measurement of myogenic tone in small resistance vessels at near physiological conditions²¹³. Different vascular beds have different myogenic responses – renal and cerebral arteries have a greater response to changes in transmural pressure compared with mesenteric arteries¹⁹⁹. Therefore, in future studies, autoregulatory vascular beds, including cerebral arteries, will be investigated using pressure myography to assess whether the myogenic tone of these arteries are affected by chronic glucocorticoid excess.

4.4.3 Potential mechanisms behind glucocorticoid-induced alterations in renal vascular function

4.4.4.1 MR and abnormal 11 β -HSD gene expression

It is clear that glucocorticoid excess has pathophysiological implications on the function of the renal artery. However, is this a direct effect of glucocorticoids on the renal artery itself? Glucocorticoid-related genes were assessed in both renal and mesenteric arteries of ACTH-treated and control mice by qPCR. A disadvantage of this method is that changes in expression of genes could not be differentiated between VSM cells and endothelial cells. Changes in *Fkbp5* mRNA expression were seen in both arteries, which is indicative of changes in cellular glucocorticoid activity. This is due to *Fkbp5* rapid transcription following glucocorticoids activating GR^{97,214}. FKBP5 can also act as an inhibitor of GR function as it can reduce ligand binding and delay the nuclear translocation of GR therefore, decreasing GR-dependent transcription⁹⁷. Decreases in GR transcription has been shown in renal arteries, suggested to be due to increased GR activation. MR mRNA was not altered in renal arteries. However, there was a significant increase in 11 β -HSD2 gene expression, suggesting protection of MR from glucocorticoids but not mineralocorticoids. Although, this may be counteracted by increases in gene expression of 11 β -HSD1. This could have detrimental effects on the function of the renal artery as abnormal 11 β -HSD expression has been suggested to play a pathogenic role in vascular dysfunction¹¹⁸.

Preliminary data suggests a role of MR (endothelial and/or VSM) in contributing to renal vascular dysfunction. Vascular MR activation has been shown to contribute to BP control and vascular dysfunction and remodelling in response to hypertension^{215–218}. Future experiments are necessary to determine whether glucocorticoids are predominately acting via MR to contribute to the renal vascular dysfunction, and in turn salt-sensitive hypertension, seen in glucocorticoid excess. This is explored in Chapter 5. Furthermore, the role of intravascular 11 β -HSDs, specifically in the renal artery of ACTH-treated mice, needs to be addressed. Dysfunction in vasodilatory

responses in the mesenteric artery were shown following a longer duration of ACTH (28 days). Therefore, it would also be of interest to investigate if the changes in GR/MR balance and intravascular 11 β -HSDs were also seen in mesenteric arteries following 28 days ACTH.

4.4.3.2 Vascular remodelling

Elastin and emilin1 mRNA was significantly reduced with an increase in *Tgf- β* in the renal arteries of ACTH-treated mice, which was not seen in mesenteric arteries.

Elastin insufficiency can cause vascular stiffness²¹⁹. Glucocorticoids have been shown to reduce elastin synthase in the skin²²⁰. However, there is limited information on the effect of glucocorticoids on elastin at a vascular level. Emilin-1 has been shown to be associated with elastin in the extracellular matrix as it has a role in elastogenesis, maintenance of vascular cell morphology and VSM cell adhesion to elastic fibres²²¹. Emilin-1 is an elastic extracellular matrix protein which is expressed in endothelial cells, VSM cells, endocardium and right ventricle myocytes. Emilin-1, specifically in VSM cells and not in endothelial cells, have been shown to be involved in regulation of systemic BP and regulates myogenic response by inhibiting TGF- β biosynthesis. Loss of Emilin-1 in the VSM increases vascular TGF- β signalling, leading to rises in BP with structural changes of elastic arteries including VSM cell growth and vascular remodelling^{222,223}. This is suggested to be due to increases in TGF- β signalling in the VSM stimulating heparin-binding epidermal growth factor to activate its receptor - epidermal growth factor receptor. This then signals the recruitment of transient receptor potential channels to activate voltage-dependent calcium channels to potentiate myogenic tone²²³. This could be a potential mechanism for renal vascular dysfunction in our model of glucocorticoid excess. However, only preliminary data have been obtained and future work will be needed to explore this further including firstly measuring the myogenic tone of the arteries of ACTH-treated mice and stain for extracellular proteins in arteries including elastin.

Furthermore, future work into the potential association between glucocorticoids and emilin-1 should be explored.

4.4.4 Summary of chapter findings

- The acute PN response was unaltered with glucocorticoid excess before and after a high salt challenge. However, future studies need to include BSA to reduce fluid extravasation.
- RBF was significantly higher following glucocorticoid excess. RBF increased following pressure ramps, suggesting impaired renal autoregulation.
- Renal artery reactivity was significantly reduced with glucocorticoid excess, with no change in mesenteric artery reactivity.
- Renal artery reactivity did not differ with a longer duration of glucocorticoid excess. There was decreased sensitivity to endothelium-dependent and -independent vasodilation, with a longer duration of glucocorticoid excess in mesenteric artery reactivity.
- Renal and mesenteric artery reactivity did not differ comparing glucocorticoid excess before and after a high salt challenge.
- Chronic glucocorticoid excess decreased GR mRNA with no change in MR mRNA in the renal artery, suggesting that glucocorticoids are primarily acting via MR in the renal artery.
- Chronic glucocorticoid excess decreased elastin mRNA in the renal artery.

**Chapter 5: Mineralocorticoid
receptor antagonism improved
renal vascular function in
glucocorticoid excess, independent
of blood pressure changes**

5.1 Introduction

In Chapter 4, it was suggested that MR could contribute to the alterations in renal vascular dysfunction in this model of ACTH-dependent Cushing's syndrome, as MR mRNA was not altered unlike GR mRNA which was downregulated in the renal artery. MR could be activated by either corticosterone or aldosterone in this setting as both were elevated. Expression of renal artery 11 β -HSD2 was increased, protecting MR from activation from corticosterone. However, saturation of 11 β -HSD2 cannot be discounted. Furthermore, pathophysiological activation of MR by glucocorticoids has previously been found in rodent models of salt-sensitive hypertension^{128,224}. I hypothesise that overactivation of MR contributed to salt-sensitive hypertension and vascular abnormalities. Therefore, the objective of this chapter was to determine the impact of MR antagonism, using spironolactone, on BP homeostasis and renal vascular reactivity in glucocorticoid excess following a high salt challenge.

5.2 Methods

Details of each procedure used in this chapter are outlined in the main methods section (Chapter 2). The longitudinal study in this chapter was carried out on adult male C57BL/6JCrI mice, aged 8-10 weeks, with implanted minipumps releasing 2.5 µg/day of ACTH and given *ad libitum* access to control salt diet (0.3% Na⁺ by weight) or high salt diet (3% Na⁺ by weight) and water (see Materials and methods 2.2). The design of this experiment is described below.

5.2.1 Experimental design

Single-housed adult male mice ($n = 8$) were implanted with radio telemetry devices (see Materials and methods 2.3). Following a recovery period, SBP and DBP were recorded over a 3-day period, where mice were given *ad libitum* access to control diet and water. An Alzet model 2006 minipump was implanted into mice for a continuous infusion of ACTH over 41 days. BP was not recorded on the day of implantation as mice were allowed time to recovery from the anaesthesia. Following 21 days of ACTH treatment, mice were placed on a high salt diet for 14 days before administration of a daily dose of spironolactone (20 mg/kg/day in jelly²²⁵) for 6 days (Figure 5.1). To ensure the consumption of the jelly, mice were acclimatised to the jelly prior to the study commencing.

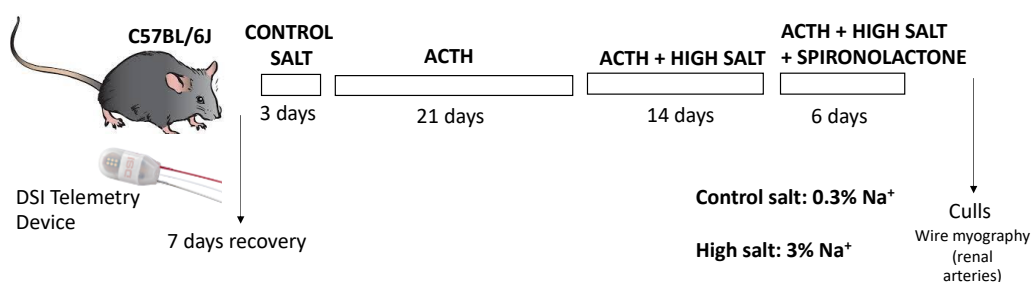


Figure 5.1: Timeline of the experimental design of spironolactone telemetry study.

Adult male C57BL/6J mice ($n = 8$) were implanted with radio telemetry devices, with a 7-day recovery period. Blood pressure (BP) recordings were collected in these mice on a control salt diet (0.3% Na⁺ by weight) for 3 days before implantation of minipumps releasing 2.5 µg/day ACTH over 41 days. Following 21 days of infusion, mice were then placed on a high salt diet (3% Na⁺ by weight) for 20 days. Spironolactone (20 mg/kg/day in jelly) was administered daily for the remaining 6 days. Mice were then culled, renal arteries dissected, and vascular reactivity assessed by wire myograph.

At the end of the study, mice were culled between 7-8 AM, via decapitation and renal arteries were dissected and mounted on a wire myograph (see Materials and methods 2.8). As a control group for this part of the experiment, a separate group of mice received ACTH and high salt as per the protocol but were not treated with spironolactone. KPSS was used as a depolarising contraction. Vascular reactivity was tested by generating cumulative concentration (1nM to 30 μ M) response curves to the vasoconstrictor PE and vasodilators ACh and SNP.

5.3 Results

5.3.1 Mineralocorticoid antagonism had no effect on blood pressure

The first part of the longitudinal study demonstrated that a prolonged ACTH duration (21 days) did not alter BP compared with 14 days ACTH, evaluated in Chapter 3. In this chapter, the primary focus was to evaluate whether spironolactone (spiro) had an effect on BP and diurnal variation that were previously shown to be altered by ACTH + high salt.

Figure 5.2 gives an overview of the entire experiment, plotting 24 h average SBP and DBP in conscious unrestrained mice. After baseline measurements of a control salt diet, averaged over 3 days, SBP was 123.0 ± 4.3 mmHg and DBP was 87.1 ± 2.7 mmHg. All mice were implanted with minipumps containing ACTH. After 21 days of ACTH infusion, SBP was 130.7 ± 5.2 mmHg and DBP was 90.0 ± 4.9 mmHg, as previously mentioned in Chapter 3. Adding high salt as an additional challenge increased SBP to 140.2 ± 4.0 mmHg and DBP to 97.1 ± 4.6 mmHg. Mice were then treated with spiro for 6 days and BP remained similar with SBP at 143.1 ± 5.5 mmHg ($p = 0.192$) and DBP at 98.6 ± 7.6 mmHg ($p = 0.539$).

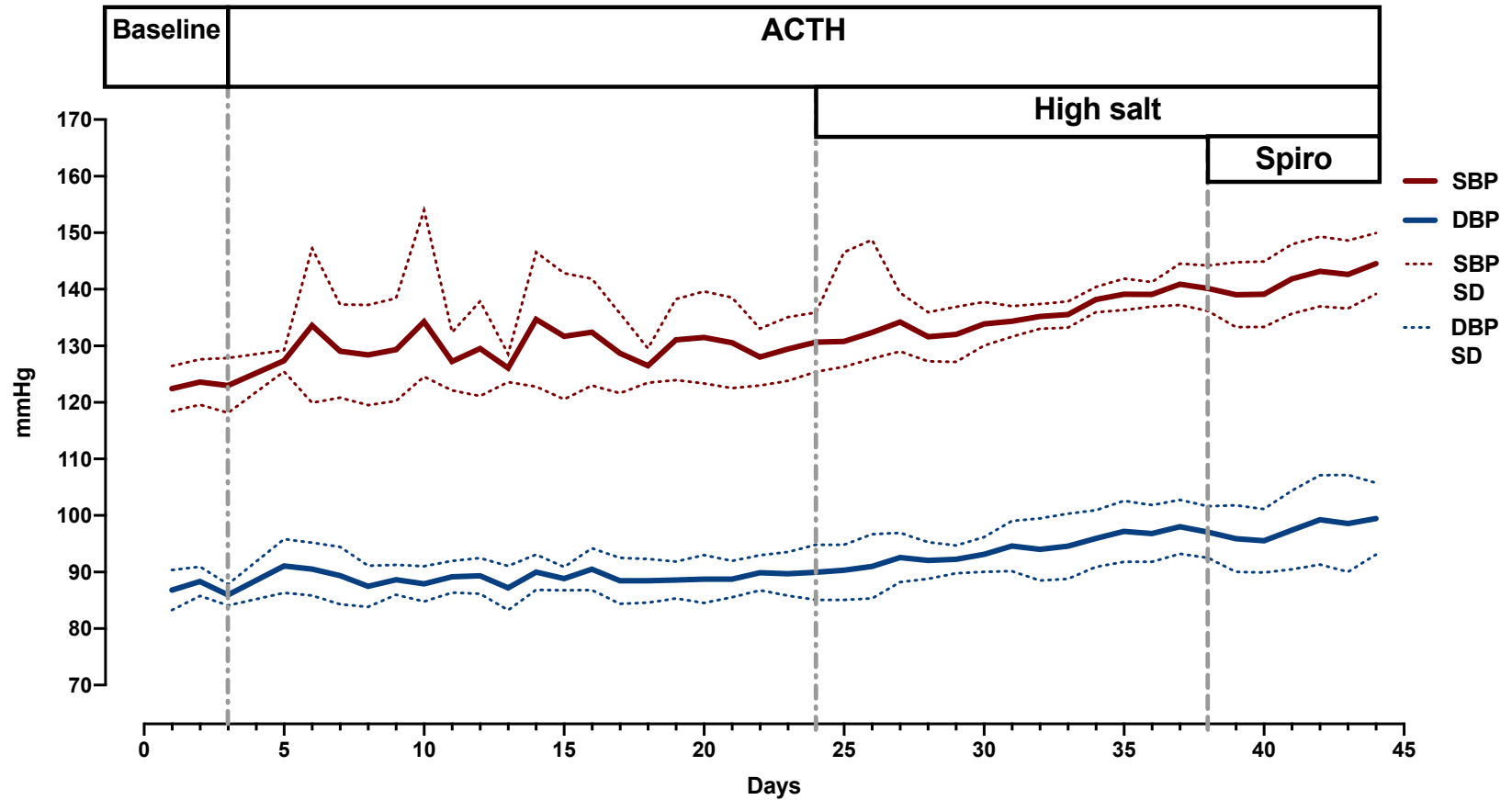


Figure 5.2: Systolic and diastolic blood pressure after treatment of spironolactone following ACTH infusion and high salt. Averaged 24 h systolic blood pressure (SBP; red line) and diastolic blood pressure (DBP; blue line), measured by radio telemetry, of male C57BL/6J mice ($n = 4$). Data were acquired every 20 min over a longitudinal protocol (Figure 5.1) incorporating control diet (0.3% Na⁺) baseline, treatment with ACTH for 41 days with the latter 20 days on high salt diet. The remaining 6 days mice were treated with spironolactone (Spiro; 20 mg/kg/day in jelly). Data are presented as group mean (solid line) $\pm$ SD (dashed lines).

Night:day variation of SBP throughout the protocol is illustrated in Figure 5.3 A. Similar to the previous study, mice following ACTH + high salt treatment displayed reduced night-day SBP variation (3.9 ± 2.5 mmHg). The SBP change was not different following spiro treatment (4.9 ± 4.8 mmHg). This was also seen during the last 24 h cycle in both treatments (Table 5.1). To further explore the daily rhythms of SBP, data were smoothed using a 5 h rolling average and aligned into 3-day bins (Figure 5.3 B) for cosinor analysis (Table 5.2).

Table 5.1: Night:day systolic blood pressure variation following spironolactone. Data shown as mean $\pm$ SD ($n = 4$) and comparisons made by paired t-test.

<i>SBP</i>	ACTH + high salt	Spiro
<i>Active or night (mmHg)</i>	142.4 $\pm$ 2.7	147.5 $\pm$ 6.3
<i>Inactive or day (mmHg)</i>	138.0 $\pm$ 5.5	141.6 $\pm$ 6.0
<i>Night-day variation (mmHg)</i>	4.4 $\pm$ 3.3	5.9 $\pm$ 5.9
<i>% Dip</i>	3.1%	3.9%

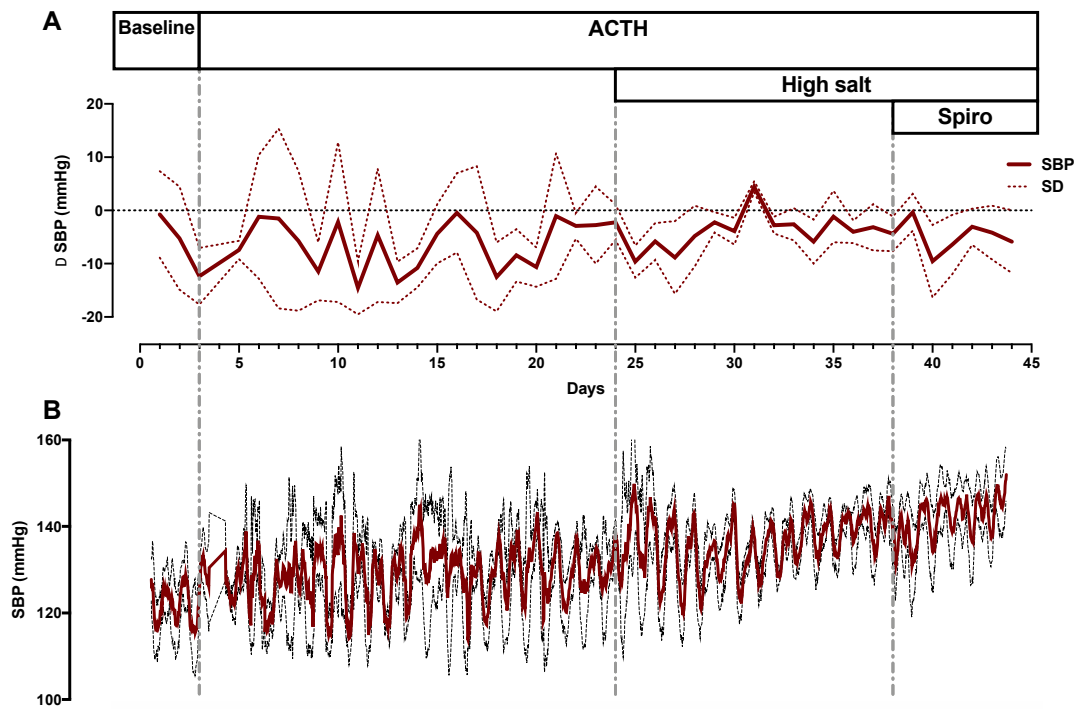


Figure 5.3: Night:day systolic blood pressure variation following spironolactone.

(A) Night:day systolic blood pressure (SBP) variation and (B) 5 h rolling averages of SBP from male C57BL/6J mice ($n = 4$) over a longitudinal protocol incorporating control diet (0.3% Na^+) baseline, treatment with ACTH for 41 days with the latter 20 days on high salt diet. The remaining 6 days mice were treated with spironolactone (Spiro; 20 mg/kg/day in jelly). Data are presented as group mean (solid line) $\pm$ SD (dashed lines).

Cosinor analysis for SBP was performed for each treatment. Spiro did not change period, acrophase (time of the peak of the rhythm), amplitude (difference between peak and MESOR), MESOR and robustness (strength of the rhythm). This level of robustness is still indicative of rhythm as levels above 20% are accepted as a signal¹⁴⁹.

Table 5.2: Cosinor analysis of systolic blood pressure (SBP) following spironolactone.

Data shown as mean $\pm$ SD ($n = 4$) and comparisons made by paired t-test.

<i>SBP</i>	ACTH + high salt	Spiro
<i>Period (hours)</i>	22.0 $\pm$ 3.5	15.1 $\pm$ 5.1
<i>Acrophase (ZT)</i>	06h50 $\pm$ 09h28	09h04 $\pm$ 08h38
<i>Amplitude (mmHg)</i>	4.4 $\pm$ 1.4	4.3 $\pm$ 2.2
<i>MESOR (mmHg)</i>	140.0 $\pm$ 3.2	143.6 $\pm$ 5.5
<i>Robustness (%)</i>	45.0 $\pm$ 14.6	48.0 $\pm$ 7.7

Night:day variation of DBP throughout the protocol is illustrated in Figure 5.4 A. During ACTH + high salt treatment, DBP change was 3.7 $\pm$ 2.3 mmHg. The DBP change was not different following spiro treatment (3.7 $\pm$ 3.7 mmHg). This was also seen during the last 24 h cycle in both treatments (Table 5.3). To further explore the daily rhythms of DBP, data were smoothed using a 5 h rolling average and aligned into 3-day bins (Figure 5.4 B) for cosinor analysis (Table 5.4). Spiro treatment did not influence period, acrophase, amplitude, MESOR or robustness.

Table 5.3: Night:day diastolic blood pressure variation following spironolactone.

Data shown as mean $\pm$ SD ($n = 4$) and comparisons made by paired t-test.

<i>DBP</i>	ACTH + high salt	Spiro
<i>Active or night (mmHg)</i>	99.1 $\pm$ 5.0	101.6 $\pm$ 4.7
<i>Inactive or day (mmHg)</i>	95.1 $\pm$ 4.6	97.2 $\pm$ 8.3
<i>Night-day variation (mmHg)</i>	4.0 $\pm$ 2.9	4.4 $\pm$ 4.6
<i>% Dip</i>	4.0%	4.5%

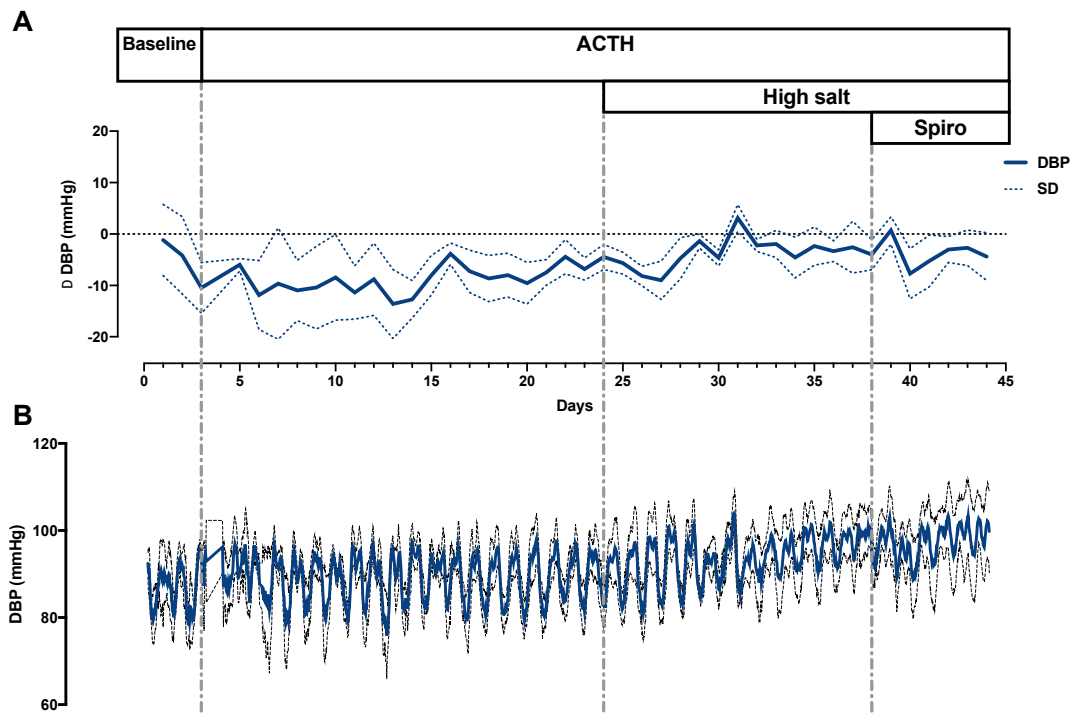


Figure 5.4: Night:day diastolic blood pressure variation following spironolactone.

(A) Night:day diastolic blood pressure (DBP) variation and (B) 5 h rolling averages of SBP from male C57BL/6J mice ($n = 4$) over a longitudinal protocol incorporating control diet (0.3% Na⁺) baseline, treatment with ACTH for 41 days with the latter 20 days on high salt diet. The remaining 6 days mice were treated with spironolactone (Spiro; 20 mg/kg/day in jelly). Data are presented as group mean (solid line) $\pm$ SD (dashed lines).

Table 5.4: Cosinor analysis of diastolic blood pressure (DBP) following spironolactone.

Data shown as mean $\pm$ SD ($n = 4$) and comparisons made by paired t-test.

<i>DBP</i>	ACTH + high salt	Spiro
<i>Period (hours)</i>	21.9 $\pm$ 3.7	15.1 $\pm$ 5.1
<i>Acrophase (ZT)</i>	07h04 $\pm$ 08h46	08h26 $\pm$ 08h34
<i>Amplitude (mmHg)</i>	3.6 $\pm$ 1.4	3.6 $\pm$ 1.3
<i>MESOR (mmHg)</i>	97.4 $\pm$ 4.7	99.2 $\pm$ 7.5
<i>Robustness (%)</i>	42.3 $\pm$ 15.7	50.6 $\pm$ 11.0

To test the impact of spiro treatment on activity, radio telemetry signal was used to calculate an activity count. Activity from the study above in Figure 5.2 was presented in Figure 5.5 A. The night:day variation of activity was calculated for each 24 h period throughout the protocol (Figure 5.5 B). During ACTH + high salt, the activity night:day variation was 30.1 ± 28.1 counts per min. This was reduced, but not significant, with spiro treatment (-3.1 ± 12.0 counts per min; $p = 0.08$). Table 3.11 illustrates the diurnal activity at the end of each period. There was a significant reduction in inactive or day activity following spiro treatment compared with ACTH + high salt.

Table 5.5: Night:day activity variation following spironolactone.

Data shown as mean $\pm$ SD ($n = 4$) and comparisons made by paired t-test.

<i>Activity</i>	ACTH + high salt	Spiro
<i>Active or night (counts per min)</i>	85.3 ± 39.7	53.6 ± 16.2
<i>Inactive or day (counts per min)</i>	59.6 ± 13.9	41.7 ± 15.4 ($p = 0.032$)
<i>Night-day variation (counts per min)</i>	25.7 ± 37.1	12.0 ± 20.4

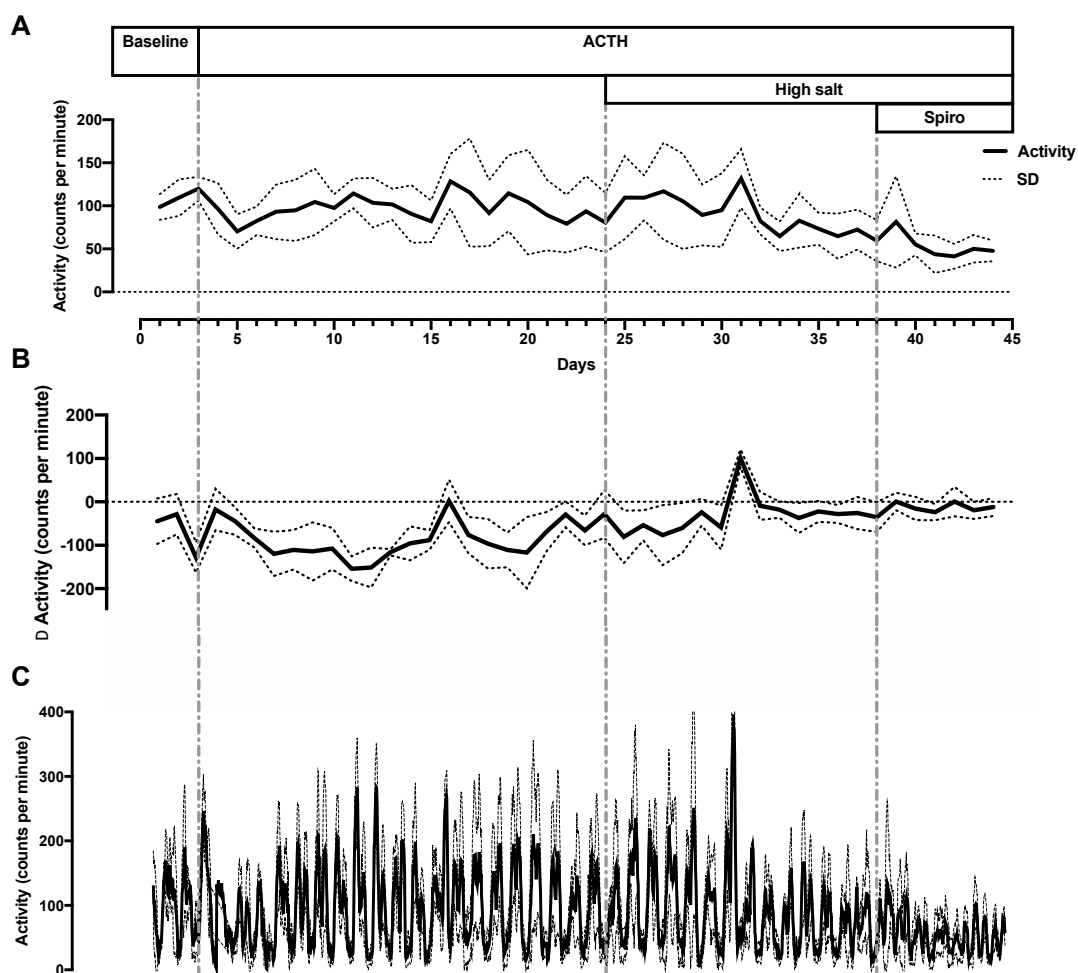


Figure 5.5: Night:day activity variation following spironolactone.

(A) Average 24 h activity, (B) night:day activity variation and (C) 5 h rolling averages of activity from male C57BL/6J mice ($n = 4$) over a longitudinal protocol incorporating control diet (0.3% Na^+) baseline, treatment with ACTH for 41 days with the latter 20 days on high salt diet. The remaining 6 days mice were treated with spironolactone (Spiro; 20 mg/kg/day in jelly). Data are presented as group mean (solid line) $\pm$ SD (dashed lines).

Activity data were smoothed using a 5 h rolling average and aligned into 3-day bins (Figure 5.5 C) for cosinor analysis (Table 5.6). Spiro did not influence period, acrophase, amplitude or MESOR. However, spiro did significantly increase robustness.

Table 5.6: Cosinor analysis of activity.

Data shown as mean $\pm$ SD ($n = 4$) and comparisons made by paired t-test.

<i>Activity</i>	ACTH + high salt	Spiro
<i>Period (hours)</i>	17.6 $\pm$ 6.8	14.9 $\pm$ 5.3
<i>Acrophase (ZT)</i>	14h34 $\pm$ 07h35	15h07 $\pm$ 04h56
<i>Amplitude (counts per min)</i>	39.2 $\pm$ 23.0	23.0 $\pm$ 11.8
<i>MESOR (counts per min)</i>	67.1 $\pm$ 25.2	69.6 $\pm$ 41.9
<i>Robustness (%)</i>	36.1 $\pm$ 9.4	50.7 $\pm$ 6.3 ($p = 0.049$)

5.3.2 Mineralocorticoid antagonism improved renal vascular dilatory reactivity

Concentration response curves for PE in renal arteries of ACTH + high salt mice (ACTH infusion for 28 days and fed a high salt diet for latter 14 days) or Spiro mice (following the telemetry study described above) is shown in Figure 5.6 A. Renal arteries from ACTH + high salt mice and Spiro mice had a similar mean KPSS and %KPSS. There was no change in logEC50 or %Emax in renal arteries between groups (Figure 5.6 B).

Concentration response curves to ACh in renal arteries of mice following Spiro treatment had improved maximum dilatory response of ACh (%Preconstriction), with no change in sensitivity (logIC50) to ACh in renal arteries between ACTH + high salt and Spiro groups (Figure 5.6 C, D).

Similar to the response to ACh, concentration response curves to SNP in renal arteries of mice following Spiro treatment had improved maximum dilatory response of SNP with no change in sensitivity to SNP in renal arteries between ACTH + high salt and Spiro groups (Figure 5.6 E, F).

The dilatory response to ACh following spiro treatment in renal arteries compared to control renal arteries were similar, showing complete rescue of function (control renal logIC50 = 7.27 $\pm$ 0.14, %Preconstriction = 5.42 $\pm$ 3.6).

The dilatory response to SNP following spiro treatment in renal arteries compared to control renal arteries was still reduced, suggesting a partial rescue of function (control renal $\log\text{IC}_{50} = 7.78 \pm 0.10$, $\%\text{Preconstriction} = -1.09 \pm 2.28$).

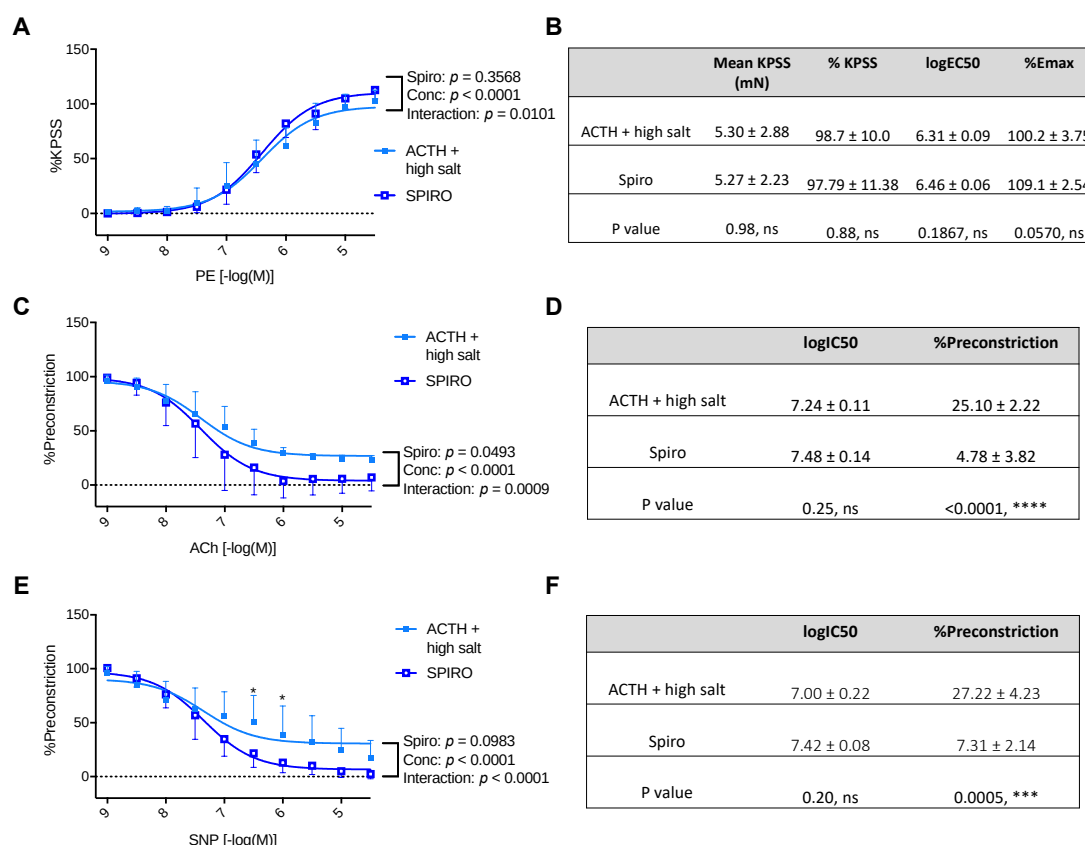


Figure 5.6: Vascular response to phenylephrine, acetylcholine and sodium nitroprusside in renal arteries following spironolactone.

Arteries were isolated at 7 AM from adult male C57BL/6J mice treated with ACTH for 28 days on high salt diet for the latter 14 days (3% Na^+ ; blue squares; $n = 8$) or ACTH for 21 days before a high salt challenge for 20 days with the remaining 6 days treated with spironolactone (Spiro; 20 mg/kg/day in jelly). (A) Concentration response curves to phenylephrine (PE) were obtained from renal arteries. Constriction was normalised to the maximum response induced by a high potassium physiological salt solution (KPSS) and concentration response curves generated by non-linear regression. Mean KPSS, %KPSS, $\log\text{EC}_{50}$ and %EMax were calculated for each concentration response curve for renal arteries. Concentration response curves to (C) acetylcholine (ACh) and (E) sodium nitroprusside (SNP). Dilation was normalised to a percentage of each vessels response to a half maximal concentration of PE (%Preconstriction) and concentration response curves shown as non-linear regression. $\log\text{IC}_{50}$ and %Preconstriction were calculated for each concentration response curve for ACh (D) and SNP (F). Data are group mean $\pm$ SD and were analysed by two-way ANOVA with repeated measures and Holm-Šidák's *post hoc* test. Conc, concentration. * $p < 0.05$.

5.4 Discussion

In this chapter, MR antagonism, via treatment with spironolactone, improved the maximal dilatory response for both endothelium-dependent and -independent vasodilation in renal arteries following glucocorticoid excess and a high salt challenge. This suggests glucocorticoid activation of MR contributes to renal vascular dysfunction. However, future work will be necessary to discriminate between effects of endothelial and VSM MR on vascular function. Moreover, MR blockade had no effect on BP over 6 days of treatment. Therefore, suggesting renal vasodilatory dysfunction does not contribute to salt-sensitive hypertension in our model of ACTH-dependent Cushing's syndrome.

5.4.1 Mineralocorticoid receptor activation in cardiovascular disease and hypertension

MR activation promotes renal sodium retention, hypokalaemia, SNS activation, and vascular damage and therefore, is implicated in CVD and hypertension²²⁶. Spironolactone and eplerenone are clinically approved MR antagonists that have proven to be beneficial in patients with heart failure. The Randomized Aldactone Evaluation Study (RALES) demonstrated remarkable effects of MR blockade by spironolactone which reduced mortality by 30% in patients with congestive heart failure²²⁷. The Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS) also showed reduced mortality with eplerenone treatment in patients who suffered from an acute myocardial infarction²²⁸. Alongside cardio-protective effects of MR blockade, it also has antihypertensive effects. Although, spironolactone's usefulness in treatment of hypertension was thought to be limited due to its progestational and antiandrogenic side effects and risk of hyperkalaemia²²⁹. That was until the PATHWAY-2 trial, which showed spironolactone to be the most effective BP lowering add-on (fourth-line) drug for the treatment of resistant hypertension²³⁰. Eplerenone is highly specific for MR, reducing the progestational and antiandrogenic side effects, and has also been shown to

be effective in lowering BP in hypertensive patients – although has been shown to not be as effective as spironolactone at the same dose^{231,232}.

In the RALES and EPHESUS trials, plasma aldosterone levels were not raised in patients^{228,233}. Furthermore, the antihypertensive effects of spironolactone occurred regardless of the aldosterone status²³⁴. This indicates that MR activation, rather than excess aldosterone, is critical in the pathogenesis of hypertension and CVD. Activation of MR can be increased without aldosterone excess through increased MR expression levels, increased MR sensitivity or MR stimulation by other factors such as glucocorticoids²³⁵. This is relevant in the context of Cushing's syndrome. Spironolactone is recommended to Cushing's patients to control hypokalaemia and used as an add on drug to lower BP^{229,236}.

5.4.2 Vascular mineralocorticoid receptor

It has emerged over the past two decades that MR has a role in modulating vascular function and there has been growing interest in the concept of MR activation contributing to CVD. MR in endothelial and VSM cells were first identified in the aorta and pulmonary arteries in rabbits in 1992²³⁷, before being confirmed in human arteries later in the decade^{238,239}. This led to the discovery of MR functioning as a transcriptional regulator of genes involved in vascular fibrosis, inflammation, calcification and oxidative stress²⁴⁰. Vascular MR, both in the endothelial and VSM cells, have also been shown to have a direct role in BP regulation. BP was seen to rise with impaired vascular reactivity in a mouse model of inducible overexpression of human MR in endothelial cells²⁴¹. In rodent models of hypertension, including angiotensin II-induced and DOCA/salt, endothelial MR has been shown to contribute to endothelial dysfunction and inflammation^{215,216}. Although, deletion of endothelial MR did not impact BP or vascular function in healthy mice²¹⁶. Regarding VSM MR, an inducible VSM cell MR knockout mouse was used to demonstrate its role in BP control, which has been suggested to contribute to BP via changes in vascular L-type calcium channel expression and function to alter

vasoconstriction²¹⁸. VSM MR has also been demonstrated to have a role in vascular stiffness, a sign of vascular remodelling, in response to hypertension²¹⁷. Therefore, endothelial and VSM MR blockade can be cardioprotective²⁴². This was supported by the RALES and EPHESUS trials, as the positive outcomes were suggested to be in part due to vascular MR antagonism^{228,233}.

5.4.3 Mineralocorticoid receptor antagonist selection

Spironolactone was discovered over 60 years ago and is orally available with a high potency but low selectivity for MR. Spironolactone is a competitive antagonist as it binds at the same site as aldosterone where it dissociates rapidly to destabilise the active conformation of MR. Spironolactone also has several active metabolites, including canrenone, resulting in a longer half-life of up to 17 h and requiring only a daily dose²⁴³. Spironolactone has a slow onset, with a peak response around 48 h following the first dose and up to 72 h for its active metabolites to reach steady state. The natriuretic effect of gradually declines during this period with the duration of spironolactone effect differing relative to natriuresis and antikaliuresis²⁴⁴. The maximal hypotensive effect of spironolactone has been reported to takes 3 weeks in hypertensive patients²⁴⁵. It can cause off-target side effects as it can also bind to progesterone and androgen receptors. Therefore, a more selective and equally or more potent MR antagonist was desirable. Eplerenone was discovered in 1987 and clinically approved in 2002 as a more selective MR antagonist. Although, it is less potent than spironolactone. Eplerenone has no active metabolites and has a shorter half-life of 4 h, therefore requiring dosing twice a day²⁴⁶.

Spironolactone was selected for these initial studies as dosing was only required once a day due to its longer half-life and I wanted minimal disruption during the telemetry recording. The daily dose of 20 mg/kg/day was selected as it was previously used in the laboratory and detailed in MacDonald et al²²⁵, although subcutaneously administered. Furthermore, it has been reported to

lower BP by several groups when administered orally at this dose^{247,248}. At this dose, BP was not lowered over a 6 day period. This may be due to the duration of the treatment. Previous studies using the same dose have treated both mice and rats for 8-12 weeks, substantially longer than the treatment duration carried out. Therefore, it is possible that the BP lowering effect would take longer than 6 days to be evident. Shorter treatment periods predominately treat mice with daily oral doses of 100 mg/kg/day of spironolactone. Therefore, for future studies, it would be worth investigating whether a longer duration/higher dose of spironolactone have BP-lowering effects in our model. Although, studies using oral doses of 100 mg/kg/day of spironolactone have not reported any off-target effects, caution will have to be taken regarding off-target effects as both progesterone and androgens have been shown to affect BP and vascular function^{249,250}.

Blockade of the androgen receptor is particularly relevant in this setting as androgen deprivation therapy has been shown to enhance endothelium-dependent vasodilation²⁵¹. Furthermore, ACTH stimulates the synthesis of adrenal androgens and in Cushing's patients the specific adrenal androgen DHEA sulfate is chronically increased in both males and females. However, in male patients the pituitary gonadotrophic function accounts for 90% of circulating androgens which is suppressed by cortisol. The excess adrenal androgens has been shown not to compensate for this loss and overall circulating androgens are abnormally low which clinically can lead to infertility^{252,253}. Therefore, in the setting of male ACTH-treated mice, it is unlikely androgens play a role in this phenotype.

5.4.4 Blood pressure-independent actions of spironolactone

With the effects of spironolactone treatment improving vascular function, independent of BP, this led to questioning the role of endothelial and VSM MR in renal vascular dysfunction in ACTH-treated mice. Previous studies (both human and animal) have shown limited BP lowering with spironolactone in ACTH/cortisol-induced hypertension. However, they have shown to reduce

serum potassium^{254–256}. This is important, especially for control of hypokalaemia in these patients. Furthermore, emerging data have indicated MR blockade reduces the risk of cardiovascular events via vascular protective effects, independent of BP effects^{257–259}. This was apparent in these data as spironolactone had no effect on BP but improved the maximal dilatory response for both endothelium-dependent and -independent vasodilation in renal arteries in ACTH-treated mice following high salt challenge. The response to ACh was completely rescued with spironolactone treatment compared with control arteries but only partially rescued SNP response in renal arteries (summarised in Table 5.7). However, the underlying mechanisms behind this remain unclear.

A potential mechanism behind this could be associated with arterial stiffness. Arterial stiffness causes endothelial cell and VSM cell dysregulation (previously shown in ACTH-treated mice by wire myography), VSM cell growth and migration within the media and changes in collagen:elastin ratio within the vascular wall. Spironolactone has been shown to improve endothelial function and reduce arterial stiffness. These benefits have been shown to be at least in part independent of BP lowering^{260,261}. To support this, studies have indicated that patients with primary aldosteronism develop increased arterial stiffness compared with hypertensive patients with similar BP^{262,263}. Elastin transcript was significantly reduced in renal arteries of ACTH-treated mice. Experiments were planned for assessment of the collagen:elastin ratio but unfortunately could not be completed due to COVID-19. Future work should be to focus on examining the arterial structure of these mice and if arterial stiffness is present, whether spironolactone reduces arterial stiffness.

Table 5.7: Summary of renal artery vascular response to phenylephrine, acetylcholine and sodium nitroprusside.
Data shown as mean + SD (*n* = 7-8).

	PE		ACh		SNP	
	LogEC50	%Emax	LogIC50	%Preconstriction	LogIC50	%Preconstriction
Control	6.66 ± 0.04	141.9 ± 2.26	7.27 ± 0.14	5.42 ± 3.60	7.78 ± 0.10	-1.09 ± 2.28
ACTH	5.81 ± 0.21	113.1 ± 11.60	6.55 ± 0.33	42.41 ± 7.05	6.55 ± 0.30	29.90 ± 7.23
ACTH + high salt	6.31 ± 0.09	100.2 ± 3.75	7.24 ± 0.11	25.10 ± 2.22	7.00 ± 0.22	27.22 ± 4.23
Spiro	6.46 ± 0.06	109.1 ± 2.54	7.48 ± 0.14	4.78 ± 3.82	7.42 ± 0.08	7.31 ± 2.14

5.4.5 Limitations of the experimental work

Unfortunately, there were several complications throughout this experiment meaning this experiment will have to be repeated. Firstly, at the start of the study the n number was 8. However, one mouse later died one week after the telemetry surgery. Furthermore, three of the telemetry devices were not working when in protocol, potentially due to misplacement of the catheter in the carotid artery or device failure. Nevertheless, this left the study at an n of 4 for BP measurements, underpowering the experiment. Secondly, for an extended duration of the ACTH + high salt period, a light remained on throughout the whole 24 h cycle. Therefore, the circadian rhythm of the mice would be disturbed as mice would continuously be in the light, acting as their inactive phase. The lights in the telemetry suite are on their own timer but unfortunately one of the lights was no longer controlled by the timer. This issue was recognised when analysing data, which is carried out day by day and a reduction in activity was observed. Furthermore, the BP was not increasing at the same rate as the previous experiment following a high salt diet. An electrician was required to fix the problem, so the expected duration of the lighting issue was 7 days, therefore lasting a significant portion of the ACTH + high salt treatment.

Environmental light is the main input that synchronises the master circadian clock in the hypothalamic SCN, which then communicates to the clocks within the brain and peripheral clocks to regulate sleep and food intake and glucose metabolism. In rodents, light during the dark phase (active phase, 7 PM – 7 AM) has been shown to increase sleep²⁶⁴ and decrease food intake²⁶⁵ within minutes, explaining the reduction in activity in our study. This then would impact the BP of the mice and potentially the vascular reactivity. Therefore, this highlights the necessity to repeat this experiment to validate the results acquired during the telemetry and myography experiments. For future telemetry experiments, a light sensor is now available and is remotely accessible to check to ensure the lights are on between 7 AM and 7 PM and lights off between 7 PM and 7 AM.

Regarding the myography experiment, data were obtained from mice that had undergone telemetry, which was not comparable to myography data from mice treated with ACTH and a high salt challenge. Furthermore, they had varying durations of ACTH as ACTH + high salt mice were treated with ACTH over 28 days and Spiro mice from the telemetry study were treated with ACTH over 41 days. In Chapter 4, there was no change in renal vascular reactivity when comparing different durations of ACTH infusion. However, only 14 and 28 days have been explored and the effect of longer durations of ACTH on vascular reactivity are uncertain. Nevertheless, spironolactone did show to improve the maximal dilatory response for both endothelium-dependent and -independent vasodilation in renal arteries following glucocorticoid excess and a high salt challenge to levels close to controls (Table 5.7). Therefore, these preliminary data suggesting the potential role of MR in renal vascular function in glucocorticoid excess needs to be validated with an appropriate control.

5.4.6 Summary of chapter findings

- MR antagonism had no impact on BP homeostasis following glucocorticoid excess and a high salt challenge. However, this study will need to be repeated due to lighting issues during the experiment.
- MR antagonism improved the maximal dilatory response for both endothelium-dependent and -independent vasodilation following glucocorticoid excess and a high salt challenge in renal arteries. However, this study will need to be repeated with an appropriate control.

Chapter 6: The effect of salt intake on plasma glucocorticoids and HPA axis function

6.1 Introduction

In Chapter 3, I presented data showing that 7 days high salt decreased plasma corticosterone (Figure 3.12). Previous studies in humans^{146,182} and mice¹⁴¹ indicate that increased dietary salt activates the HPA axis. This chapter aimed to resolve these apparent conflicts by systematic examination of plasma corticosterone concentration, and cellular glucocorticoid action over a 14 day high salt challenge. This is relevant as data from this thesis suggest that “HPA axis status” is important in determining the cardiovascular and renal response to salt. Furthermore, stress-induced production of cortisol is enhanced in salt-sensitive individuals⁷⁷, suggesting in humans that there is a connection between stress and salt-sensitivity.

The experiments were designed to investigate:

1. Plasma corticosterone before and over the course of a 14 day high salt challenge.
2. Whether high salt impacts steroidogenesis, cellular corticosterone action, corticosterone metabolism and/or negative feedback of corticosterone.
3. Whether high salt diet alters the response to restraint stress.

6.2 Methods

Procedural details are outlined in Chapter 2. Single-housed adult male C57BL/6JCrI mice, aged 8-10 weeks, were again used and given *ad libitum* access to control salt (0.3% Na⁺ by weight) or high salt diet (3% Na⁺ by weight) and water (see Materials and methods 2.2).

6.2.1 Timed plasma corticosterone sampling in high salt treated mice

To measure the effect of high salt on plasma corticosterone levels in mice, blood samples were collected between 7.15-8.00 AM and 6.30-7.15 PM by tail venesection and plasma corticosterone was quantified by an ELISA (see Material and methods 2.7). Samples were collected in the same mouse at the end of each treatment ($n = 8$). Therefore, an AM and PM sample was taken at the end of 7 days control salt, 7 days and 14 days high salt (Figure 6.1).

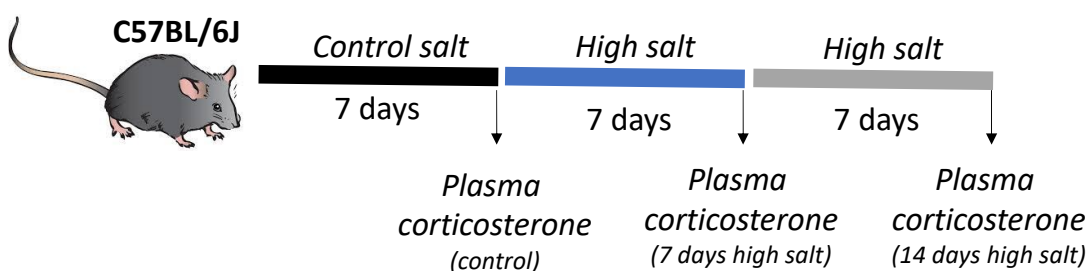


Figure 6.1: Plasma corticosterone sampling protocol.

Blood was collected from adult male C57BL/6J mice, using tail venesection, to measure plasma corticosterone levels. Samples were taken between 7.15-8 AM and 6.30-7.15 PM at the end of each treatment in the same mice ($n = 8$). Treatments included a control salt diet (0.3% Na⁺ by weight) for 7 days, a high salt diet (3% Na⁺ by weight) for 7 days then 14 days.

6.2.2 Timed dissections in control and high salt treated mice

To measure cellular corticosterone action in key tissues, a separate cohort of mice were culled between 7.15-8 AM, via decapitation, following either 7 days control salt, 7 days or 14 days high salt. Terminal blood was collected for measurement of CBG binding capacity (see Material and methods 2.7.7). Tissues, including right kidney cortex, right kidney medulla, right adrenal gland, liver, hypothalamus, hippocampus and anterior pituitary, were placed on dry

ice following dissection before storage at -80°C prior to RNA extraction (see Materials and methods 2.8).

6.2.3 Restraint stress test

To measure baseline plasma corticosterone levels, blood collection by tail nick technique was performed between 7.15-8 AM (the lowest plasma corticosterone level; $n = 24$). Blood was collected within one minute of disturbing each individual cage to ensure plasma corticosterone levels were as close to baseline as possible. After baseline blood sample collection, mice were transferred to a restraint tube where they were held for 15 min. After 15 min, another blood collection was taken using the same tail nick wound in order to measure stress levels of plasma corticosterone. The mice were returned to their individual cages and allowed to recover in a different holding room for either 30 min ($n = 8$), 60 min ($n = 8$) or 90 min ($n = 8$), after which a final blood sample was collected (recovery plasma corticosterone). The mice were then moved on to a high salt diet for 7 or 14 days, after which the stress test experiments were repeated as described above. A schematic diagram of the protocol design is illustrated in Figure 6.2. Plasma corticosterone in samples collected were quantified by an ELISA (see Material and methods 2.7).

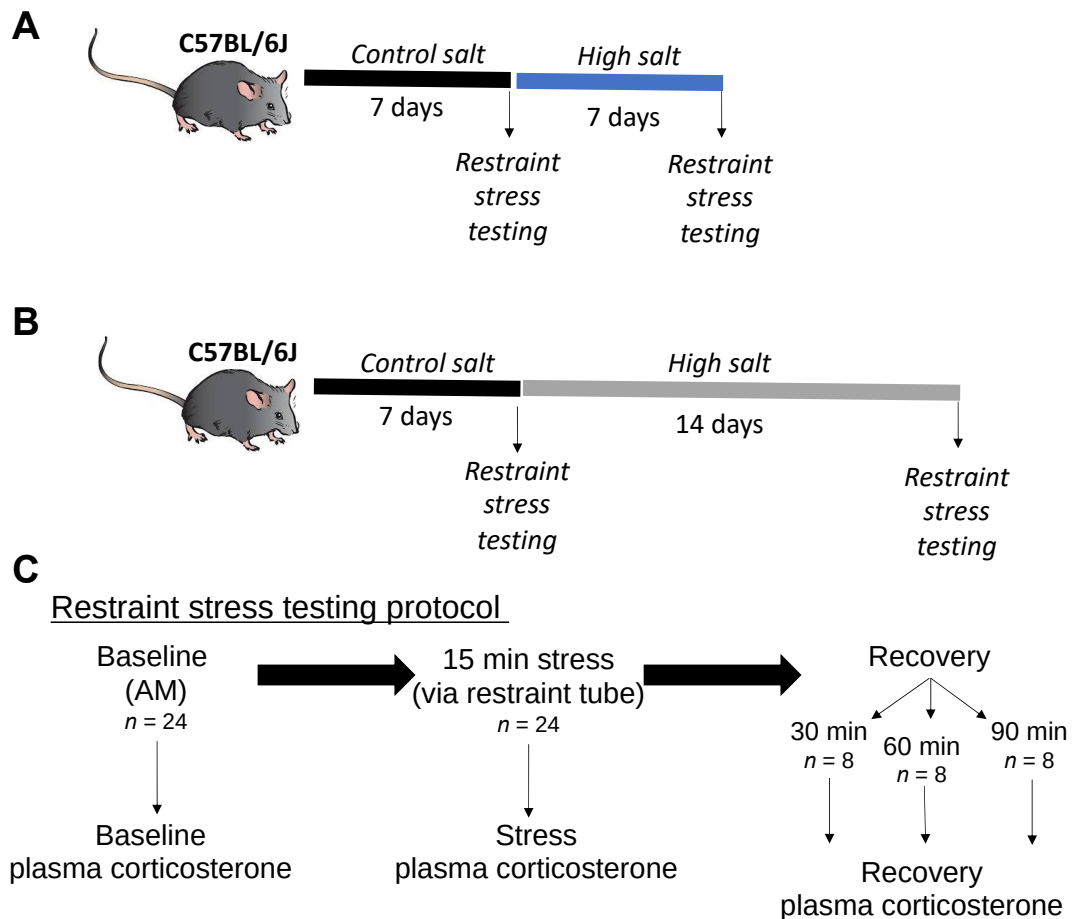


Figure 6.2: Timeline of the experimental design of restraint stress test study.

A restraint stress test was carried out on adult male C57BL/6J mice on a control salt diet (0.3% Na⁺ by weight) and following a high salt diet (3% Na⁺ by weight) for either 7 days (A) or 14 days (B). (C) The restraint stress test protocol involved collecting blood samples, using tail venesection, to measure plasma corticosterone levels at baseline (collected between 7.15-8 AM) and following a 15-min stress via a restraint tube ($n = 24$). Mice were split into 3 groups and plasma corticosterone levels were measured following recovery of either 30 min, 60 min or 90 min ($n = 8$ each group).

6.3 Results

6.3.1. Salt-induced a biphasic response in total plasma corticosterone levels

Figure 6.3 A tracks plasma corticosterone concentration before, during and after exposure to 14 days high salt diet. After 7 days high salt, there was a reduction in plasma corticosterone followed by a significant increase compared to both control and 7 days high salt. Although, diurnal variation was preserved (ANOVA main effect of diurnal variation $p < 0.0001$; ANOVA main effect of salt $p < 0.0001$; ANOVA interaction of diurnal variation and salt $p < 0.0001$). Plasma corticosterone (AM) was measured as part of the restraint stress testing protocol in a separate cohort of mice before and after either 7 days or 14 days high salt. Plasma corticosterone followed a similar pattern with decreasing following 7 days high salt compared with control (Figure 6.3 B) and increased following 14 days high salt compared with control (Figure 6.3 C).

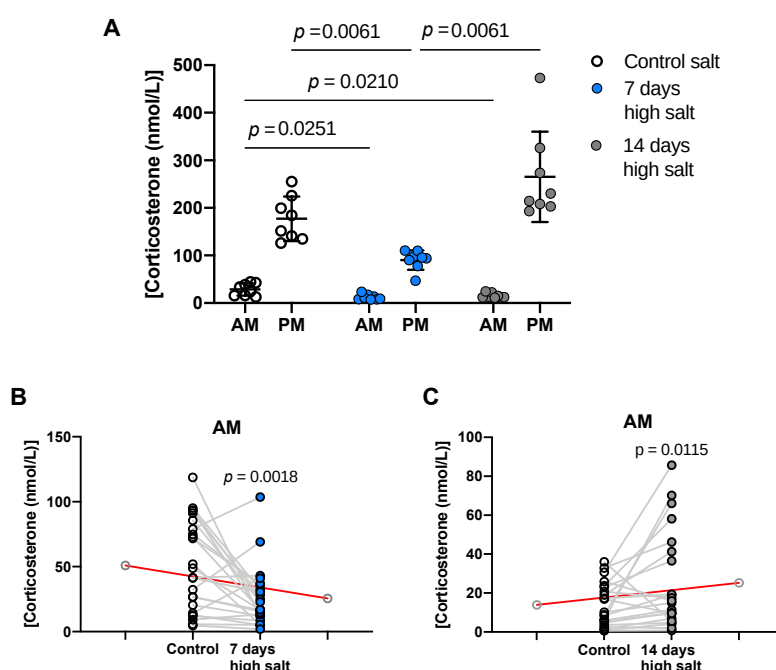


Figure 6.3: Diurnal plasma corticosterone levels following a high salt challenge.

(A) Paired measurements of corticosterone in C57BL/6J mice ($n = 8$) on control salt diet (0.3% Na^+ ; open black circles) and after 7 days (blue circles) and 14 days of a high salt challenge (3% Na^+ ; grey circles). Data are mean $\pm$ SD and analysed by two-way ANOVA with Holm-Sidak's *post hoc* test. (B & C) Paired measurements of corticosterone in mice before and after 7 days high salt ($n = 23$) and before and after 14 days high salt ($n = 22$). Data are mean $\pm$ SD and analysed by Wilcoxon test. The red line represents the general trend of averaged plasma corticosterone before and after high salt diet.

Plasma corticosterone assays measure total corticosterone; *in vivo* over 90% of corticosterone is bound to plasma proteins, predominately CBG⁸⁴. Therefore, I measured the gene expression of CBG in the liver and the binding capacity of CBG in the plasma (Figure 6.4 A). Expression of *Cbg* was significantly decreased following both 7 and 14 days high salt compared with control salt (ANOVA main effect of diet $p = 0.0003$), with no change compared with 7 and 14 days ($p = 0.190$). Although, the CBG binding capacity was only reduced after 14 days of high salt, compared with both control and 7 days high salt (ANOVA main effect of diet $p = 0.009$; Figure 6.4 B).

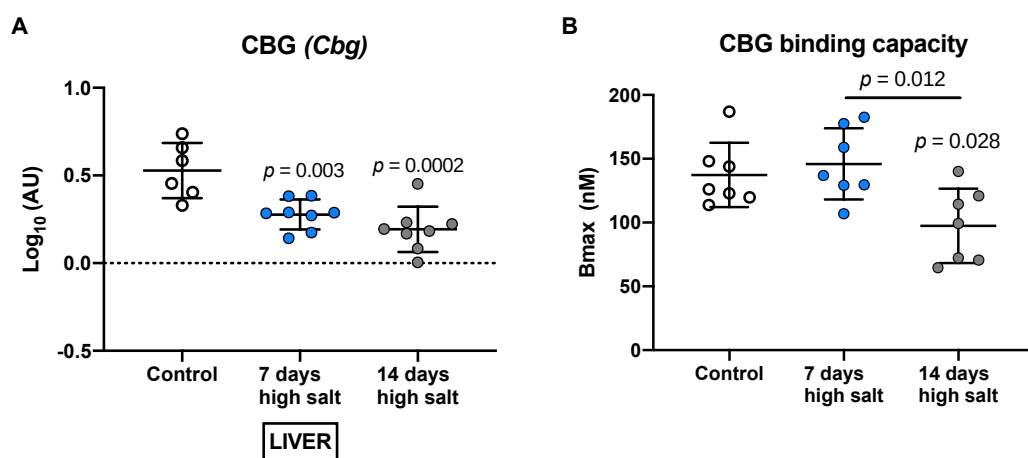


Figure 6.4: Gene expression and binding capacity of corticosteroid binding globulin following high salt challenge.

Male C57BL/6J mice on either control salt diet (0.3% Na⁺; open black circles; $n = 6-7$), 7 days (blue circles; $n = 7-8$) and 14 days high salt (3% Na⁺; grey circles; $n = 7-8$). (A) Gene expression of *Cbg* in liver tissue and normalised to the average expression of reference genes *Actb* and *Hprt*. Protein the gene transcribes in brackets. (B) Plasma CBG binding capacity. Data are mean $\pm$ SD and analysed by one-way ANOVA with Holm-Šidák's *post hoc* test. AU, arbitrary units.

Overall, these data suggest that 14 days high salt diet causes a sustained increase in free (bioactive) corticosterone. This led to questioning whether high salt impacted the synthesis of corticosterone, as illustrated in Figure 6.5. Genes investigated transcribe the proteins in blue circles in the schematic diagram.

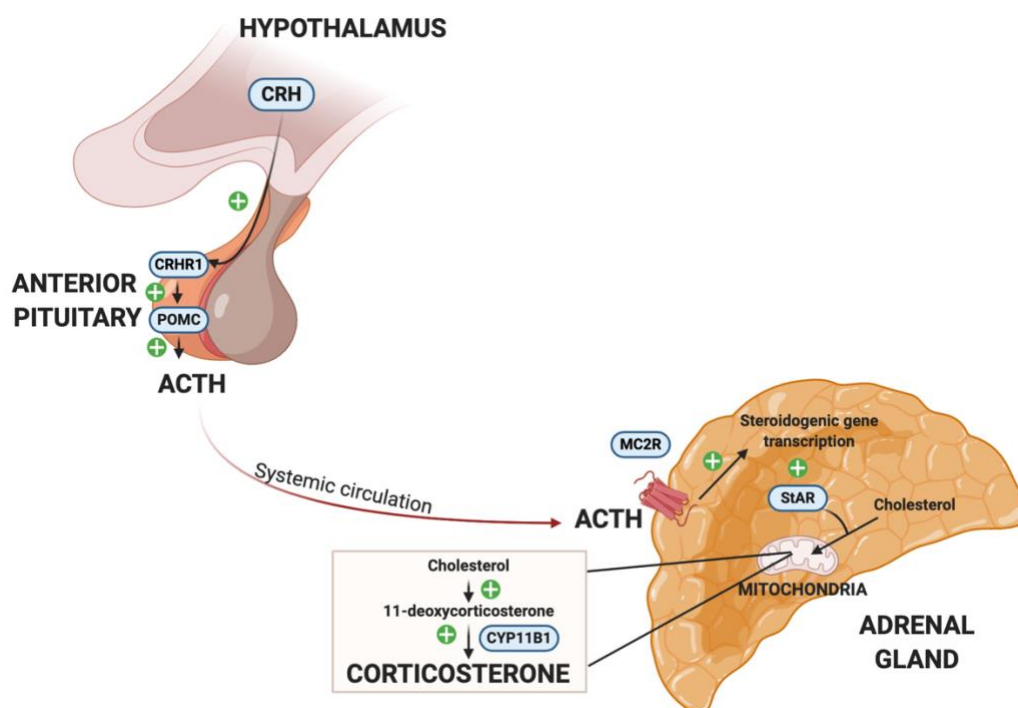


Figure 6.5: Steroidogenesis of corticosterone.

Corticotropin-releasing hormone (CRH) binds to its receptor, CRHR1, in the anterior pituitary corticotrophs which stimulates the activation of pro-opiomelanocortin (POMC) transcription for the secretion of POMC-encoded adrenocorticotrophic hormone (ACTH). ACTH activates the G-protein-coupled receptor melanocortin type 2 receptors (MC2R) of the adrenal cortex to induce the transcription of steroidogenic acute regulatory protein (StAR). StAR mobilises cholesterol for its transfer into the mitochondria. Once cholesterol has entered the mitochondria, a cascade of processes trigger the synthesis of corticosterone. Diagram created with BioRender.com.

High salt increased gene expression of CRH in the hypothalamus (ANOVA effect of diet $p = 0.044$) although, this was not reflected in *post hoc* analysis. However, there were no differences in expression of its receptor (*Crhr1*). *Pomc*, encoding the precursor of ACTH, in the anterior pituitary was significantly increased following both 7 and 14 days high salt (ANOVA effect of diet $p < 0.0001$), suggesting increased ACTH synthesis. Expression of ACTH receptor (MC2R) was not changed with high salt. However, high salt significantly increased *StAR* in the adrenal gland (ANOVA effect of diet $p = 0.018$). Finally, gene expression of the enzyme CYP11B1 responsible for synthesis of corticosterone, from 11-deoxycorticosterone, was unchanged following high salt (Figure 6.6). Overall, this could suggest high salt induces

drive in the feed-forward mechanism of the HPA axis to drive increased synthesis of corticosterone.

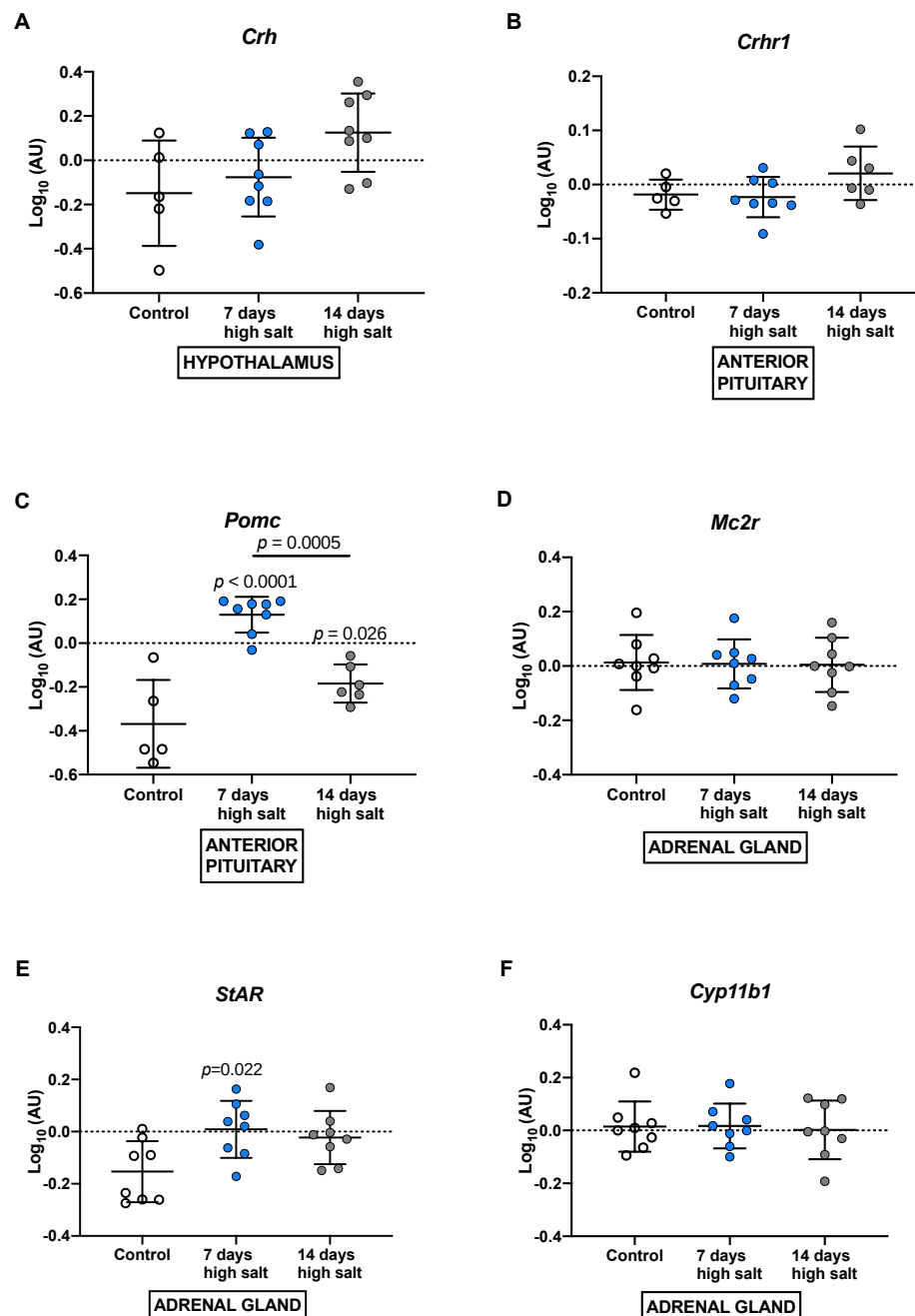


Figure 6.6: Expression of genes regulating steroidogenesis following high salt challenge.

Male C57BL/6J mice on either control salt diet (0.3% Na⁺; open black circles; $n = 5-8$), 7 days (blue circles; $n = 8$) and 14 days high salt (3% Na⁺; grey circles; $n = 6-8$). (A) Gene expression of *Crh* in the hypothalamus, normalised to the average expression of reference genes *Gapdh* and *Tbp*. (B&C) Expression of *Crhr1* and *Pomc* in the anterior pituitary and (C-E) *Mc2r*, *StAR* and *Cyp11b1* in the adrenal gland, normalised to the average expression of reference genes *Actb* and *Tbp*. Data are mean $\pm$ SD and analysed by one-way ANOVA with Holm-Šidák's *post hoc* test. AU, arbitrary units.

Next, I assessed whether this increase in free corticosterone was associated with an enhanced corticosterone action in key tissues, highlighted in blue circles in Figure 6.7, which are involved in metabolism and negative feedback of corticosterone.

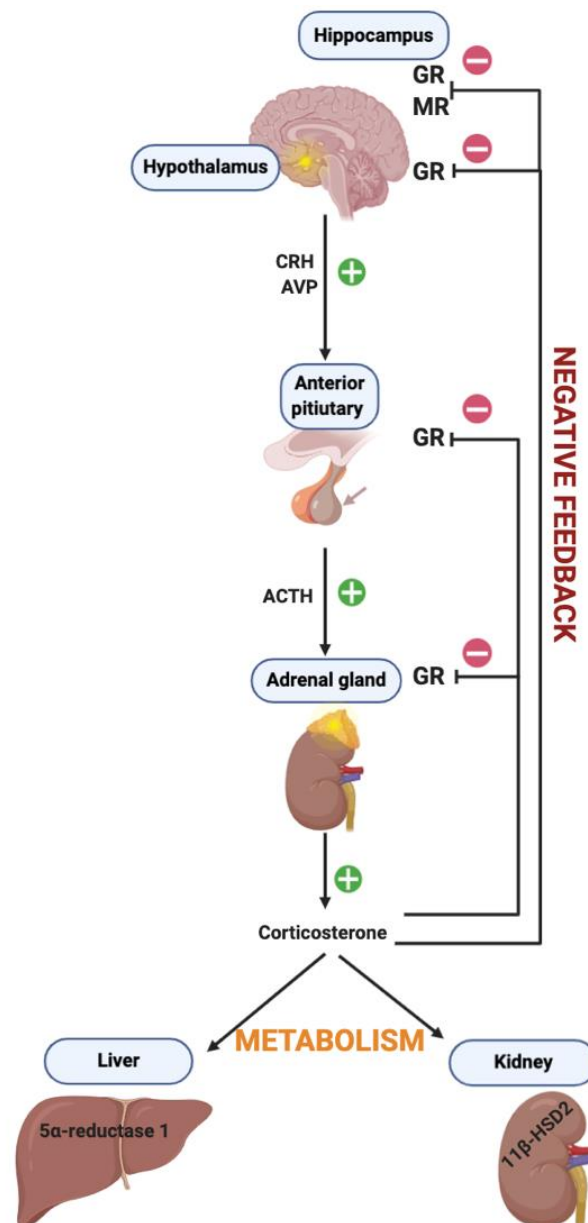


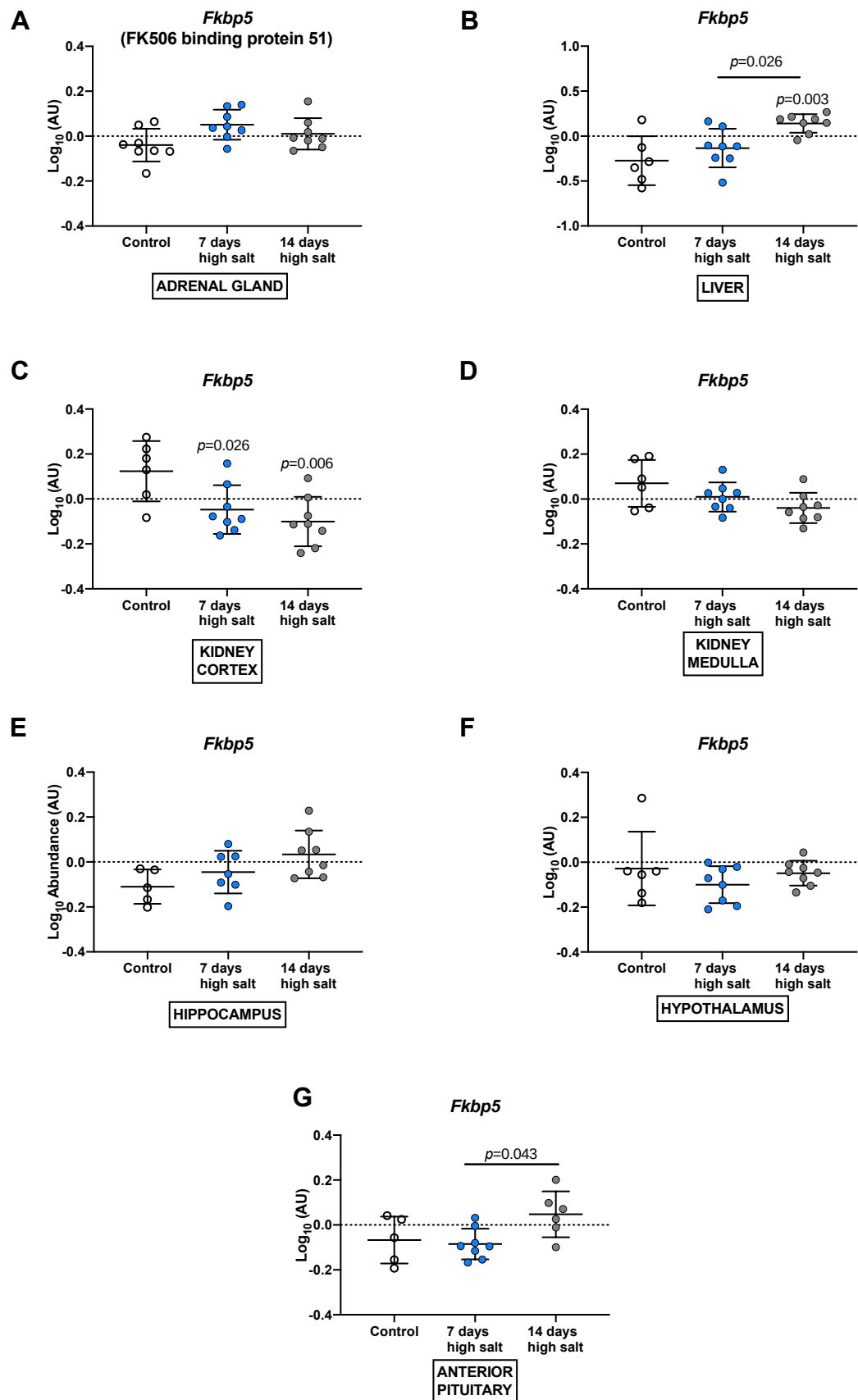
Figure 6.7: Simplified mechanisms of metabolism and negative feedback of corticosterone.

The major routes of glucocorticoid metabolism involve the inactivation of corticosterone by renal 11 β -HSD2 and corticosterone reduction by hepatic 5 α -reductase 1. Corticosterone regulates the activity of the HPA axis through feedback mechanisms by binding to its receptors, glucocorticoid receptor (GR) and/or mineralocorticoid receptor (MR), in the anterior pituitary, hypothalamus, hippocampus and adrenal gland. Diagram created with BioRender.com.

Cellular corticosterone action was assessed by measuring expression of *Fkbp5* in these tissues (Figure 6.8). *Fkbp5* is a glucocorticoid target/responsive gene, indicative of cellular corticosterone activity^{97,214}. Alterations in cellular corticosterone activity following high salt was varied among tissues. Liver *Fkbp5* was significantly increased following 14 days high salt (ANOVA main effect of diet $p = 0.003$). So too was *Fkbp5* in the anterior pituitary, which was significantly increased after 14 days of high salt compared with 7 days high salt. Although, unchanged compared with control salt (ANOVA effect of diet $p = 0.037$). Following 7 and 14 days of high salt, kidney cortex *Fkbp5* was significantly reduced compared with control salt (ANOVA main effect of diet $p = 0.006$), with no change in expression in the adrenal gland (ANOVA main effect of diet was $p = 0.053$), kidney medulla, hippocampus (ANOVA effect of diet $p = 0.054$) or hypothalamus.

Figure 6.8: Expression of glucocorticoid responsive gene, *Fkbp5*, following high salt challenge.

Male C57BL/6J mice on either control salt diet (0.3% Na⁺; open black circles; $n = 5-8$), 7 days (blue circles; $n = 7-8$) and 14 days high salt (3% Na⁺; grey circles; $n = 6-8$). Gene expression of *Fkbp5* in the adrenal gland (A), hippocampus (E) and anterior pituitary (G) normalised to the average expression of reference genes *Actb* and *Tbp*. Protein the gene transcribes in brackets. (B) Expression of *Fkbp5* in the liver, normalised to the average expression of reference genes *Actb* and *Hprt*. (C-D) Expression of *Fkbp5* in the kidney cortex and medulla, normalised to the average expression of reference genes *Actb* and *Rn18s*. (F) Expression of *Fkbp5* in the hypothalamus, normalised to the average expression of reference genes *Gapdh* and *Tbp*. Data are mean $\pm$ SD and analysed by one-way ANOVA with Holm-Šidák's *post hoc* test. AU, arbitrary units.



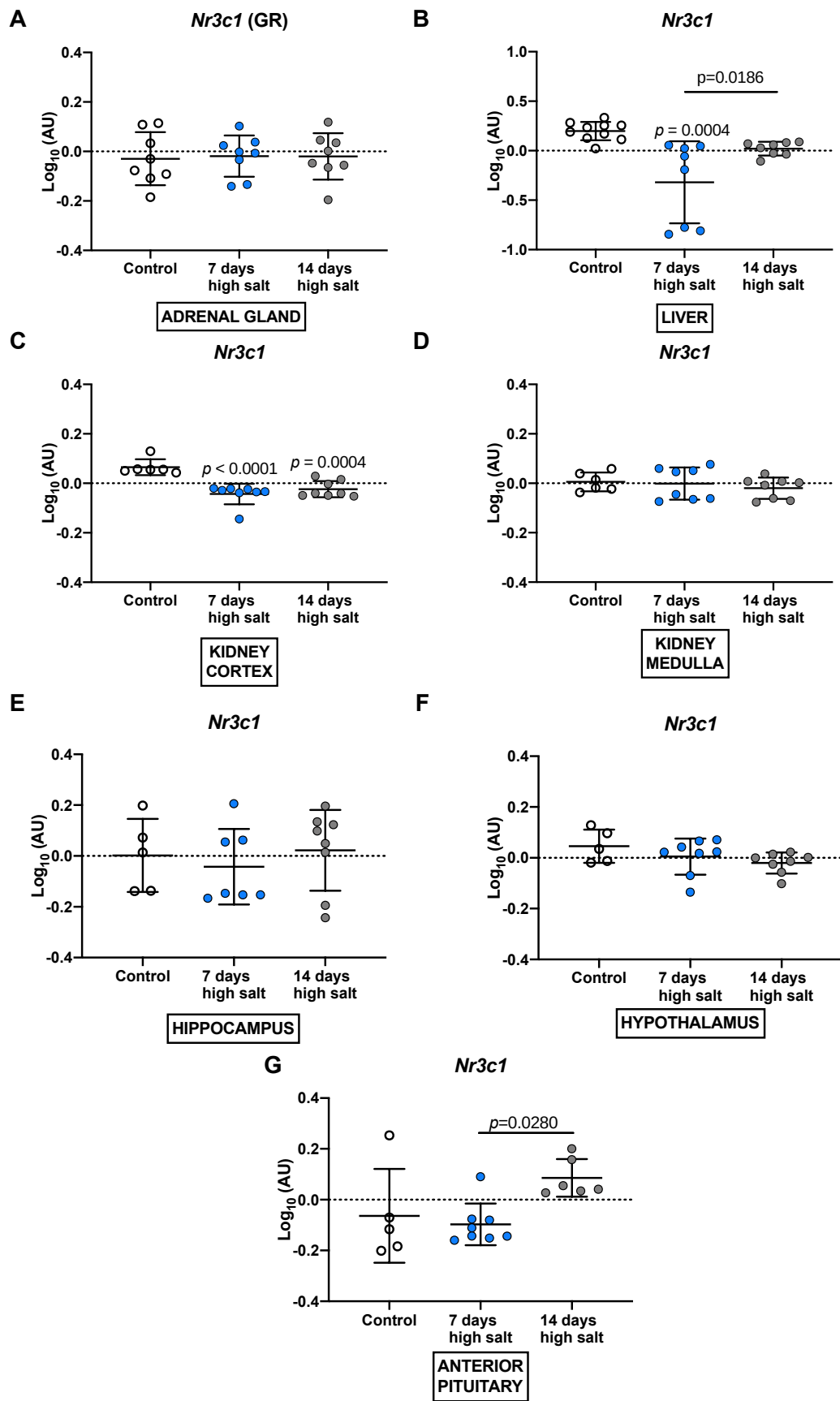
FKBP51 regulates the affinity of GR and MR for glucocorticoids and the ligand-receptor complex translocation into the nucleus⁸⁸. Increases in circulating glucocorticoids can shift the balance between MR and GR activation. However, this is dependent on the tissue as only some tissues express both receptor types. Unlike GR, which is ubiquitously expressed, MR is localised to specific organs including the kidney and regions of the brain⁸⁹. Therefore, the genes encoding GR (*Nr3c1*) and MR (*Nr3c2*) were measured in tissues previously mentioned.

Nr3c1 was significantly decreased following a high salt challenge in both the kidney cortex (ANOVA effect of diet $p < 0.0001$) and liver (ANOVA effect of diet $p = 0.0006$). Salt had an opposing effect in the anterior pituitary, where expression of *Nr3c1* was increased (ANOVA effect of diet $p = 0.026$). However, salt had no effect of GR gene expression in the adrenal gland, kidney medulla, hippocampus and hypothalamus (Figure 6.9).

Analysis of *Nr3c2* was focussed to the kidney and various regions of the brain (Figure 6.10). Similar to GR, MR gene expression in the kidney cortex was significantly downregulated following high salt (ANOVA effect of diet $p < 0.0001$), with anterior pituitary *Nr3c2* increased (ANOVA effect of diet $p = 0.022$). Furthermore, high salt caused decreases in hippocampal *Nr3c2* (ANOVA effect of diet $p = 0.041$). However, salt had no effect on *Nr3c2* expression in the kidney medulla and hypothalamus.

Figure 6.9: Gene expression of GR following high salt challenge.

Male C57BL/6J mice on either control salt diet (0.3% Na⁺; open black circles; $n = 5-8$), 7 days (blue circles; $n = 7-8$) and 14 days high salt (3% Na⁺; grey circles; $n = 6-8$). Gene expression of *Nr3c1* (GR) in the adrenal gland (A), hippocampus (E) and anterior pituitary (G) normalised to the average expression of reference genes *Actb* and *Tbp*. Protein the gene transcribes in brackets. (B) Expression of *Nr3c1* in the liver, normalised to the average expression of reference genes *Actb* and *Hprt*. (C-D) Expression of *Nr3c1* in the kidney cortex and medulla, normalised to the average expression of reference genes *Actb* and *Rn18s*. (F) Expression of *Nr3c1* in the hypothalamus, normalised to the average expression of reference genes *Gapdh* and *Tbp*. Data are mean $\pm$ SD and analysed by one-way ANOVA with Holm-Šidák's *post hoc* test. AU, arbitrary units.



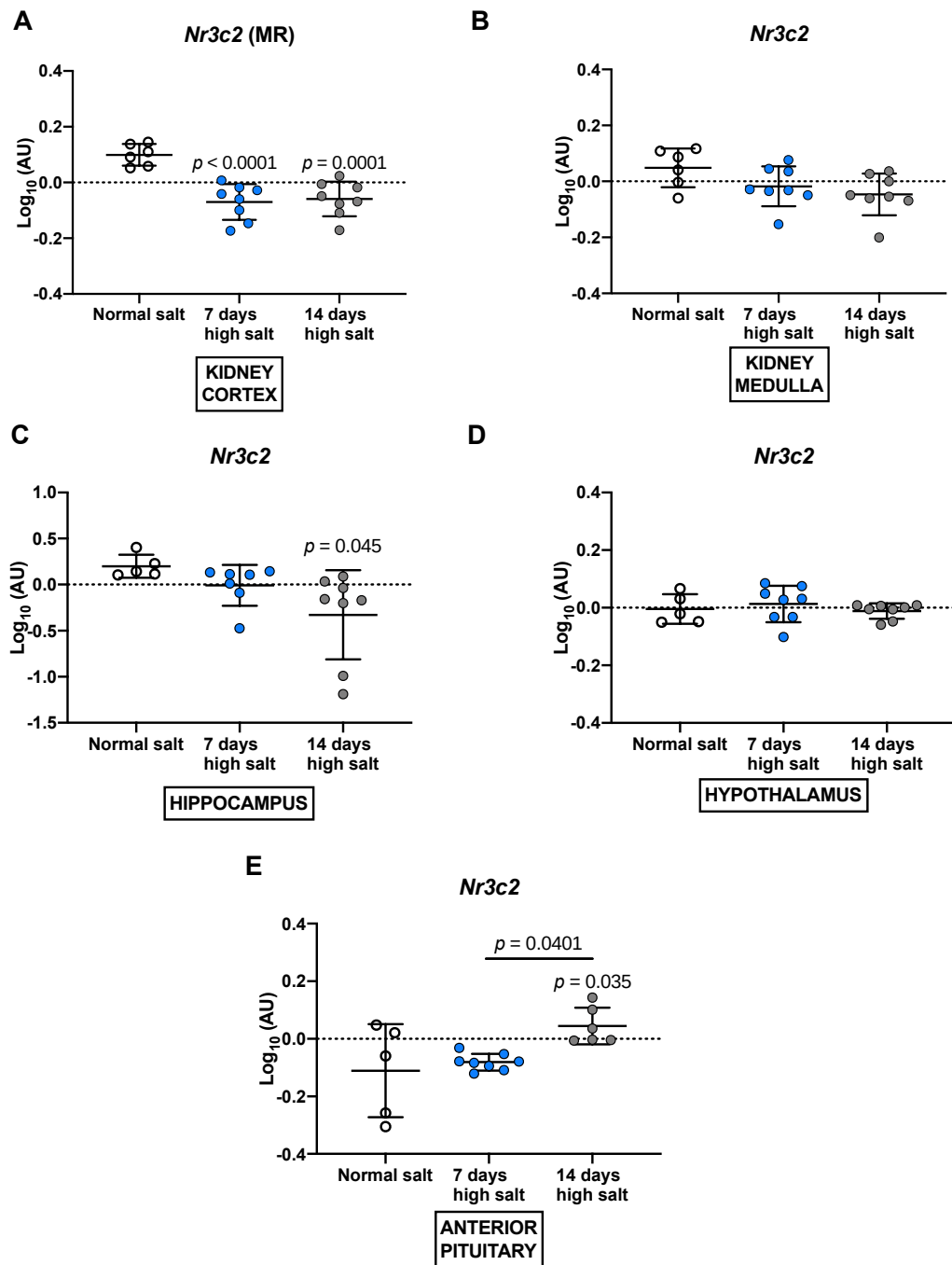


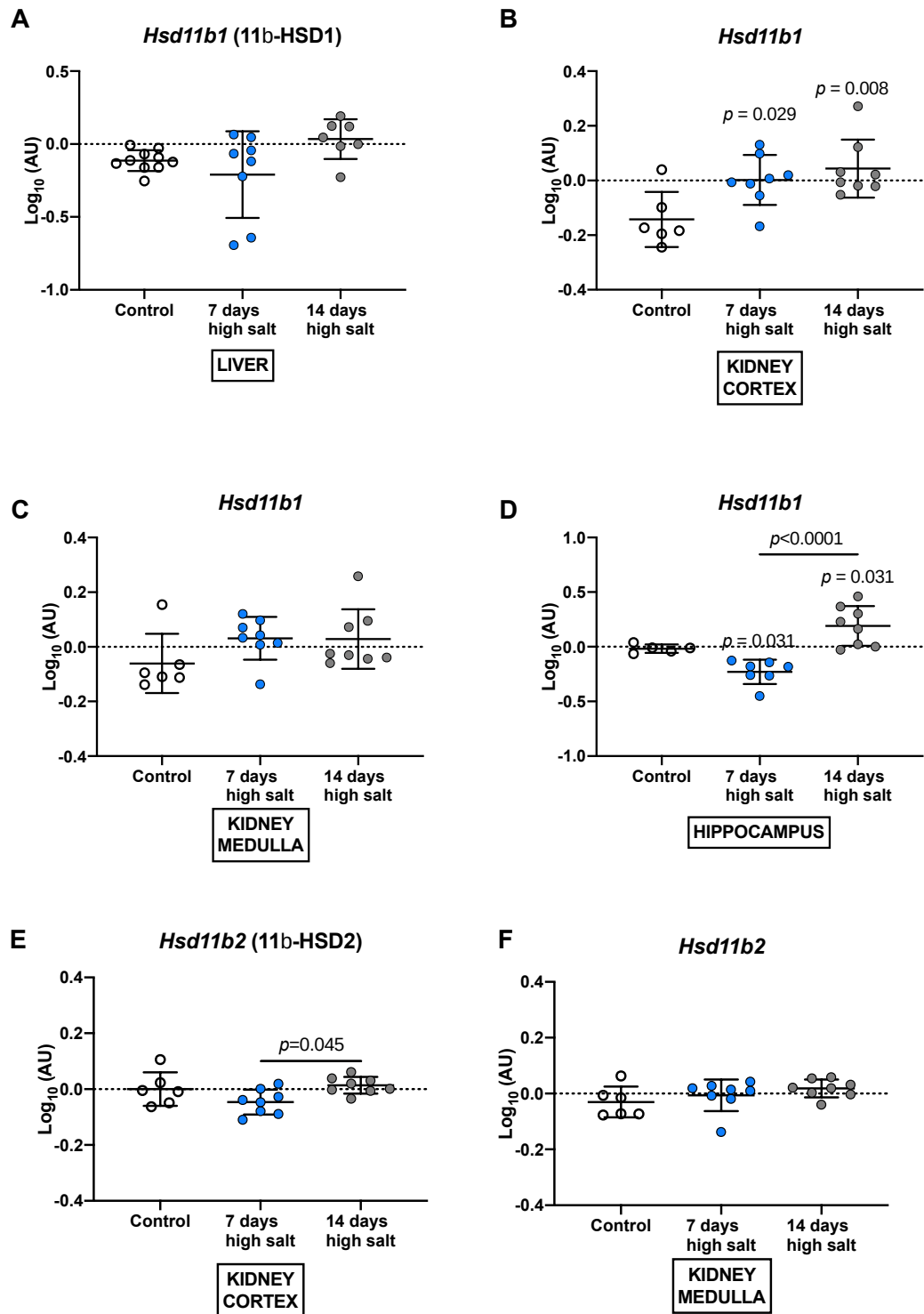
Figure 6.10: Gene expression of MR following high salt challenge.

Male C57BL/6J mice on either control salt diet (0.3% Na⁺; open black circles; $n = 5-8$), 7 days (blue circles; $n = 7-8$) and 14 days high salt (3% Na⁺; grey circles; $n = 6-8$). Gene expression of *Nr3c2* (MR) in the the kidney cortex (A) and medulla (B), normalised to the average expression of reference genes *Actb* and *Rn18s*. Protein the gene transcribes in brackets. Expression of *Nr3c2* in the hippocampus (C) and anterior pituitary (E) normalised to the average expression of reference genes *Actb* and *Tbp*. (D) Expression of *Nr3c1* in the hypothalamus, normalised to the average expression of reference genes *Gapdh* and *Tbp*. Data are mean $\pm$ SD and analysed by one-way ANOVA with Holm-Šidák's *post hoc* test. AU, arbitrary units.

Cellular glucocorticoid action can also be influenced by 11 β -HSDs, where 11 β -HSD1 re-activates corticosterone and 11 β -HSD2 inactivates corticosterone into inactive metabolites. 11 β -HSD2 also acts as a protective barrier against MR activation by corticosterone⁹³. Next, the effect of salt on gene expression of 11 β -HSD1 (*Hsd11b1*) and 11 β -HSD2 (*Hsd11b2*) was assessed (Figure 6.11). *Hsd11b1* following high salt was unchanged compared with control salt in liver. Hippocampal expression was significantly increased following a high salt challenge (ANOVA effect of diet $p < 0.0001$). Expression was increased in the kidney cortex (ANOVA effect of diet $p = 0.078$), with no change in the kidney medulla. Although, *Hsd11b2* was increased comparing 7 and 14 days high salt (ANOVA effect of diet $p = 0.039$), there was no effect on kidney medulla *Hsd11b2*.

Figure 6.11: Gene expression of 11 β -hydroxysteroid dehydrogenases following high salt challenge.

Male C57BL/6J mice on either control salt diet (0.3% Na⁺; open black circles; $n = 5-10$), 7 days (blue circles; $n = 7-8$) and 14 days high salt (3% Na⁺; grey circles; $n = 7-8$). (A) Gene expression of 11 β -HSD1 (*Hsd11b1*) in the liver, normalised to the average expression of reference genes *Actb* and *Hprt*. Protein the gene transcribes in brackets. Expression of *Hsd11b1* in the kidney cortex and medulla (B&C), normalised to the average expression of reference genes *Actb* and *Rn18s*. (D) Expression of *Hsd11b1* in the hippocampus, normalised to the average expression of reference genes *Actb* and *Tbp*. *Hsd11b2* in the kidney cortex and medulla (E&F), normalised to the average expression of reference genes *Actb* and *Rn18s*. Data are mean $\pm$ SD and analysed by one-way ANOVA with Holm-Šidák's *post hoc* test. AU, arbitrary units.



Along with 11 β -HSD2, hepatic 5 α -reductase 1 (*Srd5a1*) is a major route for glucocorticoid clearance. The expression of hepatic *Srd5a1* (5 α -reductase 1) was not different following high salt (Figure 6.12).

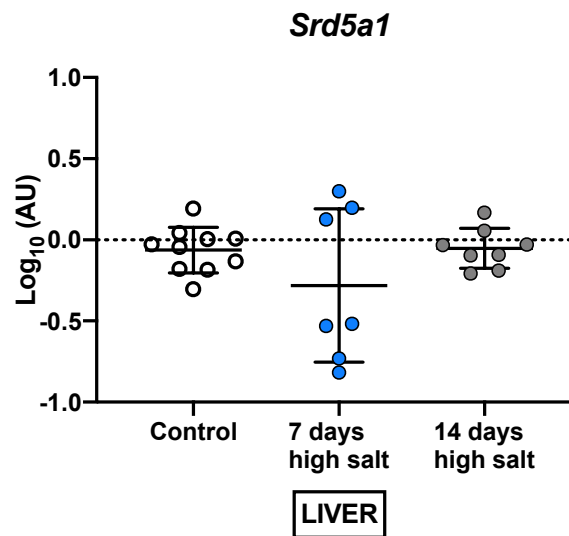


Figure 6.12: Gene expression of 5 α -reductase 1 following high salt challenge. Male C57BL/6J mice on either control salt diet (0.3% Na⁺; open black circles; $n = 10$), 7 days (blue circles; $n = 7$) and 14 days high salt (3% Na⁺; grey circles; $n = 8$). Gene expression of 5 α reductase 1 (*Srd5a1*) in the liver, normalised to the average expression of reference genes *Actb* and *Hprt*. Data are mean $\pm$ SD and analysed by one-way ANOVA with Holm-Šidák's *post hoc* test. AU, arbitrary units.

6.3.2. Salt-induced reduction in plasma aldosterone levels

During high dietary salt intake, RAAS activity is inhibited, decreasing levels of aldosterone²⁶⁶. To confirm this response, plasma aldosterone and some key genes involved in aldosterone synthesis were assessed. High salt suppressed plasma aldosterone in a time-dependent manner (ANOVA main effect of diet $p < 0.0001$; Figure 6.13 A). This was reflected in the gene expression of *Agt* in the liver and *Cyp11b2* in the adrenal gland which are involved in aldosterone synthesis, as these genes were significantly downregulated following a high salt challenge (both ANOVA main effect of diet $p < 0.0001$).

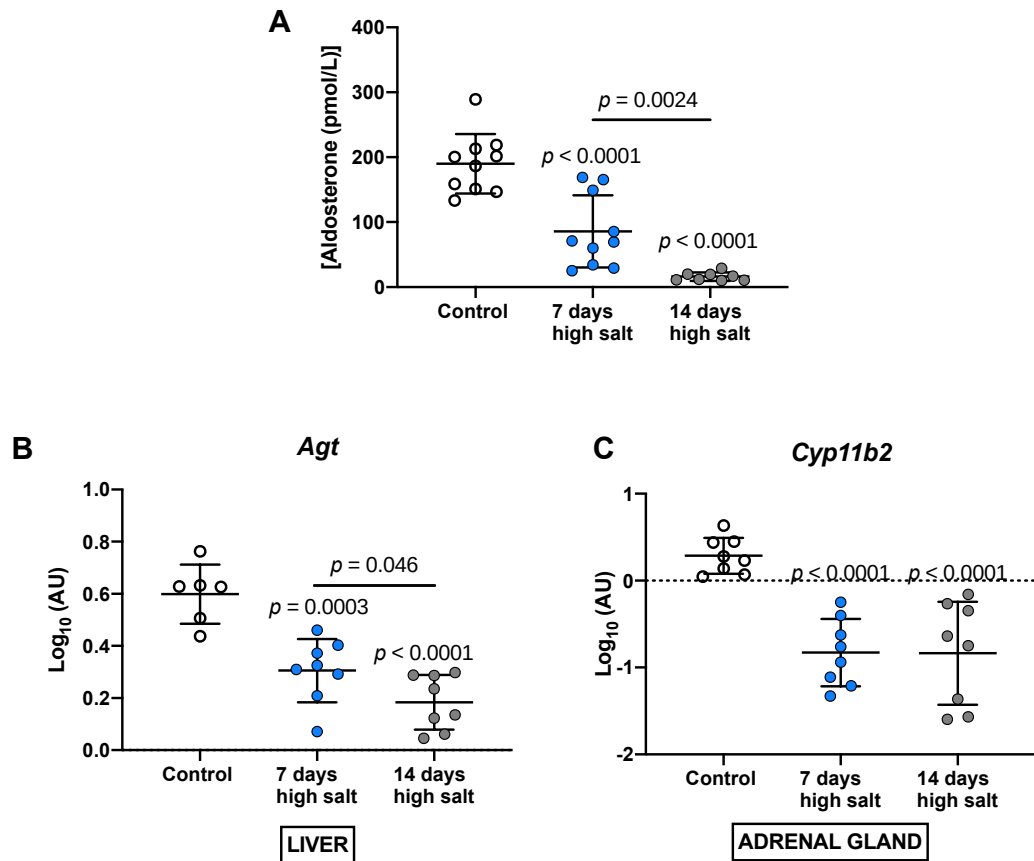


Figure 6.13: Plasma aldosterone levels following high salt challenge.

Male C57BL/6J mice on either control salt diet (0.3% Na⁺; open black circles; $n = 6-10$), 7 days (blue circles; $n = 8-10$) and 14 days high salt (3% Na⁺; grey circles; $n = 8$). (A) Plasma was collected at 8 AM for measurements of aldosterone (B) Gene expression of *Agt* in the liver, normalised to the average expression of reference genes *Actb* and *Hprt*. (C) Gene expression of *Cyp11b2* in the adrenal gland, normalised to the average expression of reference genes *Actb* and *Tbp*. Data are mean $\pm$ SD and analysed by one-way ANOVA with Holm-Šidák's *post hoc* test. AU, arbitrary units.

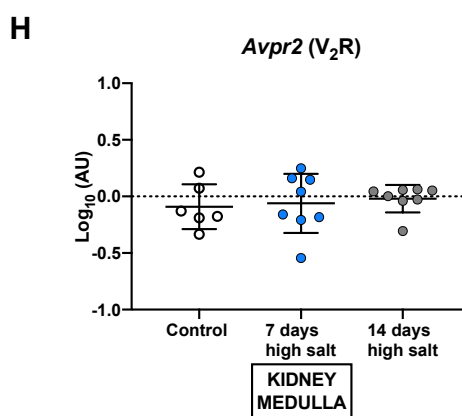
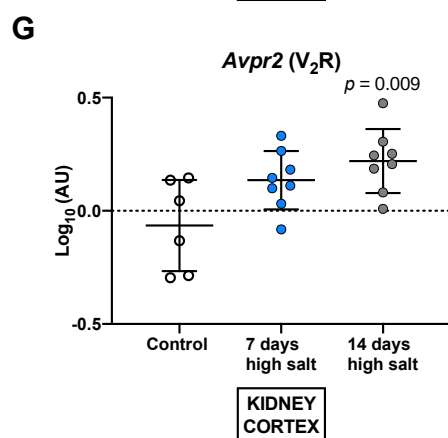
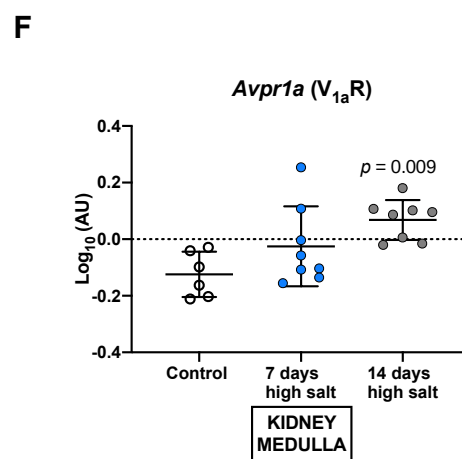
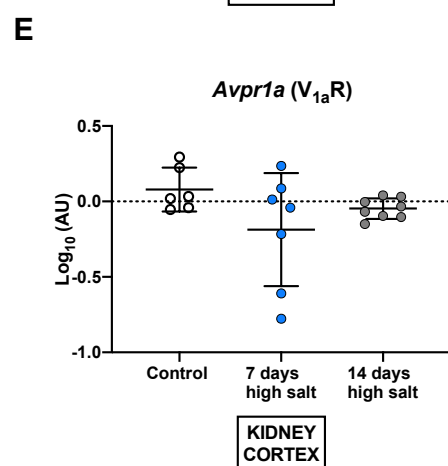
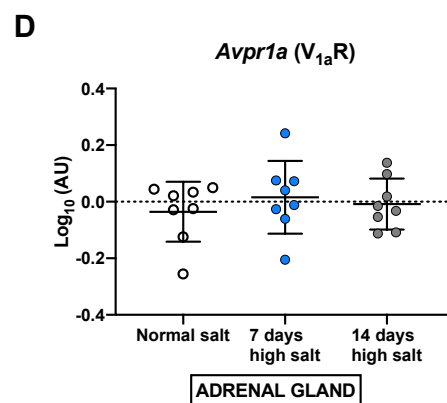
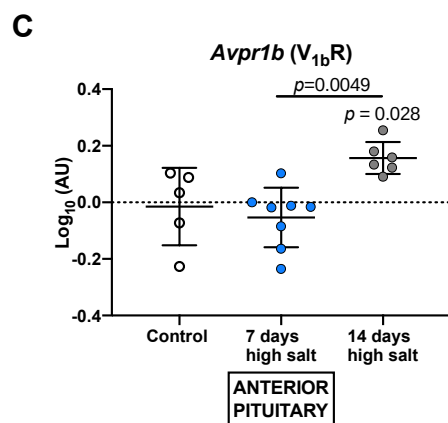
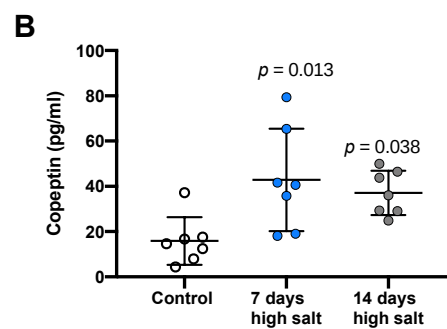
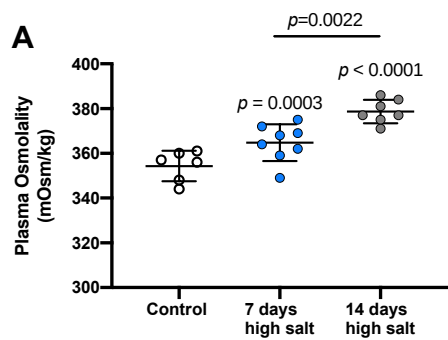
6.3.3. Salt-induced increases in plasma copeptin levels

The physiological response to increased dietary salt is increases in plasma osmolality, inducing activation of AVP. Here, plasma osmolality, plasma copeptin levels (surrogate marker of AVP) and genes encoding vasopressin receptors (*Avpr1a*, *Avpr1b* and *Avpr2*) were measured in mice following a high salt challenge (Figure 6.14). Plasma osmolality was increased with salt (ANOVA main effect of diet $p < 0.0001$) along with plasma copeptin levels (ANOVA main effect of diet $p = 0.011$). Gene expression of V_{1b}R in the anterior pituitary was significantly increased (ANOVA main effect of diet $p = 0.005$).

High salt induced no changes in *Avpr1a* in the adrenal gland and kidney cortex but was increased in the kidney medulla (ANOVA main effect of diet $p = 0.011$). *Avpr2* was increased in the kidney cortex (ANOVA main effect of diet $p = 0.010$), with no change in the kidney medulla.

Figure 6.14: Plasma copeptin levels following high salt challenge.

Male C57BL/6J mice on either control salt diet (0.3% Na⁺; open black circles; $n = 6-8$), 7 days (blue circles; $n = 7-8$) and 14 days high salt (3% Na⁺; grey circles; $n = 6-8$). Plasma was collected at 8 AM for measurements of osmolality (A) and copeptin (B) on normal salt diet (0.3% Na⁺) and after 7 and 14 days of a high salt challenge (3% Na⁺). (C) Gene expression of *Avpr1b* in the anterior pituitary, normalised to the average expression of reference genes *Actb* and *Tbp*. (D) Gene expression of *Avpr1a* in the adrenal gland, normalised to the average expression of reference genes *Actb* and *Tbp*. (E&F) Gene expression of *Avpr1a* in the kidney cortex and medulla, normalised to the average expression of reference genes *Actb* and *Rn18s*. (G&H) Gene expression of *Avpr2* in the kidney cortex and medulla, normalised to the average expression of reference genes *Actb* and *Rn18s*. Data are mean $\pm$ SD and analysed by one-way ANOVA with Holm-Šidák's *post hoc* test. AU, arbitrary units.



6.3.4. Salt-induced increases in sensitivity to stress

To test the impact of salt on the stress response, plasma corticosterone was measured throughout a restraint stress test in mice before and after either a 7 day or 14 day high salt challenge. Figure 6.15 A gives an overview of the plasma corticosterone levels at baseline, following a 15 min stress and varying recovery times in mice on a control salt diet then a 7 day high salt diet (ANOVA main effects of salt $p = 0.246$; stress response and recovery response $p < 0.0001$; interaction of salt and stress and recovery response $p = 0.058$). The stress response was calculated by subtracting the stress corticosterone levels by the baseline levels (Figure 6.15 B). There was no change in stress response following 7 days high salt diet. The recovery response was calculated by subtracting the recovery corticosterone levels for each recovery time by the stress corticosterone levels (Figure 6.13 C-E). Recovery response after 30 min had a tendency for a bigger recovery from stress following 7 days high salt. This was also reflected in the recovery response after 60 min. However, this was not the case for the recovery response after 90 min where there was a smaller recovery from stress following 7 days high salt. Overall, there was no strong evidence for 7 days high salt impacting the stress response in C57BL/6J mice.

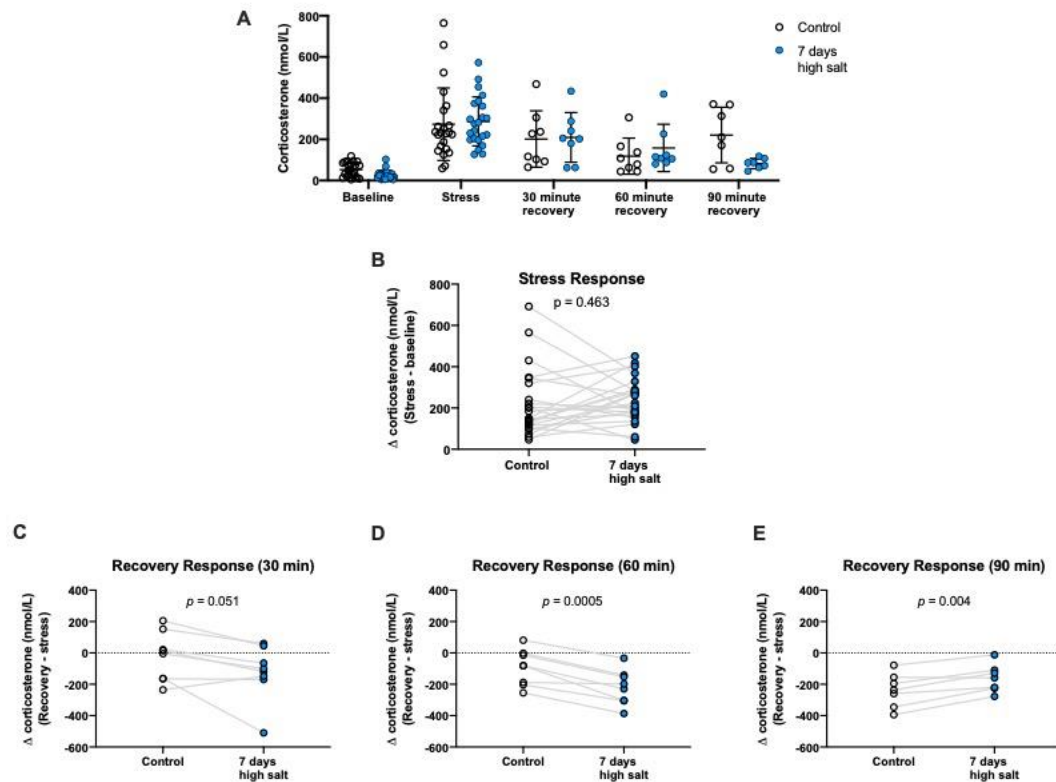


Figure 6.15: Stress response before and after 7 days of a high salt challenge. Paired measurements of the stress response in male C57BL/6J mice ($n = 23$) on control salt diet (0.3% Na^+ ; open black circles;) and after 7 days high salt (blue circles). (A) Plasma corticosterone measured at baseline (collected between 7.15-8 AM), following 15-min stress and after either 30, 60 or 90 min recovery before and after 7 days high salt challenge. Data are mean $\pm$ SD ($n = 23$ for baseline and stress; $n = 7$ -8/recovery time point) and were analysed by two-way ANOVA. (B) The change in stress response following control and 7-day high salt diet with data analysed by Wilcoxon test. The change in recovery response at (C) 30, (D) 60 and (E) 90 min recovery following control and 7-day high salt diet. Data were analysed by paired t test.

Figure 6.16 A gives an overview of the plasma corticosterone levels at baseline, following a 15 min stress and varying recovery times in mice on a control salt diet then a 14 day high salt diet (ANOVA main effects of salt $p = 0.0002$; stress response and recovery response $p < 0.0001$; interaction of salt and stress and recovery response $p = 0.122$). There was a significant increase in the Δ stress response following 14 days high salt diet (Figure 6.16 B). There was no change in recovery response at either 30, 60 or 90 min of recovery to the stressor between control and high salt diet (Figure 6.16 C – E).

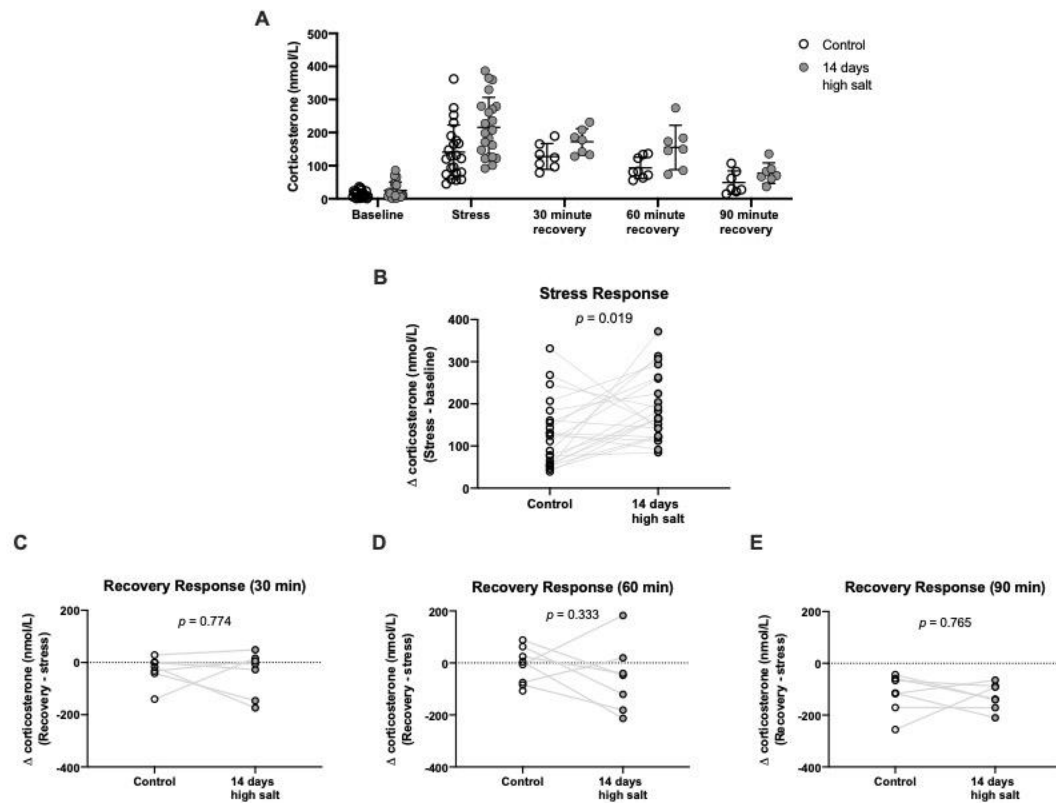


Figure 6.16: Stress response before and after 14 days of a high salt challenge. Paired measurements of the stress response in male C57BL/6J mice ($n = 22$) on control salt diet (0.3% Na^+ ; open black circles;) and after 14 days high salt (grey circles). (A) Plasma corticosterone measured at baseline (collected between 7.15-8 AM), following 15-min stress and after either 30, 60 or 90 min recovery before and after 14 days high salt challenge. Data are mean $\pm$ SD ($n = 22$ for baseline and stress; $n = 7-8$ /recovery time point) and were analysed by two-way ANOVA. (B) The change in stress response following control and 14-day high salt diet with data analysed by Wilcoxon test. The change in recovery response at (C) 30, (D) 60 and (E) 90 min recovery following control and 14-day high salt diet. Data were analysed by paired t test.

6.4 Discussion

In this chapter, high salt increased free (bioactive) plasma corticosterone with increased tissue specific corticosterone exposure following a 14-day high salt challenge. Potential mechanisms behind salt increasing HPA activity is thought to involve increasing circulating copeptin, indicative for the activity of AVP-secreting magnocellular neurons, and/or changes in MR:GR expression within the hippocampus, which alter the set point of stress system activity²⁶⁷. The impact of salt altering the regulation of the HPA axis has been shown to affect the stress response by increasing the sensitivity to stress.

6.4.1 High salt increases bioactive plasma corticosterone

Under basal conditions, glucocorticoids are transported in the circulation predominately bound to CBG (> 80%), a glycoprotein synthesised and secreted predominately in the liver, with only a small percentage of circulating glucocorticoids being free and able to enter the tissue to exert biological effects. Thus CBG plays a role in free corticosterone control²⁶⁸. Only total plasma corticosterone (including both bound and free glucocorticoids) measurements in the same mice before and after a high salt challenge were possible. Free plasma corticosterone concentration was unable to be measured due to limited sample volume. However, expression and binding capacity of CBG can indicate concentrations of free corticosterone. Following a high salt challenge, both hepatic *Cbg* mRNA and binding capacity was reduced after 14 days of high salt. A change in CBG binding capacity can reflect a change in cleavage of CBG but with reductions in its mRNA, it is likely due to a decrease in production itself²⁶⁹. Furthermore, glucocorticoids and stress have been shown to downregulate CBG²⁷⁰. Therefore, it is thought that salt alters glucocorticoid levels and rises in glucocorticoids then downregulates CBG, impacting free corticosterone concentration. With increased total plasma corticosterone and decreased expression and binding capacity of CBG, this suggests high salt increases free, biologically active plasma corticosterone.

So, does the increase in free corticosterone have implications on cellular corticosterone action? FKBP5/FKBP51 (*Fkbp5*) belongs to the family of FK506 binding proteins and has multiple roles in GR signalling and responses to stress. *Fkbp5* has been reported to interact with MR in a similar manner to GR²⁷¹. However, its interaction with MR has not been as well documented.

Fkbp5 mRNA expression was used here to indicate cellular corticosterone activity. This is due to FKBP5 rapid transcription following glucocorticoids activating GR^{97,214}. FKBP5 acts as an inhibitor of GR function as it reduces ligand binding and delays the nuclear translocation of GR therefore, decreasing GR-dependent transcription. Given that glucocorticoids mediate transcription of *Fkbp5*, this process acts as an ultra-short negative feedback loop^{96,97}. This is reflected in hepatic *Fkbp5* following a high salt diet as this was increased with a decrease in GR gene expression. The liver is a major target organ for glucocorticoids in control of glycogen metabolism and gluconeogenesis. Furthermore, it is also a major site for glucocorticoid metabolism, primarily via 5 α -reductase 1⁸³. With increased plasma free corticosterone, it was hypothesised that the increased exposure of corticosterone to the liver was associated with an increased glucocorticoid metabolism. However, gene expression of 5 α -reductase 1 was not altered following high salt. Therefore, the link between salt and increased corticosterone exposure to the liver remains unclear. The kidney is also a key site for glucocorticoid metabolism. Although, renal *Fkbp5* was reduced with a high salt challenge. This suggests this could be a protective mechanism as both GR and MR are involved in sodium reabsorption⁷⁵ and were reduced.

FKBP5 has also been reported to regulate the sensitivity of GR and therefore, is a key modulator of GR activity in the stress response. Enhanced FKBP5 expression following GR activation leads to an increased GR resistance and decreased efficiency in the negative feedback of glucocorticoids²⁷². FKBP5 gene expression was increased in the anterior pituitary, a site of glucocorticoid negative feedback. This negative feedback involves corticosterone-induced inhibition of POMC transcription⁸⁸. With increased corticosterone exposure

and potentially increased GR resistance, this could have implication on the termination of the HPA axis. This was supported as POMC transcript levels were not suppressed, it was significantly increased following 14 days high salt compared with control.

Measuring FKBP5 expression is informative for assessing cellular glucocorticoid action. However, there are other glucocorticoid responsive genes that would be for interest for future assessment such as serum and glucocorticoid-regulated kinase 1 (SGK1) as this is known to be central to the stress response as it enhances cell survival following exposure to apoptotic stress such as hyperosmolality^{273,274}. SGK1 also regulates renal sodium homeostasis so would be of interest to investigate expression in the context of a high salt challenge^{275,276}. Furthermore, glucocorticoid action on target tissues is determined by the density of GR and MR and the intracellular metabolism by 11 β -HSDs. Expression of these genes were assessed here but within the tissue pool of corticosterone, it is unclear whether it originates from plasma pool or from corticosterone regenerated intracellularly by 11 β -HSD1. Previously, this was impossible to distinguish between. This has now been made possible using mass spectrometry imaging where mice were infused with deuterated cortisol and its distribution measured in tissues over time. Its inactivation to cortisone (by loss of 11-deuterium) and regeneration to cortisol (by 11 β -HSD1) could be assessed using this method^{277,278}. This could be useful in future investigations to explore the equilibration of corticosterone in different tissue depots following a high salt challenge.

These data support the growing literature that the HPA axis is activated by a high salt diet^{114,141,143,146,182}. However, the mechanisms behind this phenomenon have not been previously described.

6.4.2 Potential mechanisms behind salt-induced increases in plasma glucocorticoids

6.4.2.1 Enhanced glucocorticoid synthesis via AVP

Glucocorticoid synthesis is activated via ACTH, which can be synthesised by CRH or AVP. AVP is often a forgotten stress hormone. AVP is a neuropeptide that is cleaved from preprovasopressin. Copeptin, as well as neurophysin II, is also cleaved from the COOH-terminal of the precursor. Although the biological role of copeptin is not fully understood, copeptin has been useful as a stable surrogate marker for the activity of AVP-secreting magnocellular neurons^{279,280}. In this study, plasma osmolality was increased in mice following a high salt challenge, which would normally activate AVP synthesis and secretion from magnocellular neurons. This is supported in this setting as plasma copeptin was increased. This is the classic antidiuretic response to retain water following hypernatremia²⁷⁹. High salt has also been reported to increase plasma copeptin in patients²⁸¹. Although how would an increase in AVP impact glucocorticoids?

Copeptin levels are indicative of circulating AVP and therefore the activity of magnocellular neurons in the supraoptic nucleus and PVN that terminate at the median eminence and the posterior lobe of the pituitary gland. These neurons are activated by stress and salt status. However, the HPA axis is regulated primarily by the parvocellular cells in the PVN, where AVP is colocalised with CRH. Unlike rats that have only half of their CRH containing neurons in the PVN releasing AVP, in mice all CRH-containing neurons are immunopositive for AVP^{282,283}. A plethora of studies over the past several decades have confirmed that both CRH and AVP are hypothalamic neurohormones involved in ACTH synthesis and secretion from the pituitary. CRH is required to stimulate the transcription of the precursor for ACTH, POMC (proopiomelanocortin), with AVP amplifying the effect of CRH to stimulate ACTH secretion^{283,284}. Interestingly, in certain conditions leading to high magnocellular AVP, AVP can be released from the magnocellular axons passing through the median eminence and enter the hypophyseal portal blood

going to the anterior pituitary^{279,283}. AVP can then bind to its receptor, V_{1b} receptor in the anterior pituitary to stimulate ACTH secretion and, in turn, glucocorticoid synthesis from the adrenal cortex. The mRNA expression of V_{1b} receptor was significantly increased following 14 days high salt. With an increase in both “AVP”, in terms of copeptin, and its receptor in the anterior lobe of the pituitary, this could explain salt-induced rise in plasma corticosterone.

Under normal conditions, magnocellular AVP release to the posterior lobe of the pituitary is up to 10-fold higher than parvocellular AVP release to the anterior lobe. Although unlike magnocellular neurons, release of AVP by parvocellular neurons is regulated by adrenal glucocorticoids. Following adrenalectomy, AVP release from the parvocellular neurons is at a similar magnitude to magnocellular neurons²⁸⁵. The mechanisms behind the decreases in plasma corticosterone in mice following 7 days high salt is not fully understood, but this could explain the subsequent rise in *Pomc* if AVP release from parvocellular neurons is increased. However, this is not reflected in the circulating glucocorticoid levels. Furthermore, since magnocellular neurons are not regulated by glucocorticoids, they can bypass glucocorticoid feedback and induce “escape from glucocorticoid feedback” in the pituitary, resulting in sustained HPA activation²⁷⁹.

To further support this hypothesis, previous studies using either a V_{1b} receptor antagonist (SSR149415)^{286,287} or mice lacking V_{1b} receptor showed lower circulating ACTH and corticosterone levels under basal conditions²⁸⁸. To test this, a V_{1b} receptor antagonist was planned to be administered to mice following a high salt challenge and diurnal plasma corticosterone would be measured as previously described. However, due to COVID-19, this was unable to be completed. However, glucocorticoids have been shown to induce increases in V_{1b} receptor mRNA but downregulate V_{1b} receptor at the protein level possibly by inhibiting translation of the V_{1b} receptor mRNA. Although, CRH and AVP can overcome this inhibition and cause V_{1b} receptor

upregulation²⁸⁹. Since AVP from the magnocellular neurons are not regulated by glucocorticoids and is suggested to be high following a high salt challenge, this could overcome the proposed inhibition of V_{1b} receptor mRNA translation. Therefore, the protein level of the V_{1b} receptor would also have to be explored prior to the V_{1b} receptor antagonist experiment.

In conclusion, high salt increases plasma copeptin, which is indicative of increases in activity of the AVP-secreting magnocellular neurons. We propose AVP is released from the magnocellular neurons into the hypophysial portal blood and enters the anterior pituitary where it stimulates ACTH secretion, via V_{1b} receptor, increasing glucocorticoid synthesis. Since magnocellular neurons are not regulated by glucocorticoids, this process escapes glucocorticoid negative feedback. This is supportive of the actions of 14 days high salt as circulating corticosterone is increased in mice. However, the mechanisms behind the reduction in circulating corticosterone remains elusive.

6.4.2.2 Hippocampal MR:GR balance altering negative feedback

The primary regions in the brain responsible for the negative feedback of glucocorticoids are the hypophysiotropic neurons of the PVN and pituitary corticotropes. In both these regions, glucocorticoids binding to GR is responsible for the termination of this neuroendocrine response. Furthermore, MR is present in the PVN, where it is involved in salt homeostasis. It is also present in the pituitary, although whether MR is involved in glucocorticoid negative feedback remains unclear^{267,290,291}. A plethora of studies have described GR and MR in limbic structures, notably the hippocampus, to have a role in feedback control of the HPA axis^{290,292–296}. Although, hippocampal MR and GR have been shown to have different functions. Since the hippocampus is absent of 11 β -HSD2, glucocorticoids binds with a 10-fold higher affinity to MR than GR. Therefore, basal glucocorticoids predominately occupies hippocampal MR to mediate the maintenance of tone of basal HPA activity. This has been shown to stabilise hippocampal excitability and is of relevance

for the sensitivity of the stress response^{267,290}. Hippocampal GR becomes occupied following glucocorticoid increase at circadian peak or following a stressor. Therefore, both hippocampal MR and GR facilitate the termination of stress-induced neuroendocrine response. Interestingly, following 14 days high salt diet, C57BL/6J mice have decreased hippocampal MR mRNA levels, with no changes in GR. This could potentially explain the increase in plasma corticosterone seen following 14 days high salt diet. Deficiency in MR has been predicted to increase a corticosteroid response²⁹⁰. Studies investigating reduced hippocampal MR, via centrally administered MR blockade by intracerebroventricular (ICV) injection, showed elevated diurnal plasma corticosterone and increased night ACTH levels^{293,297}, with an enhanced ACTH response to stress and changes in adrenocortical sensitivity to ACTH²⁹⁸. Furthermore, similar results have been described following a hippocampal MR blockade²⁹⁹.

6.4.3 Salt-induced increased sensitivity in the stress response

Following 14 days of high salt, there was an increase in sensitivity to the restraint stress, so salt induced a significant increase in plasma corticosterone following restraint. Both CRH and AVP have been reported to simultaneously increase following a restraint stress^{300,301}. Furthermore, there is a good correlation between V_{1b} receptor abundance and ACTH responses. Therefore, regulation of V_{1b} receptor in the anterior pituitary has been shown to have an important role in response to a novel stimulus and to chronic stress³⁰². Since both plasma copeptin and the anterior pituitary V_{1b} receptor expression were significantly increased following 14 days high salt, this could implicate the role of AVP in the increased sensitivity to a stress. Furthermore, it has been hypothesised that shifts in the balance of MR and GR in the hippocampus can alter the set point of the stress response and can lead to an inability to maintain homeostasis, resulting in neuroendocrine dysregulation²⁹⁰. With decreases in hippocampal MR mRNA expression, this could also implicate the role of hippocampal MR:GR balance in this response.

6.4.4 Gene transcript analysis vs protein analysis

The bulk of this chapter used the technique of qPCR to investigate mechanisms underlying certain physiological and pathophysiological changes in multiple models. The positive of this is the ability to screen multiple genes over multiple tissues in a timely and inexpensive way. However, there are negatives solely using this method. Changes in gene expression does not necessarily reflect the function of the protein. Gene and protein analysis complement each other³⁰³. Therefore, for certain target genes, such as the V_{1b} receptor, protein expression should also be investigated in future work.

6.4.5 Summary of chapter findings

- High salt induced an increase in free (bioactive) plasma glucocorticoid concentration.
- High salt increased plasma copeptin levels, indicative of magnocellular AVP-secreting neurons, which could bypass glucocorticoid feedback and support sustained activation of the HPA axis.
- High salt decreased hippocampal MR mRNA expression which could have implications on the tone of the HPA axis.
- High salt amplified the stress response sensitivity with the potential mechanisms behind this being AVP driven and/or salt shifting hippocampal MR:GR balance.

Chapter 7: Discussion

This thesis aimed to investigate the relationship between salt, glucocorticoids and BP, as illustrated in Figure 7.1. In order to address this, several interactions were explored including:

1. The impact of salt on BP in C57BL/6JCrI mice.
2. The impact of glucocorticoids on BP in C57BL/6JCrI mice.
3. The impact of salt and glucocorticoids on BP in C57BL/6JCrI mice.
4. The impact of salt on glucocorticoids in C57BL/6JCrI mice.

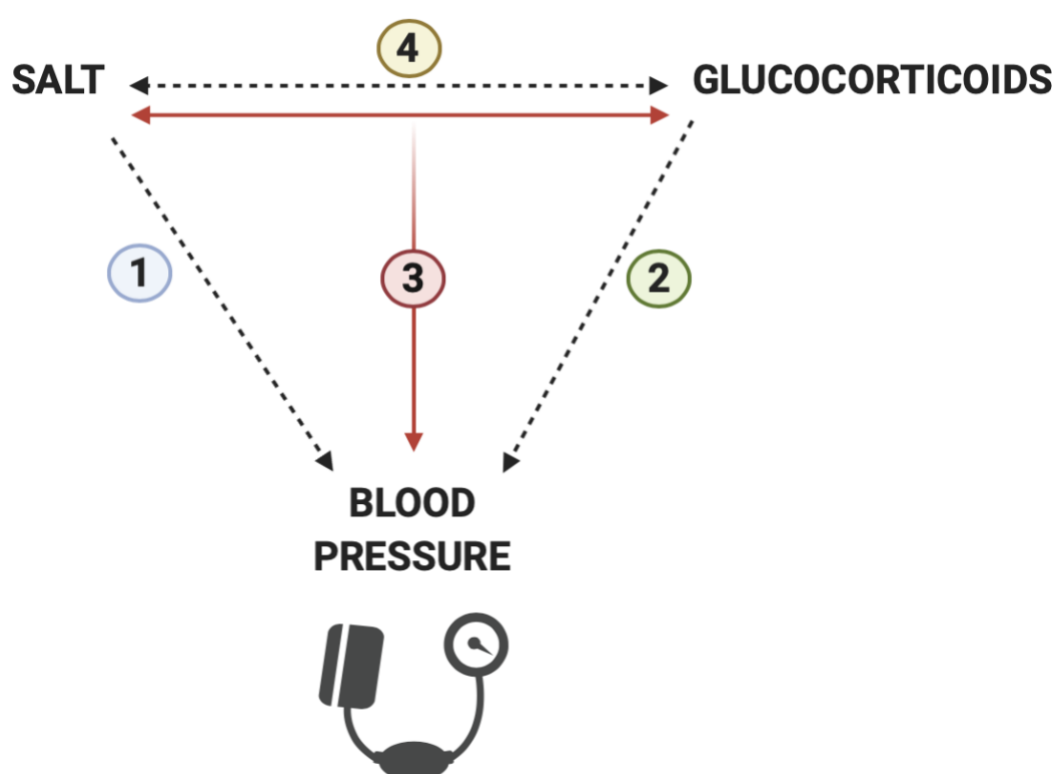


Figure 7.1: Exploring the interaction between salt, glucocorticoids and blood pressure.

A schematic diagram of the interactions addressed in this thesis including the impact of salt on blood pressure (BP) (1), the impact of glucocorticoids on BP (2), the impact of salt and glucocorticoids on BP (3) and finally the impact of salt on glucocorticoids (4). Diagram created with BioRender.com.

The major findings of this thesis was that I uncovered a relationship between salt and glucocorticoids; increased dietary salt drives rises in circulating glucocorticoids; glucocorticoid excess amplifies the abnormal cardiovascular response to high salt intake. This vicious cycle may be implicated in hypertension and CVD.

7.1 Salt-sensitivity in the ‘salt-resistant’ C57BL/6JCrI mouse

C57BL/6 mice have often been reported as salt-resistant, with studies relying on this mouse strain for investigating salt-sensitivity and hypertension^{44,165}. However, others have showed they were salt-sensitive¹⁶⁶. Therefore, the BP response of the C57BL/6JCrI mouse strain used throughout this thesis to high salt had to be investigated. In our hands, C57BL/6JCrI mice are salt-sensitive. The mechanisms behind salt-sensitivity in these animals was not a focus of this thesis. However, I was involved in the work by Dr Ralph in the lab¹⁶⁷ showing salt-sensitivity was due to elevated levels of epinephrine, increased vascular sensitivity to α adrenoceptor activation and increased sympathetic activity.

7.2 The ACTH-dependent Cushing's syndrome mouse model

The main finding when exploring the impact of glucocorticoids on BP in this model of ACTH-dependent Cushing's syndrome mouse model was that these mice were not hypertensive but exhibited non-dipping BP. Non-dipping BP profile would be missed during regular GP appointments but an important diagnosis to make as individuals with normotensive non-dipping BP have an elevated risk for CVD compared with normotensive dippers^{177,304}. Further characteristics of this model were found including increased plasma glucocorticoid concentration, increased plasma copeptin concentration (surrogate marker of plasma AVP concentration), increased RBF and renal vascular dysfunction (Figure 7.2).

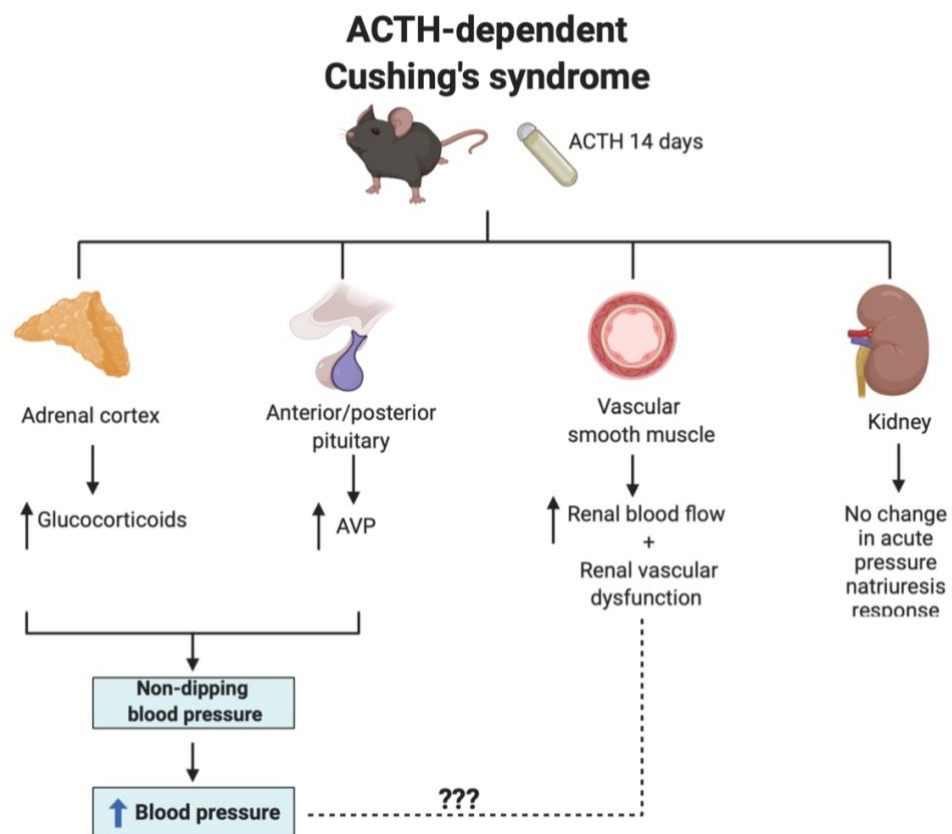


Figure 7.2: Proposed characteristics of the ACTH-dependent Cushing's syndrome mouse model.

Modelling the ACTH-dependent Cushing's syndrome involved minipump infusion of 2.5 µg/day of ACTH in C57BL/6J adult male mice. Non-dipping blood pressure leading to increased blood pressure is proposed to be due to increased plasma glucocorticoids and increased plasma AVP levels. Increased renal blood flow and renal vascular dysfunction has been found in the model. However, whether this is involved in increasing blood pressure remains unclear. Diagram created with BioRender.com.

The increase in plasma glucocorticoid and copeptin concentration during the inactive period of the mice is thought to contribute to the non-dipping BP and leading to increased overall 24 h BP. This is supported by reports of GR-dependent contraction of volume depletion, hyperosmolality and hypernatremia in ACTH-treated mice, which is known to stimulate AVP release¹²⁸. This study suggested AVP was involved in sustaining the increase in BP seen with chronic ACTH. Furthermore, as previously mentioned, high magnocellular AVP can stimulate ACTH secretion from the anterior pituitary, leading to increased glucocorticoid synthesis. This could mean that the HPA axis is in a viscous loop of glucocorticoids altering plasma osmolality which increases AVP which could then ultimately increase plasma glucocorticoids, dysregulating these systems and impacting BP homeostasis. To test this hypothesis, the gene and protein expression of the V_{1b} receptor in the anterior pituitary will be assessed in ACTH-treated mice. If altered, a V_{1b} receptor antagonist will be used and plasma glucocorticoids measured. Following this, if plasma glucocorticoids are reduced with V_{1b} receptor antagonism, radiotelemetry will assess if V_{1b} receptor activation plays a role in non-dipping BP.

BP is reported to increase by ~20 mmHg in Cushing's patients¹⁶⁸. Furthermore, mouse studies have shown ACTH infusion to increase BP by a similar magnitude using telemetry^{115,169–171} and under anaesthetic¹²⁸. However, this model caused BP to increase only by ~8 mmHg. The reason behind this is not fully understood. Differences were seen in renal haemodynamics, renal and vascular function compared to what has been previously reported. There have been variable results of the effects of chronic ACTH (with subsequent increases in glucocorticoids) and glucocorticoids on RBF and RVR. RBF was increased in some studies^{125,197} but not in all studies¹²⁸. However, it is thought that increases in RVR play a major role in elevations in BP by ACTH/glucocorticoids in humans and rats. There is a vast literature supporting the potentiation of vasoconstrictor action by chronic ACTH/glucocorticoids^{118,119}, potentially explaining the increases in RVR. This

could explain the BP differences in our model as I report reduced maximal contraction and sensitivity to PE in renal arteries with decreases in RVR in our ACTH-treated mice. Furthermore, ACTH infusion has also been associated with reduced sodium excretion, likely contributing to elevated BP^{111,128}. This was not found in our model as there were no changes in renal sodium excretion, although there did appear to be a flattening of the diurnal sodium excretion.

Regardless of the differences seen in our model of ACTH-dependent Cushing's syndrome, glucocorticoid excess was accomplished and was used to investigate the relationship between glucocorticoids, salt and BP homeostasis.

7.3 Proposed mechanism behind amplification of salt-sensitivity by glucocorticoid excess

Chronic glucocorticoid excess amplified salt-sensitivity in male C57BL/6JCrI mice. This major finding supports emerging evidence that abnormal glucocorticoid activity plays a major contributory role in salt-sensitivity. The potential mechanisms behind this amplification of salt-sensitivity were investigated throughout this thesis. The mechanisms underlying salt-sensitivity remain elusive but two fundamental mechanisms are thought to be involved – impaired renal salt excretion and vascular dysfunction.

7.3.1 Renal vs vascular dysfunction

Guyton proposed that hypertension, including salt-sensitive hypertension, can only develop if there is impairment of renal salt excretion as the PN response is shifted to the right at a higher arterial pressure^{66–68}. Furthermore, Hall proposed impaired PN is required for a salt-sensitive phenotype³⁴. Renal sodium excretion was measured via metabolic cages and by measuring the acute PN response to determine if this contributed to the salt-sensitive phenotype. Renal sodium excretion in chronic glucocorticoid excess was unchanged, although there did appear to be a flattening of the diurnal sodium

excretion, with no change in the acute PN response. Therefore, based on these data, renal dysfunction was unlikely to be involved in salt-induced BP increases seen in this model of chronic glucocorticoid excess, moving the focus on to the vasculature. Although, the assessment of the acute PN response will be repeated using the optimised protocol explained in Chapter 4. This optimised protocol is in the process of being published (Stewart et al). Measuring medullary blood flow, using a laser-Doppler needle flow probe, would be of interest here as an additional approach to assess sodium excretion as medullary blood flow has been described as a major determinant of sodium excretion and thought to play an important role in determining the set point and slope of the PN relationship³⁰⁵. This is due to an incomplete medullary autoregulation which increases both medullary and papillary blood flow to increase medullary interstitial hydrostatic pressure and inhibit sodium reabsorption¹⁹⁹.

My initial data indicated that renal, not mesenteric, vascular dysfunction may contribute to amplified salt-sensitivity in glucocorticoid excess. The renal artery is responsible for perfusing the kidney, with RBF accounting for approximately 25% of CO¹⁹⁹. In normal conditions, RBF remains relatively constant over a range of pressures due to myogenic responses. Alteration in renal haemodynamics were present in glucocorticoid excess, with increases in RBF and decreases in RVR. Surprisingly, this had no impact on GFR. Furthermore, renal autoregulation appeared to be impaired. The changes in renal autoregulation could be explained by reductions in sensitivity and maximal contraction/dilation to known vasoconstrictors/dilators in the renal artery, likely impacting myogenic tone. Myogenic responses to changes in transmural pressure are greater in renal arteries (and also cerebral) than mesenteric arteries, which may explain the impairment responses seen solely in the renal artery¹⁹⁹. However, it is important to note that the vascular reactivity of only renal and mesenteric arteries were assessed in this thesis and would be of interest to measure the myogenic response using pressure myography in

these vascular beds including other vascular beds, including the cerebral artery.

Does vascular function contribute to salt sensitivity? Glucocorticoids have different cardiovascular effects, including vascular remodelling, tone and inflammation, depending on the receptor they bind⁹⁸. Glucocorticoids binding to VSM GR have been shown to increase contractility to norepinephrine in the forearm³⁰⁶, decrease proliferation³⁰⁷ and migration³⁰⁸. Bound to endothelial GR, it can cause decreases in endothelium-dependent vasodilation²⁰⁹ and decreases in angiogenesis³⁰⁹. However, when bound to VSM MR, they can increase perivascular inflammation³¹⁰ and have variable effects on vasoconstriction³¹¹. In the endothelium, MR activation has variable effects on vasodilation³¹¹. Overall, glucocorticoids binding to either GR or MR in the vasculature can result in hypertension and the balance between expression and ligand access to MR and GR can impact the outcome. By looking at gene expression levels in both renal and mesenteric arteries, GR gene expression was significantly reduced with no change in MR expression in renal arteries of ACTH-treated mice. Unlike the mesenteric arteries, which had no changes in GR and MR expression. The potential role of MR in salt-sensitivity and renal vascular dysfunction following glucocorticoid excess was assessed. MR blockade improved renal vascular sensitivity and maximal dilatory response to ACh and SNP. However, had no effect on BP. This suggests potentially an alternative mechanism is contributing to amplification of salt-sensitivity in our mouse model of ACTH-dependent Cushing's syndrome. However, as previously explained, this study needs to be repeated with appropriate controls. Preliminary data began to investigate the arterial structure of ACTH-treated mice. Gene transcript levels of elastin and emilin-1 were both reduced following ACTH treatment. However, planned studies to examine the arterial structure further by staining renal and mesenteric arteries for extracellular proteins including elastin were unable to be complete due to COVID-19. Overall, the mechanism(s) involved still remain unclear.

The role of PVAT in vascular regulation and remodelling was not investigated but could potentially be relevant in this setting. PVAT has been shown to regulate vascular tone via contractile and anticontractile effects. Furthermore, PVAT secretes adipokines, cytokines and growth factors, including TGF- β , which regulates vascular remodelling. PVAT has also been shown to modulate BP *in vivo*, as mice lacking PVAT were hypotensive and resulted in an extreme-dipper phenotype. Excessive accumulation of dysfunctional PVAT leads to increased vascular tone and arterial stiffness and is related to the development of obesity-associated hypertension. There have been reports of impaired anticontractile effects of PVAT and PVAT inflammation in these models. Since PVAT is involved in vascular physiology and pathophysiology, BP regulation and BP rhythm, the role of PVAT in vascular dysfunction seen in glucocorticoid excess should be studied^{312,313}.

7.3.2 A role of renin-angiotensin-aldosterone system in salt-sensitivity?

The non-modulation of aldosterone following salt-loading in ACTH-treated mice might contribute to amplification of salt-sensitivity. RAAS is involved in the pathogenesis of hypertension and has been implicated in salt-sensitive hypertension^{72,314}. Hepatic *Agt* gene expression was investigated in ACTH-treated mice and was significantly downregulated (data not shown), suggesting reduced Ang I, Ang II and aldosterone synthesis. Although, the proposed Rac1-MR-NCC/ENaC pathway should be investigated in this model to determine whether this could contribute to salt-sensitivity in chronic glucocorticoid excess. Circulating RAAS is only one entity of the overall RAAS system. Elements of RAAS are present in the vasculature, kidney and adipose depots including PVAT. RAAS within both the vasculature and PVAT influences vascular function³¹⁴ and RAAS within the kidney influences sodium reabsorption³¹⁵. Overall, the role of RAAS in amplification of salt-sensitivity in this model of glucocorticoid excess cannot be ruled out. Further studies to investigate the role of Rac1 and tissue RAAS would be crucial in understanding the mechanisms behind this phenomenon.

7.3.3 A role for the sympathetic nervous system in salt-sensitivity?

The SNS is also considered an important regulator of BP, with an inability to modulate the SNS linked with contributing to salt-sensitivity^{71,72}. The SNS influences renal function by affecting renal haemodynamics and renal tubular sodium and water handling. Sympathetic hyperactivity leads to decreased RBF, GFR and urinary sodium excretion, thought to contribute to the development of salt-sensitivity³¹⁶. There are several methods used to investigate SNS activity including, measuring urinary excretion of catecholamines³¹⁷, inhibiting SNS activity pharmacologically via use of ganglionic blockers (trimethaphan and hexamethonium)³¹⁸ and renal denervation. Renal denervation in animal models is accomplished either chemically (20% phenol in absolute ethanol) or mechanically stripping all visible nerves along the renal artery and vein³¹⁹. In this thesis, the role of the SNS in amplifying salt-sensitivity in the ACTH-dependent Cushing's syndrome mouse model was not investigated since RBF was increased in our model, with no change in GFR and urinary sodium excretion. Therefore, this suggests that the SNS would play a limited role in amplification of salt-sensitivity in the ACTH-dependent Cushing's syndrome mouse model.

7.4 The relationship between glucocorticoids and salt

This thesis describes how increased dietary salt over a 14 day period increased plasma glucocorticoids, specifically during their active phase, in adult male C57BL/6JCrI mice. The proposed mechanisms are illustrated in Figure 7.4. Here, I propose salt drives increases in glucocorticoids via increased circulating AVP. Since magnocellular neurons are not regulated by glucocorticoids, they can bypass glucocorticoid feedback and induce "escape from glucocorticoid feedback" in the pituitary, resulting in sustained HPA activation²⁷⁹. Furthermore, there were decreases in expression of hippocampal MR, which have implications on the set point of the stress response. There are several components of this hypothesis that have not been demonstrated experimentally. Future experiments are required to assess the

role of AVP in salt driving glucocorticoids, via the use of a V_{1b} receptor antagonist.

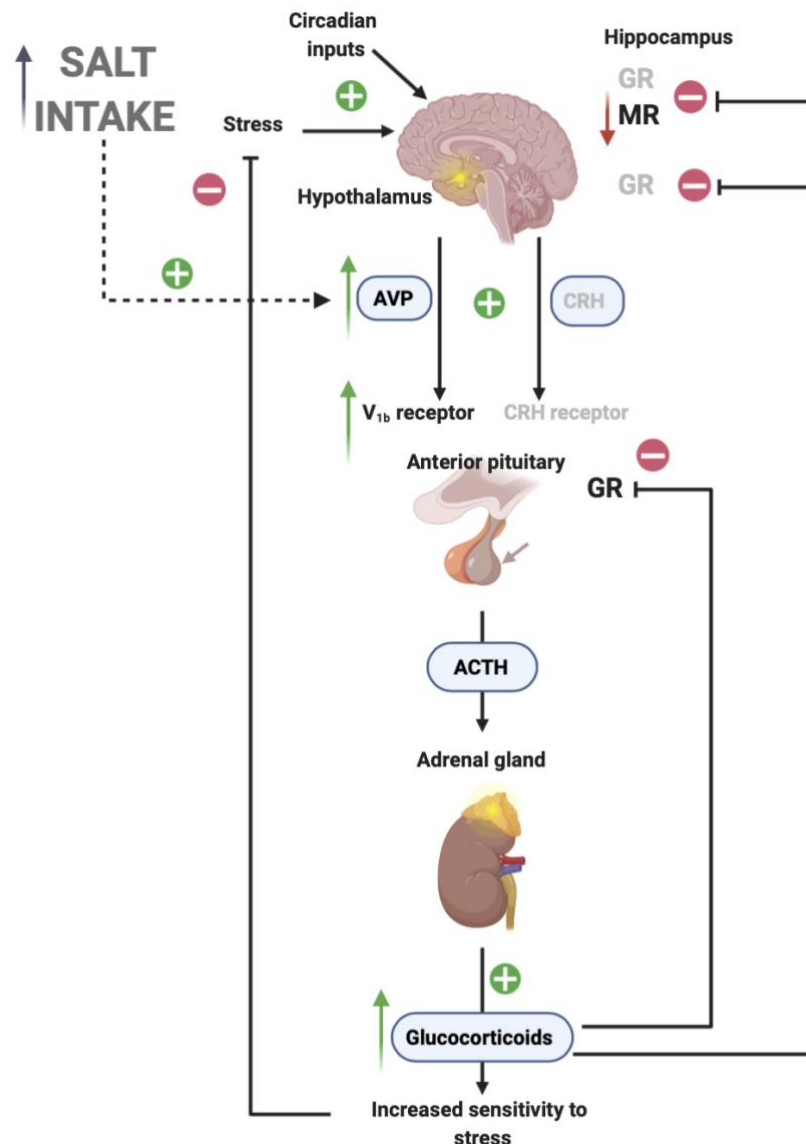


Figure 7.3: Proposed relationship between increased dietary salt and glucocorticoids.

A schematic diagram of the proposed influence of increased salt intake on the hypothalamic-pituitary-adrenocortical (HPA) axis to influence glucocorticoids. Increased salt intake acts as an osmotic stressor leading to increases in circulating AVP and increases in its receptor (V_{1b} receptor) in the anterior pituitary. This increases the synthesis of ACTH to increase glucocorticoid synthesis. With decreases of hippocampal MR expression, this can influence tone of the HPA axis, resulting in increases in the sensitivity to a stressor. Diagram created with BioRender.com.

Interestingly, in a state of glucocorticoid excess, high salt caused a rise in both day and night plasma glucocorticoids. The mechanisms behind this doubling

of plasma glucocorticoids following a high salt challenge during glucocorticoid excess has not been explored. Although, a question that has arisen from this work was does glucocorticoid excess cause a shift in the behavioural drive to consume salt? Salt appetite is driven by central pathways in response to negative salt balance. Salt appetite can be enhanced by glucocorticoids³²⁰. A link between MR activation in the NTS and salt appetite has previously been shown, where reduced 11 β -HSD2 activity in the brain promoted salt-appetite and a transition from salt-resistance to salt-sensitivity in mice¹³⁹. With reduced 11 β -HSD2 activity, MR is no longer protected from glucocorticoid binding, suggesting that glucocorticoids could be associated with salt-appetite. In a model of glucocorticoid excess, it would be of interest to investigate whether increased glucocorticoids promotes a hunger for salt and if this contributes to the drastic rise in plasma glucocorticoids, and potentially the amplification of salt-sensitivity.

Glucocorticoids are intrinsic in restoring homeostasis following exposure to a stressor. Therefore, I wanted to investigate whether salt impacted the stress response. I observed that 14 days high salt increased the sensitivity to an acute stressor. The physiological and psychological impact and mechanisms behind this were not explored in this thesis. However, a link between salt and stress has been suggested, where psychological stress has been shown to increase salt appetite, which could then enhance overall vulnerability to stress³²¹. This link has been further supported by a study which proposed that high salt increased stress responsivity by enhancing stress coping behaviour. This was thought to involve increased number of AVP positive PVN neurons with increased activation. It is important to note that this study reported increased serum corticosterone levels in mice following 4% saline consumption³²². It was first suggested by this study that this was due to hypovolemia but mice consuming 2% saline also presented with hypovolemia without hyperosmolality or elevated corticosterone, highlighting hypovolemia was not sufficient to increase glucocorticoids in this study. It has been suggested that this long-term activation of AVP-PVN neurons could increase

the likelihood of developing stress-related mood/psychiatric disorders. A study following on from this work by the same group found that high salt did not have any effect on anxiety-related behaviours, but nest building was reduced with increased voluntary social interaction. This study led on to suggest that prolonged high salt intake enhances maladaptive behavioural and cardiovascular responses to perceived stressors³²³.

7.4.1 Potential role of salt-inducible kinase?

Salt-inducible kinases (SIKs; SIK1-SIK3) are a family of related serine/threonine kinases. SIK1 plays an important role in regulating adrenocortical function, specifically ACTH-mediated induction of steroidogenic enzymes, under the stimulation of high salt and ACTH³²⁴. SIK1 also represses steroidogenic gene transcription. It has been suggested that SIK1 determines the precise timing of steroidogenic gene expression following initial ACTH stimulation³²⁵. Further work will be required to assess whether SIK1 could contribute to salt-induced increases in glucocorticoids.

7.5 Implications for a salt-saturated and stressed society

Salt is essential for normal physiological function as it is critical in BP management. However, its other so-called essential role is catering to our salt preference and need to preserve food. This has led salt to be saturated in our society with our hunger and intake for salt substantially increasing over time. This high salt intake has a major impact on public health. Evidence has shown that elevated dietary salt and salt-sensitivity of BP has been associated with an increased risk of hypertension and CVD^{11,55,59}.

Stress is another major issue in our society, with stress contributing to many psychiatric disorders. CVD is closely linked with multiple psychiatric disorders including depression and anxiety. Psychosocial stress, a major contributor of depression and anxiety, and increased salt intake has been linked with salt-sensitive BP. It has been suggested that the interactions between stress and salt contribute to the close association between psychiatric disorders and

CVD^{322,323}. Furthermore, it is thought that behavioural disruptions, potentially emerge prior to changes in cardiovascular parameters. High dietary salt intake is a known physiological osmotic stressor, which I have shown to increase plasma glucocorticoids and alters the response to stress. Therefore, could our salt-saturated society be contributing to our stressed society? Well, as previously mentioned, glucocorticoids can enhance salt-appetite³²⁰. Therefore, this suggests a cyclical relationship between salt and glucocorticoids.

Our digital use has increased substantially driving an addictive behaviour. There is a body of evidence now linking screen time effect with physiological and psychological adverse effects. Excess screen time has been linked with poor sleep, hypertension and HPA dysregulation. Furthermore, psychosocial stress is associated with digital use at night and sleep problems³²⁶. Sleep disturbances are also implicated in non-dipping BP³²⁷.

Overall, salt consumption is high, stress is common and digital use at night (environmental circadian disruptors to glucocorticoids and the HPA axis) is increasing, together increasing the risk of high BP (Figure 7.5). This ties together the key findings of this thesis – there is a reciprocal relationship between glucocorticoids and salt, where high salt intake increases glucocorticoids and when glucocorticoids are in excess, with disruptions in circadian rhythm, this can have implications on salt-sensitive BP. Together, increasing the risk for CVD. Evidence has suggested that BP response to salt and stress should be considered in strategies to prevent and control BP³²⁸. Future work in assessing the impact of prolonged high salt intake on behavioural disruptions relevant to salt-related rises in BP should be a focus.

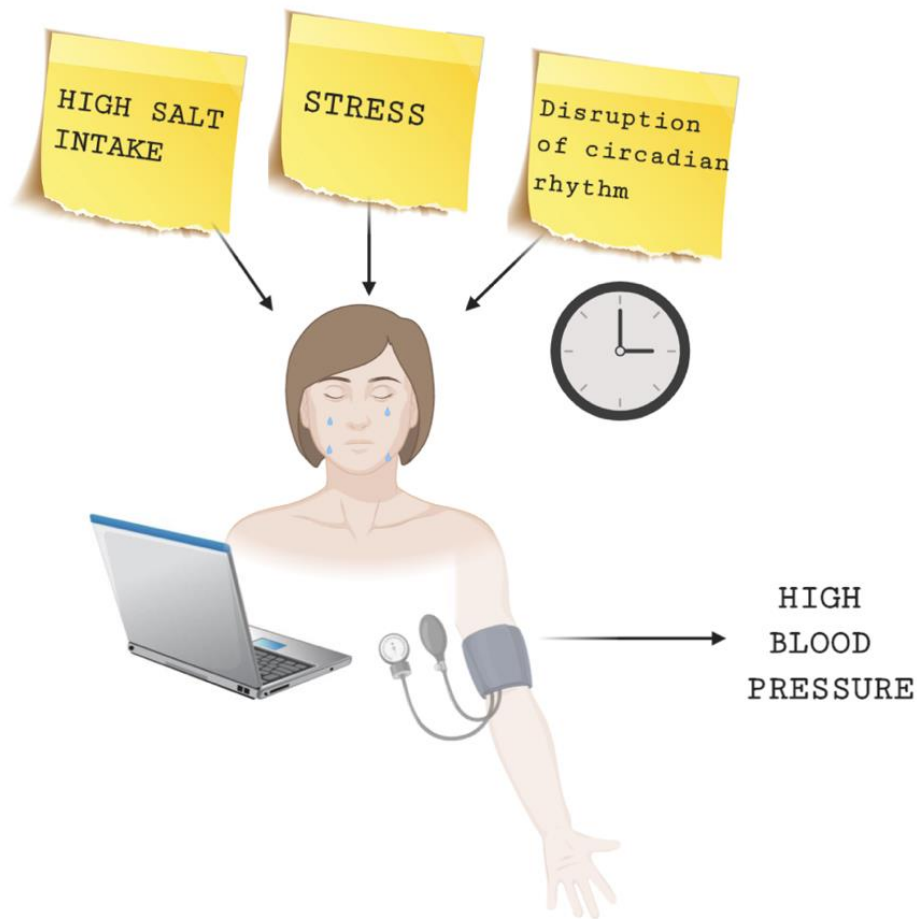


Figure 7.4: Implications for a salt-saturated and stressed society.

The interaction between high salt intake, stress with disruption of the circadian rhythm ultimately leading to high blood pressure, the leading modifiable risk factor for cardiovascular disease. Diagram created with BioRender.com.

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