Circadian clocks, glucocorticoids and the gated inflammatory response

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

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List of contents

Final word count: 84,684

List of Figures

Chapter 1:

Chapter 2:

Chapter 3:

List of Tables

Abstract

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In mammals endogenous, self sustained oscillators, known as circadian clocks, have evolved as a result of day night cycles, with a period close to 24 hours, and are involved in many physiological processes; such as sleep wake cycles, metabolic and hormonal activity. The suprachiasmatic nucleus (SCN), is the central oscillator, and is synchronised to the external environment by light, via the eye. It has been demonstrated that peripheral clocks, too, contain the circadian oscillator, with tissues such as the lung, liver, heart and kidney as well as many isolated cell types remaining rhythmic, in culture, for many days. However, these peripheral oscillators require a signal from the central oscillator in order to co-ordinate a synchronised time. Leading candidates in the relay of this information are the circulating glucocorticoid hormones corticosterone (rodents) or cortisol (man), which are known to have potent effects on the peripheral clock, both *in-vivo* and *in-vitro*. Further to this, glucocorticoids have been used for many decades to suppress the symptoms of inflammation, a by product of many human diseases.

This thesis aims to address the temporal regulation of the peripheral clock by the endogenous glucocorticoid, corticosterone, using a transgenic mouse harbouring a luciferase conjugated clock reporter, and circadian reporter cell lines. It also aims to address the relative contribution of the two closely related nuclear hormone receptors, the glucocorticoid and mineralocorticoid receptors. A further aim of the work with glucocorticoid signalling was to design a flow-though culture system, in order to address the effects of the endogenous pulsatile release of glucocorticoids on the peripheral oscillator. This thesis also aims to characterise the inflammatory response in relation to its circadian characteristics; its relationship with corticosterone and the effect of inflammation on the central clock components. Finally, this thesis aims to investigate a potential input/output of the clock, a member of the family of C/EBP transcription factors, C/EBP alpha, and whether it is under endogenous circadian control and regulated by glucocorticoids.

Work in this thesis has shown that glucocorticoids dynamically regulate the peripheral clock at all phases of the circadian cycle and that this regulation occurs mainly through the glucocorticoid receptor; yet the mineralocorticoid receptor does have a function in the immediate response to glucocorticoid administration. Furthermore, as a result of the initial temporal profile after corticosterone addition, on the clock protein PERIOD2, I have shown transient regulation of the clock through Caveolin-1 based signalling. There is also a significant circadian component to the inflammatory response, which appears, at least in part, to be REV-ERB alpha mediated, and the inflammatory response also has profound effects on circadian gene expression in the periphery. A functional flow-through system was designed and a working model produced, albeit with technical difficulties, to address glucocorticoid pulsing and circadian timing but much more work is needed for effects to be fully understood. C/EBP alpha appears not to be under circadian regulation nor under direct glucocorticoid regulation, at least in peripheral models used here.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Stephen Beesley

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Abbreviations

~	approximate
-/-	knock out
+ve	positive
-ve	negative
α	alpha
AA-NAT	arylalkylamine-N-acetyltransferase
act D	actinomycin D
ACTH	adrenocorticotropic hormone
AMP	adenosine monophosphate
Amp	ampicillin
AR	androgen receptor
AP-1	activator protein-1
ARNT	aryl hydrocarbon receptor nuclear translocator
ATP	adenosine 5’ triphosphate
AVP	arginine vasopressin
β	beta
BAT	brown adipose tissue
BCA	bicinchoninic acid assay
bHLH	basic helix loop helix
BMAL	brain and muscle ARNT- like
bp	base pair
BSA	bovine serum albumin
bZIP	basic leucine zipper
c-Fos	immediate early gene
c-Jun	immediate early gene
CAMKII	calmodulin kinase II
cAMP	cyclic adenosine monophosphate
casein kinase 1	CK1
cav-1	caveolin-1
CCL2/MCP1 (JE)	monocyte chemoattractant protein 1
cDNA	complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancer binding protein
cGMP	cyclic guanosine monophosphate
CHX	cycloheximide

CKII	casein kinase II
CLOCK	circadian locomotor output cycles kaput
con	control
COPD	chronic obstructive pulmonary disease
CORT	corticosterone
CRBP1	cellular retinal binding protein 1
CRE	cAMP response element
CREB	cAMP response element binding protein
CRH	corticotropic releasing hormone
CRY	cryptochrome
CT	circadian time
Ct	cycle threshold
CXCL1	KC
δ	delta
DAB 3-3’	diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA binding domain
DBP	albumin-D-site binding protein
DD	12hr dark: 12hr dark (constant darkness)
DEX	dexamethasone
DMH	dorsalmedial nucleus of the hypothalamus
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
D. melanogaster	Drosophila melanogaster
ε	epsilon
Ec50	half maximal effective concentration
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme linked immunosorbant assay
EMS	ethyl methane sulphonate
ENU	N-ethyl-N-nitrosurea
ER	estrogen receptor
ERRβ	estrogen related receptor β

F1	forward primer
FACS	fluorescence activated cell sorting
FAM	6-carboxyfluorescein
fASPS	Familial advanced sleep phase syndrome
FBS	fetal bovine serum
FEO	food entrainable oscillator
FRQ	Frequency
GABA	γ- aminobutyric acid
GAPDH	glyceraldehyde phosphate dehydrogenase
Gc(s)	glucocorticoid(s)
GCNF	germ cell nuclear factor
GFP	green fluorescent protein
GI tract	gastro-intestinal tract
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GSK3β	glycogen synthase kinase 3β
h	hour
HAT	histone acetyl transferase
HBSS	Hanks' balanced buffer solution
H and E	hematoxylin and eosin
HEK293	human embryonic kidney cells 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HDAC	histone deacetylase
HIOMT	hydroxyindole-O-methyltransferase
HPA	hypothalamic pituitary adrenal axis
HRP	horseradish peroxidase
11-β HSD	11-β hydroxysteroid dehydeogenase
HSP	heat shock protein
Hyg^+	hygromycin ($^+$ = hyg positive)
IgG	immunoglobulin G
IFNγ	interferon gamma
IL (6)	interleukin (6)
IMS	industrial methylated spirit
Inhib	inhibitor
IL-(6)	interleukin-(6)

i.p.	intraperitoneal (injection)
LD	12h light: 12h dark (light: dark cycle)
LPA	linear polyacrylamide
kbp	kilo base pair
KC	keratinocyte chemoattractant or keratinocyte-derived chemokine
Kd	dissociation constant
KO	knock out
LB	Luria-Bertani broth
LB Amp	Luria-Bertani broth with ampicillin
LBD	ligand binding domain
LD	12hr light: 12hr dark
LDL	low density lipoprotein
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MEFS	mouse embryonic fibroblasts
methyl	methylcyclodextrin
mGR	membrane GR
mins	minutes
MMTV	mouse mammary tumour virus
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
mut	mutant
NcoR1	nuclear co-repressor 1
N. Crassa	Neurospora crassa
NFκB	nuclear factor kappa b
nGRE	negative glucocorticoid response element
NLD	nuclear localisation domain
norpA	gene encoding phospholipase C in D.*Melanogaster*
NP-40	nonident P-40
NPAS	Neuronal PAS domain protein 2
OX	over expressed
PACAP	pituitary adenylate cyclase-activating peptide
PAI-1	plasminogen activator inhibitor 1
PAS	per ARNT single minded
PBS	phosphate buffered saline

PBST	phosphate buffered saline with Tween-20
PCR	polymerase chain reaction
PE	phycoerythrin
PEC	peritoneal exudate cells
PER	PERIOD
PER2::luc	PERIOD2 luciferase
PPAR α	peroxisome proliferator-activated receptor α
PFA	paraformaldehyde
P	phosphorylated
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
PMT	photo multiplier tube
PRC	phase response curve
PVN	paraventricular nucleus
R1	reverse primer
Rat-1	Rat-1-R12 cell line
RARα	retinoic acid receptor α
RBC	red blood cells
RLU	relative luciferase units
RGC	retinal ganglion cells
RHT	retino-hypothalamic tract
RNA	ribonucleic acid
ROR	retinoic acid receptor (RAR)-related orphan receptor
RORE	retinoic acid receptor (RAR)-related orphan receptor binding element
RPM	revolutions per minute
RT	room temperature
RU	RU486
sal	saline
SCN	suprachiasmatic nucleus
SDS	sodium dodecyl sulphate
SDS PAGE	sodium dodecyl sulphate poly acrylamide gel electrophoresis

sec	seconds
Ser	Serine
Sim	single minded protein
siRNA	small inhibitory ribonucleic acid
SPE	steptavidin phycoerythrin
spir	spironolactone
$t_{1/2}$	half life
T25/75/225	tissue culture flasks (number represents the surface area, cm^2)
TAMRA	tetramethylrhodamine
TBS	tris buffered saline
TBST	tris buffered saline with Tween-20
TF	transcription factor
THα	thyroid hormone α
Thr	threonine
TIM	timeless
TIM2	timeout
Tm	melting temperature
TNFα	tumour necrosis factor alpha
TPA	12-O-tetradecanoyl-phorbol-13-acetate
TR4	testicular receptor 4
UCP-1	uncoupling protein-1
V	volts
VIP	vasoactive intestinal polypeptide
VNTR	variable number of tandem repeats
WAT	white adipose tissue
WT	wild type
Zeitgeber	German for ‘time giver’
ZF	zona fasciculata
ZG	zona glomerulosa
ZR	zona reticularis
ZT	zeitgeber time

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Finally I would like to extend my special thanks to my supervisors Andrew and David and my advisor Julian for their guidance throughout my time in Manchester.

A poem that sits on my Gran’s kitchen wall, by Robert H Smith:

The Clock of Life

The clock of life is wound but once
And no man has the power
To tell just where the hands will stop,
At late or early hour.

To lose one's wealth is sad indeed,
To lose one's health is more.
To lose one's soul is such a loss
As no man can restore.

The present only is our own.
Live, love, toil with a will.
Place no faith in 'tomorrow'
For the clock may then be still.

Chapter 1: Introduction

1.1 Overview

The circadian clock exists in virtually all forms of life, from humans and the mammalian system to the relatively simple prokaryotic system. This high conservation, throughout evolution, suggests that the clock is of critical importance for the survival of the organism and subsequently the species. In the last 20 years a great deal of progress has been made in defining the roles of individual genes and proteins within the molecular clock. There has also been a steady progression in technology to allow the wider use of transgenic animals, allowing for rapid advances in the discovery of clock gene function. This introduction aims to provide a brief synopsis of the circadian system, addressing the discovery and circuitry of the clock, and subsequent interactions. It will provide an introduction into the regulation of the core oscillator and discuss input and output pathways, such as the inflammatory response. It will also address the known regulation of the central oscillator by glucocorticoids (Gcs), and their profound effect on the clock, which provides a running theme throughout this work.

1.2 The Circadian Clockwork

1.2.1 What Are Circadian Clocks?

It has long been reported that living organisms have a daily cycle or oscillation, with one of the earliest reports credited to Alexander the Great, in the fourth century B.C. (Fu and Lee 2003). In more recent times John Wren described, in his *Herbal Treatise* of 1662, the daily flows of the four humours; blood, phlegm, cholic and melancholy. This treatise described the fluids, which were thought to be the basis of life, and at which times of the day they were most influential (Hastings et al. 2003). Even though we now know that these ‘humours’ were not the full story, Wren was thinking along the correct lines; that rhythmic cycles do control our bodies and play a vital role within them. Furthermore, the French astronomer Jean-Jaques d’Ortous de Marian observed, in the late 1720’s, that the leaves of his Mimosa plants moved on a daily basis (Schibler 2006). To investigate matters he set about answering the question, ‘Is this movement based on environmental cues such as light or on something within?’ The answer came when he placed the plants into constant darkness and discovered that the movement persisted, and this perhaps was the first experimental demonstration of an endogenous clock.

Daily oscillatory cycles are referred to as circadian rhythms coming from the Latin *circa* (about) *dies* (a day), and thus have a period of about 24 hours. These rhythms refer to a

great variety of processes across the kingdoms; such as glucose and lipid metabolism, cell cycle control and division and sleep-wake cycles (Gallego et al. 2006). Circadian rhythms are thought to have evolved in response to the earth's rotation about its axis, forming night and day, and the orbit around the sun (Paranjpe and Sharma 2005). The earth's rotation is predictable, as are the environmental changes which accompany it, most notably the changing of the seasons. Therefore, it is reasonable to assume that over the course of geological time, organisms have evolved to read the environmental cues (Paranjpe and Sharma 2005). Circadian clocks are thought to enhance the organisms ability to survive under the changing environment with the ability to anticipate the availability of food, light and mating partners (Paranjpe and Sharma 2005), and, perhaps more importantly, the presence of potential predators. Circadian clocks are also thought to influence core body temperature, mood, cortisol (man) or corticosterone (CORT, rodent), melatonin release and performance. It is, therefore, to the organisms advantage that it evolves a method of time keeping (a circadian clock or clocks) in order to synchronise itself with the external environment; and organisms that do not, will be filtered out of the gene pool (Hastings 2003). It is also interesting to point out that circadian rhythms are temperature-compensated. This means that where most chemical reactions double in rate of reaction (speed up) for ever 10^{o}C increase (referred to as a $Q_{10} = 2$), circadian oscillations remain stable over physiological range (27^{o}C - 37^{o}C) with a Q_{10} between 0.8 – 1 (Barrett and Takahashi 1995; Izumo et al. 2003) in order to provide the organism with a precise measure of time, regardless of external or internal temperature. Studies on rat and squirrel suprachiasmatic nucleus (SCN, the location of the central oscillator, section 1.4), chick pinealocytes and hamsters have all revealed temperature compensation over a physiological range (Tosini and Menaker 1998). In fact the circadian oscillator is said to be overcompensated, meaning that it will run faster (have a shorter period) at lower temperatures i.e. less than $Q_{10}= 1$ (Izumo et al. 2003).

1.3 The Molecular Clock

1.3.1 Classical Circuitry of the Oscillator

The molecular clock is made up of a number of core genes and their respective protein products, defined as, 'genes whose protein products are necessary for the generation and regulation of circadian rhythms' (Lowrey and Takahashi 2004). This is brought about through the positive and negative regulation of genes and their protein products, with an

oscillating period of about 24 hours, (Ko and Takahashi 2006). The clock comprises of both a positive and negative arm, which act to induce and repress transcription respectively (Fig 1.1). The positive loop of the clock comprises Brain and Muscle ARNT-Like protein (BMAL1*)* and Circadian Locomotor Output Cycles Kaput (CLOCK*)*. These two are members of the basic helix loop helix (bHLH) family of transcription factors (TFs), which dimerise via their Per ARNT Single-minded (PAS) domains (Gachon et al. 2004b). The BMAL1:CLOCK heterodimer binds to the nucleotide E-box sequence (CANNTG, where N stands for any base) in the promoter regions of *Cryptochrome* (*Cry1-2)* and *Period (Per1-3)* (Ko and Takahashi 2006) and activates their transcription. The protein products of *Pe*r and *Cry* form the negative arm of the clock and the heteromeric complex of PER1-2/CRY1-2 (Gachon et al. 2004b), which interacts with the kinases Casein kinase 1 epsilon and delta (CK1ε/δ) (Gallego and Virshup 2007), F-box proteins (Godinho et al. 2007), and histone deacetylases (Naruse et al. 2004). After a delay of several hours the PER:CRY complex will translocate back to the nucleus to repress their own transcription through the inhibition the CLOCK:BMAL1 heterodimer (Lowrey and Takahashi 2004; Reppert and Weaver 2002). As a consequence *Per* and *Cry* mRNA levels fall, followed by their protein products, until their nuclear concentration is no longer high enough so sustain repression; a new cycle then begins.

NR1D1 is the 'orphan' nuclear receptor known as REV-ERBα, which along with RORα adds a further dimension to the clockwork linking both the positive and negative arms (Preitner et al. 2002). Heme was recently found to be the natural ligand, and acts to enhance the thermal stability of REV-ERBα and increases its recruitment of the nuclear receptor co-repressor (NCoR1) (Raghuram et al. 2007; Yin et al. 2007), which then increases the repressive activity of REV-ERBα. The CLOCK:BMAL1 heterodimer can activate the transcription of both nuclear receptors, *Rev-erba* and *Rora* (Preitner et al. 2002), through their E-box elements. REV-ERBα and RORα then functionally compete for the receptor element, RORE, in the promoter region of *Bmal1*, and to a lesser extent *Clock*, (Sato et al. 2004b). REV-ERBα acts to repress *Bmal1* transcription through binding to the RORE and inhibiting transcriptional protein interaction, where RORα activates transcription by binding to the same element and recruiting transcriptional proteins. RORa binds to the deoxyribonucleic acid (DNA) element at all times and is removed by competitive REV-ERBα binding (Akashi and Takumi 2005). *Rev-erbα* transcription is eventually repressed by itself and the PER:CRY heterodimer complex (Adelmant et al.

1996; Triqueneaux et al. 2004). To enhance its activity REV-ERBα can recruit the co-repressor, NCoR1, which binds to REV-ERBα via its co-repressor interaction region, discovered through yeast-2-hybrid screens (Burke et al. 1998). The full cycle (Fig 1.1) takes about 24 hours to complete, and is achieved through the described feedback loop; posttranslational modifications, (such as phosphorylation and ubiquitination) and controlled degradation.

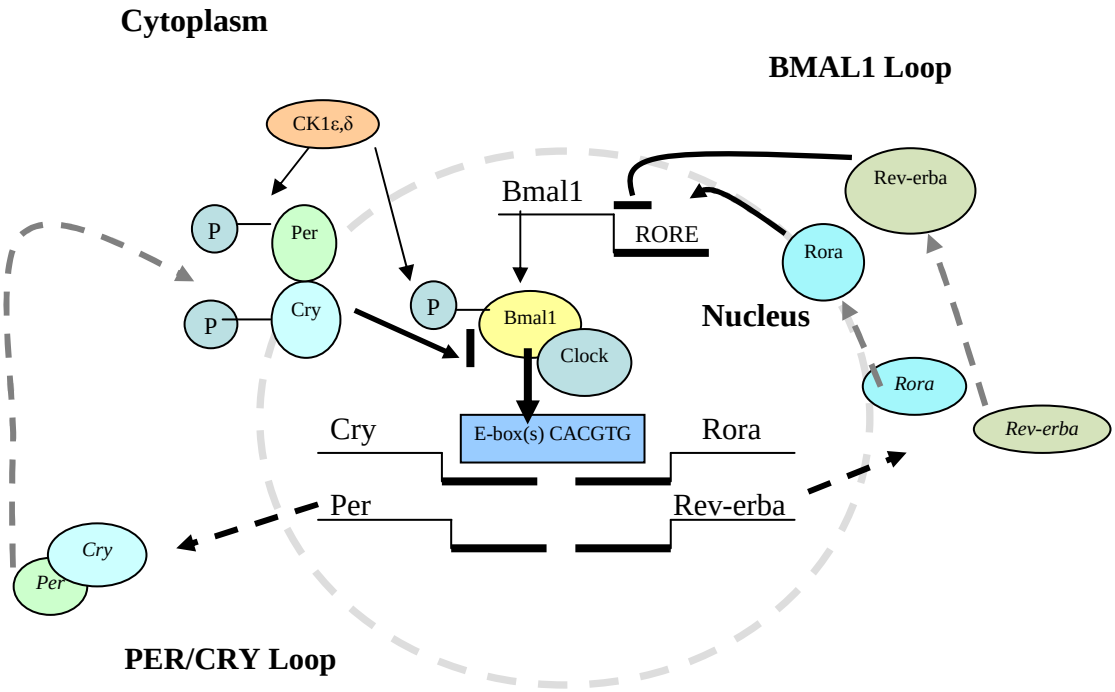

Figure 1.1: Diagram of the molecular clock: This diagram depicts a brief, simplistic overview of the circadian transcription/translational feedback loop. The dashed back arrows represent nucleus to cytoplasm translocation. The dashed grey arrows represent translation from mRNA to protein. The thick black arrows represent activation with the black bars showing repression. The thin black arrow shows phosphorylation events (P).

1.4 The Central Oscillator: the SCN

The central oscillator in the mammalian brain is a small paired bi-lateral structure called the SCN, which sits below the third ventricle and above the optic chiasm in the anterior hypothalamus (Fig 1.2). Each SCN nuclei consists of 8,000 to 10,000 neurons supported by glial cells, in the ratio of approximately 3 neurons to 1 glial, as seen in the rat (Guldner 1983). The SCN, as the central circadian oscillator, was discovered through ablation experiments carried out in the early 1970's (Moore and Eichler 1972). Surgical lesions in the rodent SCN rendered the animal arrhythmic, as seen through wheel running experiments. Interestingly though, the rhythms could be restored by the transplant of foetal SCN back into the lesioned animal. In this case the rhythm was determined by the

rhythm of the implanted SCN and not the recipient (Ralph et al. 1990; Silver et al. 1996) as was seen when a short period mutant SCN was implanted into a wild type host. These ground breaking demonstrations showed that the SCN was indeed the site of the master clock, and consequently the small structure has been well studied over the past few decades.

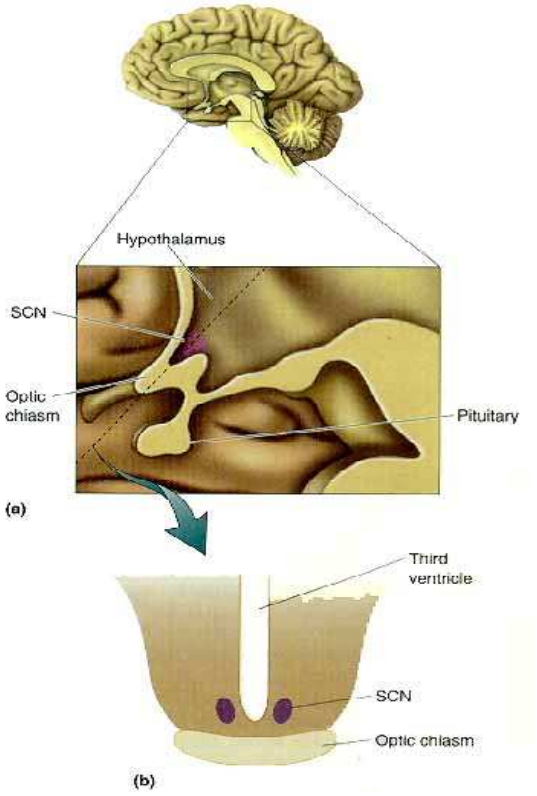

Figure 1.2: The location of the suprachiasmatic nucleus in the mammalian brain. (http://psyche.knu.ac.kr/notebook/images/c17fi16.jpg).

The mammalian SCN is composed of two distinct sub regions, both functionally and anatomically, termed the ventrolateral and dorsomedial regions, (Fig 1.3). The ventrolateral region receives information from the retino-hypothalamic tract (RHT) originating in the retina. This induces *Per1* via the MAPK pathway and occurs mainly in the ventrolateral SCN, which also contains the greatest density of retinal fibres (Hamada et al. 2001). However, using a *Per1::*luciferase transcriptional reporter assay, in cultured SCN brain slices, Nakamura et al. (2005) showed cellular desynchronisation within the SCN itself. Using this reporter, they showed that *Per1* was first transcribed in the dorsomedial region with *Per1* transcription in the ventrolateral region peaking about 1 hour later (Nakamura et al. 2005). This goes a little way to understanding how complex these structures are, as they are not one synchronised population of cells but are divided into sub

regions showing independence from each other, in terms of the molecular interactions and transcriptional events.

In addition to the SCN as the central oscillator other self-autonomous oscillators have been discovered, in the central nervous system, such as those in the retina and olfactory bulb. Semi-autonomous oscillators have been identified too, in brain regions such as the amygdale and bed nucleus of the stria terminalis (Guilding and Piggins 2007). In these semi-autonomous structures individual cells can oscillate, but are unable to maintain tissue synchronicity without input from the central oscillator, namely the SCN.

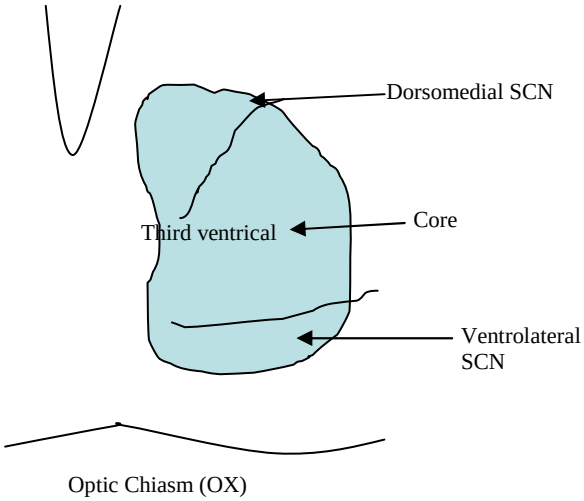

Figure 1.3: Representation of one of the paired SCN nuclei showing the three functionally separate regions. Suprachiasmatic nucleus (SCN). Reproduced from (Hirota and Fukada 2004).

1.5 Entrainment of the circadian clock

1.5.1 Overview

It is generally regarded that there are three properties that define a circadian rhythm: persisting free-running rhythms (in constant conditions), entrainment and temperature compensation (Pittendrigh 1960). In the following I will deal mainly with entrainment, but will touch on temperature compensation when reviewing temperature as a zeitgeber in its own right. Persistent free-running rhythms will not be discussed here.

The work in this thesis will focus on the ability of the endogenous Gc, CORT, to re-set the peripheral clock. However, circadian entrainment by Gcs is just one method, and although

a very potent zeitgeber there remain many more forms of entrainment. Circadian entrainment itself can be categorised into two basic groups: photic and non-photic, where photic entrainment is concerned with the circadian influence of light on the organism and non-photic entrainment concerns everything else: food, exercise, temperature, and chemical stimuli (Challet 2007; Mendoza 2007; Rensing and Ruoff 2002). Essentially, all known zeitgebers (photic and non-photic) are able to phase shift the endogenous clock of the organism and re-set internal time keeping, and will be discussed in turn in the following.

1.5.2 Photic entrainment

1.5.2.1 Light exposure

Biological clocks are endogenous oscillators that will persist under constant condition, for example, in constant darkness, with a period of about 24 hours, (Welsh et al. 1995). In reality, free-running oscillations will be slightly more or less than 24 hours, but are re-aligned with solar time via light, a process called light entrainment. Light is a predominant entraining factor for circadian clocks, in most organisms.

Light from the environment, such as the sun, is captured by the photopigments in the rods and cones in the retina but also by the photopigment melanopsin, found in a subset of retinal ganglion cells (RGC) (Hirota and Fukada 2004). Melanopsin has been show to be indispensable, as knocking out the melanopsin gene causes the RGCs to lose intrinsic photosensitivity (Lucas et al. 2003), but are unchanged by other means, such as morphology. On light stimulation, in the early night, the amino acid glutamate and the neuropeptide, pituitary adenylate cyclase-activating peptide (PACAP) are released from the terminal synapse of the RHT and bind to their respective receptors on the surface of the SCN neurons (Reppert and Weaver 2002). This triggers a cascade of phosphorylation events, which includes calcium calmodulin kinase II (CaMKII), MAPK, and cAMP response element binding protein (CREB). The phosphorylated CREB, a transcription factor, then binds to the cAMP response element (CRE) in the *Per1* promoter leading to chromatin remodelling, resulting in the up regulation of *Per1* transcription (Albrecht et al. 1997). It is also known that CaMKII can act on the *Per2* promoter, while an alternative pathway promotes the transcription/translation of *Bmal1*, so that the protein product may dimerise with CLOCK and induce *Per1 and 2* transcription. There are several other genes

activated by this series of events called intermediate early genes such as *cFos* and *cJun*, stimulated in response to entry into the cell cycle. The light activation of the *Per* genes only occurs at night and is most likely to play an important role in the phase resetting of the clock (Hirota and Fukada 2004), as the SCN only responds to light during the subjective night (Takahashi et al. 1984) . Upon a light pulse at the end of the night a different pathway is activated. Nitric oxide and cyclic guanosine monophosphate (cGMP) are activated and with the phosphorylation of CREB this activates *Per1* only (Fu and Lee 2003). This appears to be the major molecular difference between the observed clock delay, in response to a light pulse in the early night, and a clock advance, in response to a pulse in the late night.

The ability of light to phase shift the SCN (as measured by behavioural activity cycles) is time of day dependent and occurs during the subjective night (under DD) in both nocturnal (Takahashi et al. 1984) and diurnal animals (Caldelas et al. 2003). Furthermore, there is often an unresponsive 'dead-zone' (Daan S 1976; Takahashi et al. 1984) during the subjective day where light has no phase shifting effects, but this is not always the case, as work in humans has failed to reveal any form of 'dead-zone' (Khalsa et al. 2003). During the light responsive window in the SCN, very similar biochemical events occur in both nocturnal and diurnal animals. A light pulse, at night, will induce *cFos* expression in the SCN both nocturnal and diurnal animals (Kornhauser et al. 1990; Schumann et al. 2006) as well as *Per1* and *Per2* transcript and protein expression (Caldelas et al. 2003; Shigeyoshi et al. 1997; Yan and Silver 2004). The light pulse will only induce *cFos* during the light responsive window and as a result is itself under circadian regulation (Kornhauser et al., 1990). It is possible that individual species are responding to tonic levels of light intensity, as diurnal species are subject to far greater levels of light than nocturnal species (first hypothesised by Jurgen Aschoff) (Daan 2000). Recent work on the diurnal ground squirrel (*Spermophilus citellus*) has added to this hypothesis, as it becomes active more than 3h after civil twilight (the point before sunrise where there is just enough light to clearly distinguish objects and define the horizon) and becomes inactive 2-3h before civil twilight at dusk (Hut et al. 1999). This suggests that it is not just light that is required but a specific intensity, to induce a response.

1.5.2.2 Dark exposure

The re-setting effects of a light pulse in constant darkness (DD) (described above) have been extensively studied and reviewed in both nocturnal and diurnal mammalian species, but relatively few studies have focused on the reverse effect; a dark pulse in constant light (LL). This paradigm can also re-set the clock with phase advances in the subjective day and early night and delays in the subjective night and early day (Canal and Piggins 2006; Dwyer and Rosenwasser 2000). Interestingly, these dark pulse effects have two features; they induce both anti-photic and non-photic re-setting. In terms of anti-photic re-setting a dark pulse will induce phase advances during the subjective day and delays during the subjective night (opposite to light responses) as seen in the hamster (Boulos 1982), and therefore opposite to that of light pulse induction. Non-photic, dark pulse induction is said to occur when a prolonged dark pulse causes increased activity of the nocturnal species, and this increased activity contributes to the phase re-setting effects (Reebs et al. 1989). Studies between nocturnal and diurnal species have shown that, unlike light pulses where the effects are the same between the two sets of species, with regard dark pulses, re-setting occurs primarily during the rest period regardless of the active period of the animal, and therefore the re-setting effects between dark and light in nocturnal and diurnal species are different (Mendoza et al. 2007).

1.5.3 Non-photic entrainment

Non-photic entrainment can take many forms (chemical stimuli, scheduled exercise, scheduled feeding and temperature) which can be classified into two categories, arousal dependent and independent timing cues (Challet 2007). In terms of chemical stimuli melatonin, serotonin and GABA are three of the main zeitgebers which can effectively entrain the animal to a circadian rhythm.

1.5.3.1 Melatonin

Melatonin is produced and secreted at night by the pineal gland, in both nocturnal and diurnal animals (Slotten et al. 2002), from its pre-cursor amino acid L-tryptophan and the two rate limiting enzymes arylalkylamine-N-acetyltransferase (AA-NAT) and hydroxyindole-O-methyltransferase (HIOMT) (Simonneaux et al. 2003a). However, more recent studies have shown that melatonin is also synthesised and secreted into the gastro-intestinal tract (GI tract), and that pinealectomy although results in the loss of a day/night

melatonin rhythm, levels are still present in the blood due to its production in the GI tract (Konturek et al. 2007). Melatonin is secreted independently of activity patterns and is thus considered an arousal independent timing cue (Simonneaux et al. 2003b; Vivanco et al. 2007). The SCN controls pineal synthesis of melatonin, and as the SCN itself expresses a high number of high affinity melatonin receptors, it is viewed that melatonin provides a feedback loop on the SCN (Pevet et al. 2006). It has been shown that melatonin can phase advance the behavioural rhythm of mice when injected at the onset of the subjective night. Furthermore, a daily infusion of melatonin, at the onset of subjective night, has been shown also to entrain rats (Baggs et al. 2009; Benloucif 1996). It also appears that the effects of melatonin are independent of the animal being nocturnal or diurnal, as the phase shifting effects of melatonin are observed at the same time of day in the diurnal rodent (Slotten et al. 2002). The major difference between the effects of melatonin and light is seen at the molecular level. As discussed above, light induces *Per1* and *Per2* transcript and protein expression in the subjective night, yet melatonin does not do this. However, it has been shown that melatonin can phase advance *Rev-erbα* mRNA (a repressor) and induce prolonged expression of *Rorβ* mRNA (an activator), as seen in the rat SCN (Agez et al. 2007).

1.5.3.2 Serotonin

Serotonin, also known as 5-hydroxytryptamine, is a neurotransmitter important in circadian entrainment. The SCN and the intergeniculate leaflet (which have direct neuronal projections to each other) receive neuronal projections from the raphe nuclei, which transmit the serotonin signal (Meyer-Bernstein and Morin 1996). Activation of neurons by serotonin is regarded as arousal-dependent (unlike melatonin above), as activation of serotonin neurons has been shown to correlate well with behavioural responses (Jacobs et al. 1990). The expression levels of serotonin, in the SCN, are in phase with the activity pattern of the animal, shown in both nocturnal and diurnal rats (Cuesta et al. 2008; Poncet et al. 1993). Activation of serotonin neurons with serotonin agonist injections, either systemic or directly into the brain, has been shown to phase advance the activity of nocturnal rodents, when given during the subjective day, yet with no change seen with administration at other times (Bobrzynska et al. 1996; Tominaga et al. 1992). Importantly these phase shifts were not as a result of increased activity, as preventing the animal (hamsters) from moving did not abolish the observed phase shift. In contrast the action of melatonin, discussed above, serotonin agonist studies have the opposite effect in the

diurnal compared to the nocturnal rodent. The diurnal animals' sensitivity to the serotonin agonist is primarily during the subjective night rather than the subjective day (Cuesta et al. 2008). Together these data show that serotonin acts to phase shift the SCN clock during the animals' rest period.

Re-setting the SCN clock by means of serotonin agonists has been associated with a down regulation of *Per1* and *Per2* mRNA, as seen in the hamster, which occurs during the subjective day (rest period of nocturnal animals) correlating well with the phase shifting activity of the agonist (Horikawa et al. 2000). Further evidence to support this idea was presented with the knockdown of *Per1* in the hamster producing large phase shifts when administered during the subjective day, rather than the subjective night (Hamada et al. 2004). These experiments have led to the conclusion that the suppression of *Per1* mRNA is the cause, rather than the consequence, of the phase shifting effects. This method of entrainment, through the suppression of *Per1* mRNA, is in direct contrast to the effects of both light and melatonin. As of yet a molecular model to how diurnal animals are phase advanced is unknown, but data from Cuesta et al. (2008) has shown that neither *Per1*, *Per2*, *Rev-erb* α or β or *Ror* α or β transcripts are changed after serotonin agonist administration.

1.5.3.3 γ-Aminobutyric acid (GABA), Triazolam and the methamphetamine oscillator

GABA is very important neurotransmitter involved in circadian timing, and virtually all neurons of the SCN and the intergeniculate leaflet contain GABA neurons. It has been shown to be vital in the coupling of the dorsal and ventral parts of the SCN (Fig 1.3) (Albus et al. 2005; Moore and Speh 1993). The activation of GABA receptors with the known agonist muscimol will phase advance behavioural rhythms in nocturnal animals but delay diurnal animals even when administered at the same subjective time (Novak and Albers 2004; Smith et al. 1989). As such, the phase shifting effects of GABA are independent of activity and are classed as arousal independent, like that of melatonin. The phase shifting effect of GABA (as seen through agonist studies) has been associated with a decrease in *Per1* and *Per2* mRNA expression in the SCN of the nocturnal hamster, yet only *Per2* suppression in the diurnal rat when administered during the subjective day (Ehlen et al. 2006; Novak et al. 2006). Furthermore, neuropeptide Y has been shown to have non-photic phase re-setting effects in the SCN (Maywood et al. 2002). Injection of neuropeptide Y adjacent to the SCN and transfer to darkness 7h before lights out shifted

the circadian activity cycle, yet a light pulse for 1h immediately after injection blocked this behavioural response. Also *mPer1* and *mPer2* mRNA levels were suppressed 3h after injection but *mClock* mRNA and mCLOCK protein were unaltered (Maywood et al. 2002).

Triazolam is a drug often used as a mild sedative to treat insomnia, but has also been shown to have a circadian effect too. This short acting benzodiazepine can result in a PRC very similar to that of neuropeptide Y (Turek and Losee-Olson 1987), which is characterised by large phase advances during the subjective day and small if any delays during the subjective night (Albers and Ferris 1984; Smith et al. 1992). Further work showed that Triazolam phase shifting was mediated through the serotonin containing neurons of the median, but not dorsal raphe nucleus and that this pathway was independent of the pathway used for the phase shifting effects of novel wheel access (Meyer-Bernstein and Morin 1998).

Methamphetamine is a nervous system stimulant, which is similar in structure to amphetamine, and is regarded as a drug of abuse. It acts to block the reuptake to dopamine, leading to high levels in the brain. Dopamine is involved in reward, motivation and pleasure systems and as such produces a sense of euphoria when taken, therefore leading to its potential abuse (information taken from National institute on drug abuse website, http://drugabuse.gov). However, when methamphetamine was administered to rats via drinking water, a phase delay in their activity rhythm was observed with respect to the LD cycle (Honma et al. 1986). In some cases rats showed two distinct rhythms, one entrained to the LD cycle and another free-running rhythm. Once the methamphetamine was removed it took 2-3 days before the stable entrained LD rhythm persisted i.e. without the free-running methamphetamine rhythm (Honma et al. 1986). The changes observed in LD also persisted in DD, and the period of the animal was significantly shorter in the presence of methamphetamine compared to the period after its removal (Honma et al. 1986). Honma and collegues, continued with their investigations and lesioned the SCN, thereby abolishing the locomotor rhythm, yet with the presence of methamphetamine in the drinking water a locomotor rhythm appeared, which did not entrain to a LD cycle (Honma et al. 1987). The activity rhythm was also dose dependent with a lower dose producing a shorter period. In addition to this, the two components of the free-running period (activity and rest periods) were altered in a dose dependent manner. A low dose of methamphetamine produced a shorter activity time and longer rest time, however when the

drug was administered continuously through an osmotic pump an activity pattern similar to that of the control SCN lesioned rats was observed i.e. arrhythmic (Honma et al. 1987). This suggested that the observed entrainment to methamphetamine is independent of the SCN and therefore leads to the idea of an SCN independent oscillator. Despite the methamphetamine oscillator being know about for over 2 decades very little is known about it, starting with it location in the brain, yet even that is still a hypothesis, but it is presumed that it works through dopaminergic and/or serotonergic pathways (due to the action of the drug). It has been suggested that the methamphetamine oscillator works independently of the classical circadian system i.e. not through CLOCK:BMAL1 and PER:CRY. Evidence for this is two fold with mice carrying the $CLOCK^{\Delta 19}$, and double *Cry* knock out mice, both of which are arrhythmic in DD, become rhythmic when given methamphetamine in their drinking water (Honma et al. 2008; Masubuchi et al. 2001). Furthermore, NPAS2 -/-, tau and *Clock* mutants, which are rhythmic with pure water to drink lengthened their periods in the presence of methamphetamine and *Per1*/*Per2* double -/-, *Cry1*/*Cry2* double -/- and *Bmal1* -/-, which are arrhythmic with pure water, were rhythmic in the presence of methamphetamine (Mohawk et al. 2009). Together suggesting that the methamphetamine oscillator does not employ the canonical clock genes as none of the mutants above affected this new oscillator. However, much more work needs to be carried out on this novel oscillator.

In summary of the overview of photic and non-photic re-setting stimuli described above, light acts as the strongest zeitgeber within the SCN and will induce both *Per1* and *Per2* mRNA expression at the same circadian time (CT), irrespective of the animal being nocturnal or diurnal. (CT 0-12h is referred to as the subjective day i.e. when the lights would usually be on and CT 12-24h is referred to as the subjective night i.e. when the lights would usually be off). This leads to the hypothesis that animals respond to tonic levels of light rather than it acting as a binary on/off switch. In terms of a dark pulse, in constant light conditions, there are two effects termed anti-photic and non-photic. Anti-photic effects are where advances are observed during the day and delays at night (opposite to light pulses in constant darkness) and non-photic effects are a consequence of prolonged dark exposure and an increase in activity. Opposite to light pulsing, the effects are observed during the animals rest period, and therefore opposite in nocturnal and diurnal animals. Melatonin, serotonin and GABA fall in to the non-photic category of entrainment and are termed arousal independent (melatonin and GABA) and arousal dependent

(serotonin) due to their coupling (or not) with increased behavioural activity. Melatonin acts equally irrespective of the animal being nocturnal or diurnal, and phase advances *Rev-erb α* and induces *Ror β* expression rather than *Per1* and *Per2* mRNA (light pulsing), expression in the SCN. Serotonin expression levels are in phase with the behavioural activity of the animal and are therefore opposite in nocturnal and diurnal animals with phase advances observed with administration during the animals rest period (subjective day and night respectively). Furthermore, serotonin acts to suppress *Per1* and *Per2* mRNA, both opposite to that of light and involving different clock genes to melatonin. GABA will induce phase advance irrespective of the animal being nocturnal or diurnal, when administered at the same circadian time and has been shown to be associated with a decrease in *Per1* and *Per2* mRNA expression in the SCN, similar to that of serotonin action. There are also certain drugs, used in the clinic (Triazolam) and as drugs of abuse (methampthetamine) which affects the circadian clock and in the case of methamphetamine it appears that it works independently of the classical circadian network (SCN) and the classical circadian genes.

1.5.4 Food entrainment

Food is a very potent zeitgeber, which has been shown through restricted feeding experiments. In nocturnal rodents, activity and feeding primarily occur at night, however, if the availability of food is restricted to a few hours during the day the animals show anticipatory behaviour (i.e. become active prior to food delivery). This restricted feeding is also able to uncouple peripheral oscillators and other brain regions from the SCN, which remains entrained by light, and induce phase advances in tissues such as the liver, heart and kidney as well as the cerebral cortex (Damiola et al. 2000; Hara et al. 2001; Wakamatsu et al. 2001). The action of restricted feeding does change the availability of many circulating nutrients (Woods 2005), including glucose which can stimulate circadian gene expression, similar to that of Gcs or serum shock, but acts via the suppression of *Per1* and *Per2* transcription (Hirota et al. 2002). Insulin was also considered to be a circulating factor involved in uncoupling peripheral clocks from the SCN under restricted feeding, but this hypothesis was ruled out in a study using diabetic rats that were unable to produce insulin (Davidson et al. 2002). In this study *Per1*::luc expression phase shifted in both WT and diabetic rats, in response to restricted feeding and therefore ruling out insulin as synchronising factor in food restriction and anticipatory behaviour.

When animals are entrained to food restriction they become more active prior to food availability, show an increase in core body temperature, increase serum CORT levels (Krieger 1974) and an increase in duodenal disaccharides (Saito et al. 1980). However, neither adrenalectomy (Boulos et al. 1980) nor hypophysectomy (removal of the pituitary) (Davidson and Stephan 1999) prevent food anticipatory behaviour, but hypophysectomy does prevent the anticipatory rise in core body temperature. Importantly ablation of the SCN does not abolish the food anticipatory behaviour, the phase-shifted CORT or temperature rhythm, and together suggests that there is a second oscillator, the food entrainable oscillator, (FEO) (Krieger et al. 1977). This finding was met with initial scepticism but was quickly confirmed with entrainment to a 25h feeding schedule, but with a different free-running behavioural rhythm (Edmonds and Adler 1977). Later studies showed that the limits of food entrainment were between 22h and 31h, which could last up to 5 days even in the absence of food (Boulos et al. 1980; Stephan 1981).

In an attempt to locate the FEO various researchers have attempted to lesion areas of the brain with the hypothesis that this will result in the loss of anticipatory behaviour. Lesions in the ventromedial hypothalamus resulted in a temporary loss in the anticipatory behaviour, but this was recovered 14-21 weeks after ablation (Mistlberger and Rechtschaffen 1984). Further attempts to localise the FEO were again unsuccessful ruling out paraventricular nucleus and the lateral hypothalamic area (Mistlberger and Rusak 1988). An apparent breakthrough came when the ablation of the DMH resulted in the loss of anticipatory behaviour (Gooley et al. 2006), with additional evidence provided by an increase in neuronal activation (as indicated by *cFos* activation) in the DMH after ghrelin administration i.p. (Kobelt et al. 2008). Ghrelin is the only known orexigenic hormone (appetite stimulant) and plays a crucial part in regulating food intake and body weight (Cummings 2006). Furthermore, the DMH has been shown to play an important part in circadian rhythm generation and in the regulation of food intake (Chou et al. 2003; Yang et al. 2009a) making it an ideal candidate for the FEO. Unfortunately, other researchers have not been able to confirm the initial studies by Gooley et al. (2006) (Landry et al. 2007; Moriya et al. 2009), showing that after DMH ablation there is still anticipatory behaviour. So the current literature is still very much at odds with the DMH and the FEO.

The above discussed the possibility that the FEO was located in a single area of the brain, similar to that of the central oscillator located in the SCN. However, evidence from

ablating defined areas of the brain, but not successfully removing the FEO, leads to the idea that multiple nuclei could be involved. In fact when an animal is food entrained multiple brain nuclei become active (as defined by *cFos* expression) around the entrained meal time (Poulin and Timofeeva 2008). Of particular interest in the study by Poulin and Timofeeva (2008) were the nuclei that showed increased *cFos* expression up to 3h before the meal time (anticipatory behaviour at the molecular level). In this instance the paraventricular thalamic nucleus, the DMH and the anterior hippocampal continuation and septohippocampal nucleus all showed this anticipatory expression, suggesting that the FEO is the combination of signals from multiple nuclei, yet current experimental evidence cannot rule out a the existence of a single FEO oscillator (Carneiro and Araujo 2009).

In a recent review by Carneiro and Araujo (2009), they suggested quite a complex system in which food entrainment occurs. This hypothesis combines a group of connected brain oscillators, which are:

1. Directly sensitive to peripheral food related hormonal signals (such as the median eminence and the arcuate nucleus).
2. Indirectly sensitive to peripheral signals, in order to integrate the directly sensitive nuclei above (such as the brainstem and hypothalamic nuclei e.g. DMH).
3. A group of nuclei that are responsible for the variables observed during food entrainment e.g. increase in temperature, CORT and anticipatory behaviour (such as the hypothalamic and thalamic nuclei, forebrain and motor systems).

Interestingly the food anticipatory behaviour does not necessarily require a functional circadian clock, as mice with mutations in some of the core clock genes still show this behaviour, reviewed by (Mendoza 2007). $Clock^{\Delta 19/\Delta 19}$ show a longer or arrhythmic period but the food anticipation is still present. In the *Per1* -/- mice, they run with a shorter period but again food anticipation is still observed. However, in the *Per2* -/-, where the animals remain behaviourally rhythmic but run with a shorter period, food anticipation is absent, even in constant darkness (Feillet et al. 2006). Similarly, the arrhythmic *Bmal1* -/- mouse is also without food anticipation. It has been concluded that the CRY proteins are not involved in food anticipation even though in the double knockout mouse food anticipation is slow to establish and unstable (Iijima et al. 2005). *Per2*, it seems is quite important in food anticipation. Not only does PER2 oscillate in the absence of a local oscillating clock

(liver), by taking systemic timing signals (Kornmann et al. 2007) but it has a very distinct (from that of PER1) role in food entrainment. Furthermore, the loss of *Per2* in the mouse results in an increased reliance on glycolysis, with an increased expression of key enzymes in the glycolytic pathway (section 7.3). Also *Per2* -/- mice do not run as far as WT mice (Bae et al. 2006). Interestingly, in relation to the last point, in WT rats under severe food restriction (6g food/day) they will run almost continuously, despite the calorific expenditure and develop stress ulcers within days (Yi et al. 1993). These quite distinct pieces of data suggest a very prominent role for PER2 in food entrainment and the anticipatory response.

1.5.5 Temperature entrainment

Temperature, as a zeitgeber, is rather poorly understood and understudied, with respect to food and photic entrainment. This lack of knowledge is even more so when looking at temperature entrainment in the mammalian system, as the majority of studies have taken place in ectothermic organisms (animals whose temperature mirrors that of the environment) where the circadian clock can easily be manipulated by the external temperature (extensively reviewed by (Rensing and Ruoff 2002)). In the cyanobacterium Synechococcus, the protein synthesis and activity of the enzyme nitrogenase can be entrained to a temperature cycle with an range of just 5^{o}C, yet, in conjunction with a light/dark cycle light entrainment was more dominant (Lin et al. 1999). However, a protein mechanism has not been characterised but is thought to involve the rhythmic *Kai a/b/c* system (Kageyama et al. 2003) but not their phosphorylation status, and as such hypothesised to be the state of the KAI A/B/C complex formation or changes in the state of the KAI C hexamer (Yoshida et al. 2009) The fungus *N. Crassa* has been clearly shown to entrain to temperature cycles with a dynamic range of just 2-2.5^{o}C, in constant conditions (Francis and Sargent 1979; Rensing and Ruoff 2002) and a temperature range of 10 ^{o}C or more is a stronger zeitgeber than light (Rensing 1989). At the molecular level this entrainment appears to be mediated via the oscillating protein FREQUENCY (FRQ). At whatever phase of the circadian cycle the temperature is altered an increase in temperature will result in a re-set to the subjective day (CT0), as the cell will view whatever quantity of FRQ present as the minimum. Conversely, a decrease in temperature will be re-set to the subjective night (CT12) with the cell viewing the FRQ concentration as maximal, regardless of phase (Garceau et al. 1997).

The rhythmic behaviour rhythm of the fruit fly *D. melanogaster* has been shown entrain to a temperature cycle with a range of just 2^{o}C (shown in over 50% of individuals), with entrainment of blind flies to a temperature range of 3^{o}C (Rensing and Ruoff 2002; Wheeler et al. 1993). It has been shown that pulsing *Drosophila* with increasing temperatures has a dose dependent effect on the degradation rates of PER and TIM (two proteins involved in the feedback loop, (section 1.6.1) when applied at either CT15 or CT21.5 (Sidote et al. 1998). However, in this study a phase delay only occurred with a pulse at CT15, as a pulse at CT21.5 resulted in degradation but also rapid increase in both PER and TIM, which is in direct contrast to light pulsing at this time, which resulted in PER and TIM degradation and a 2h phase advance (Lee et al. 1996; Myers et al. 1996). More recent data has suggested that temperature entrainment, in *Drosophila*, may well be the product of a signalling cascade involving phospholipase C (PLC). PLC acts to splice the 3' end of the *Per* gene in response to cold temperatures, leading to an earlier accumulation of *Per* mRNA and an advance in behavioural rhythm (Majercak et al. 2004). The association between PLC and temperature entrainment was shown through mutation studies of the gene encoding PLC, *norpA.* A mutation of *norpA* led to constitutive 3' splicing regardless of temperature (Glaser and Stanewsky 2005). Furthermore in this study, a novel gene termed *no circadian temperature entrainment* (*nocte*) was isolated as it had an impact on temperature at the behavioural and molecular levels in response to constant light.

There has been relatively few studies with regard temperature entrainment in endothermic organisms (warm blooded e.g. mammals and birds) (Francis and Coleman 1988; Goldman et al. 1997; Refinetti 2010) compared to ectothermic organisms (cold blooded e.g. reptiles and fish) (Evans 1966; Foa and Bertolucci 2001; Samejima et al. 2000). This fact is not surprising considering that endothermic organisms are essentially buffered against external temperature changes and therefore temperature as a zeitgeber is far less potent in these organisms. It has been reported in a number of rodent studies that some, but not all animals, in a particular study will entrain to a temperature rhythm with a range between 4^{o}C and 14^{o}C (Rensing and Ruoff 2002). In a recent study standard domestic mice (CD-1 strain) were shown to entrain to a 12^{o}C temperature rhythm (16h at 24^{o}C:8h at 12^{o}C, or 16h at 24^{o}C: 8h at 32^{o}C). However, only 60-80% of the mice entrained to these temperature cycles, with no defined PRC, compared to 100% which entrained to light (Refinetti 2010). This confirmed previous studies which suggested that temperature is indeed a much weaker zeitgeber than light in endothermic animals. Although the

molecular mechanism for temperature entrainment has not yet been fully characterised some work has been carried out on cell lines showing that temperature changes can induce second messengers such as Ca^{2+} and cAMP (Kiang and McClain 1993; Kiang et al. 1991), but also act to lower the internal pH, Na^{+} and change the membrane potential (Skrandies et al. 1997). It has also been hypothesised (as yet without full experimental evidence) that as a positive temperature pulse and a light pulse act to phase shift the clock in the same way, using melatonin as a circadian marker (shown with dispersed chick pineal cells) (Barrett and Takahashi 1995; Robertson and Takahashi 1988). These data suggest that the two entrainment pathways could be very closely linked. Furthermore, it has been hypothesised that the small fluctuations in the internal temperature of the animal may be just another internal entrainment factor like melatonin, food and Gc, acting both as a clock output and feeding back onto the clock itself (Tosini and Menaker 1996). However, it is just a very weak internal synchronising factor. More recent work by Buhr et al. 2010 suggests that circadian changes in body temperature, in mammals, acts on the SCN which is then used to synchronise the peripheral system (Buhr et al. 2010). The authors showed that the SCN is indeed temperature compensated but network interactions are required i.e. cell to cell communication and that the heat shock pathway is essential for both temperature re-setting and compensation (section 1.5.6) (Buhr et al. 2010).

1.5.6 Temperature compensation

Temperature compensation is one of the main contributing factors to what makes an endogenous circadian clock Pittendrigh (1960). Tosini et al., (1996) showed that in the tau mutant hamster temperature compensation was lost in the retina, when assayed for melatonin release, with a Q_{10} = 1.487 compared to the WT = 1.096. The tau mutation results in a gain of function mutation in the CK1ε protein (Meng et al. 2008a) leading to a period of 20h in the homozygote mutant, as seen in both hamster and mouse (Meng et al. 2008a; Ralph and Menaker 1988). This study by Tosini et al. (1996) led to the hypothesis that CK1ε may have a role in the temperature compensation of the clock. Recent studies have shown that although chemical inhibition of CK1ε, which leads to the phosphorylation and degradation of its clock target protein PER2, lengthens the period of PER2, the activity of CK1ε WT or tau was insensitive to temperature change as was the phosphorylation of mPER2 protein (Isojima et al. 2009). This suggests that CK1ε is an important enzyme in temperature compensation of the clock, as well as more recent data is pointing to CK2 (Mehra et al. 2009).

1.6 The circadian system

1.6.1 Discovery of circadian genes

Much of the early work on circadian rhythms was undertaken using the fruit fly, *Drosphila melanogaster,* and the fungus *Neurospora crassa,* but work rapidly progressed on a number of organisms including mammals, particularly rodents, using both biochemical and genetic techniques. In 1971 Konopka and Benzer discovered the first of the clock genes, termed *per,* in *D. melanogaster,* with its locus on the X chromosome (Konopka and Benzer 1971; Sehgal et al. 1994). Using ethyl methane sulfonate (EMS) chemical mutagenesis the authors observed three altered locomotor rhythms in the flies; one which had an activity of longer than 24 hours, termed per^{L}; one which ran shorter than 24 hours, termed per^{S}; and a third which rendered the fly arrhythmic, per^{0} (Konopka and Benzer 1971). In the mid 1980's two papers showed the sequence of the *per* sequence in *Drosophila* locating it to a 90kbp region in the 3B region of the X chromosome, further work then located it to a 7.1kbp region of the gene which encoded a 4.5kbp RNA transcript (Bargiello and Young 1984). This 4.5kbp region is eliminated in *per* mutants that retain some rhythmicity but it was suggested that two novel transcripts substitute for its loss. The previously mentioned 7.1kbp fragment of DNA restored rhythms in *Drosophila* which carried mutations, when transduced back into the fly (Jackson et al. 1986). Jackson et al., 1986 also published a predicted amino acid sequence for the PER protein, which was 1127 amino acids in length. The PER amino acid sequence had high homology to the rat chondroitin sulphate proteoglycan, which was know to be distributed around the cell surface and in extracellular locations. A year later, in 1987, mutations in the *per* gene, resulting in the arrhythmic and short running flies were mapped to either a nonsense mutation in the third coding exon, or a nucleotide substitution in the forth coding exon respectively (Yu et al. 1987). The arrhythmic mutation was a glutamine to stop codon change, which resulted in a truncated protein missing the C-terminal end, and the arrhythmic phenotype. The short running fly was the result of a serine to asparagine mutation at position 589. Hardin et al., (1990) used RNase protection assays with *Drosophila* heads to determine the cycling activity of *per* mRNA. In this work the authors used the long and short mutants, discovered by Konopka and Benzer, and compared them to wild type. There was no difference in the overall amplitudes just with a shift in phase, either earlier (per^{S}) or later (per^{L}) depending on the mutant. They then postulated a model by which the *per* gene product regulated its own transcription (Hardin et al. 1990), yet with

the added uncertainty of how this occurs, either at a transcriptional or post-translational level. This then led the way to further characterisation of the biochemical clock, and a number of years later the second mutant gene, *timeless (tim)*, was discovered and mapped to chromosome 2 (Sehgal et al. 1994)**,** again in the fly. It was subsequently discovered, in the fly, that the dPER protein heterodimerised with TIMELESS and using *tim* mutant experiments dPER could not be detected in the nucleus (Vosshall et al. 1994). It was discovered that *dtim* mRNA levels show a definite circadian rhythm, in the *Drosophila* head, as do *dper* mRNA levels. It was then shown that both *dtim* and *dper* mRNA cycle in phase with each other (Sehgal et al. 1995). Following these initial breakthrough discoveries many more clock genes and their subsequent proteins followed, leading to the dissection, and subsequent understanding of the biological clock, although the understanding aspect was still very much a working progress. Genes such as *cryptochrome* (*Cry)* and *timeout (Tim2)* (Vodovar et al. 2002), were discovered. *dcycle* was another gene, first characterised using EMS mutagenesis experiments in the fly, as an essential clock protein and member of bHLH PAS family of transcription factors, with homozygote mutants becoming totally arrhythmic (Rutila et al. 1998). *dclock* was a gene, identified as a core clock component in the fly, again discovered using EMS mutation, which resulted in uniform arrhythmic phenotype in the homozygote mutant fly (Allada et al. 1998). dCLOCK was discovered to heterodimerise with dCYCLE and was co-expressed with dPER, in the fly. However, it was subsequently discovered, using a newly developed antibody, that dCLOCK did not cycle with a definite rhythm, unlike dPER, and was also found to express in non-oscillatory cells, suggesting a circadian and a non circadian role (Houl et al. 2006). However, subsequent studies revealed that this statement was wrong and dCLOCK was only expressed in oscillator cells and the mistake was made due to antibody cross reactivity with another protein BMC DACHSHUND (Houl et al. 2008)**.** Following on from these initial discoveries regulatory components were discovered, such as the kinase dDoubletime, the *Drosophila* ortholog of the mammalian CK1ε and dShaggy, the ortholog of the mammalian glycogen synthase kinase 3β (GSK3β) (Hughes et al. 1992; Ruel et al. 1993), which are involved in the posttranslational modification of the core clock proteins (Preuss et al. 2004), resulting in the period of the oscillation being roughly 24 hours. The fruit fly is an extremely useful model to use in the lab as it has a relatively simple genome with only 4 chromosomes including a sex chromosome, although each gene has mammalian homologues, and it has a generation time of 2 weeks, together making it a very useful genetic tool.

1.6.2 The mammalian PER and CRY system

1.6.2.1 The Period genes

A mammalian homolog to the *Drosophila per* gene was found simultaneously by independent labs (Sun et al. 1997; Tei et al. 1997) and Sun (1997) termed it *Rigui,* now *Per1*. It shares 44% homology to the *Drosophila per* gene and is expressed in both central and peripheral tissues. In mammals there are three *Per* genes, (*Per 1-3),* all containing PAS domains (Fu and Lee 2003), (Fig 1.4), but which do not directly bind to DNA. In the fly the dPER protein is the main oscillator and showing a rhythm independent of its mRNA rhythm i.e. the PER protein shows a rhythm even when *per* mRNA is constitutively expressed (Zhang et al. 2004). Likewise in the mouse, the mPER2 protein can oscillate even in the presence of constitutive *mPer2* mRNA (Zhang et al. 2004), this is due to posttranslational modification (Gachon et al. 2004; Iitaka et al. 2005). In the promoter region of the *mPer* genes lie a number of E-boxes, *mPer1* contains 3 and *mPer2* and *mPer3* contain 7 and 4 respectively (Travnickova-Bendova et al. 2002). This suggests tightly controlled CLOCK:BMAL1 driven E-box regulation, with a resulting peak expression at circadian time (CT) 10-12 (like that of *mCry1*; section 1.6.2.1) (Albrecht et al. 1997; Shearman et al. 1997). CT is the time of day when an animal is placed into a constant environment i.e. with no external time cues. The resulting mRNA translocates to the cytoplasm and the protein products can then complex with other proteins like PER2 and CRY (Hirota and Fukada 2004). In the cytoplasm, CRY proteins complex with the PER proteins at their C-terminal, and aid nuclear translocation (Vielhaber et al. 2001). PER2 is modified post translationally by a number of different proteins (further discussed in 1.3.6). More recent evidence suggests that both *Per 1* and *2,* rather than the CRY proteins, are essential for the functioning of the molecular clock (Chen et al. 2009), as the CRY proteins were previously shown to be the rate limiting factor in nuclear accumulation and the major component of CLOCK:BMAL1 transcriptional inhibition (Griffin et al. 1999; Kume et al. 1999). It has been demonstrated that the PER proteins bind to the CLOCK:BMAL1 complex, that is itself bound to the DNA (Lee et al. 2001), rather than CRY binding directly to repress E-box transcription. This model proposed by Chen (2009) suggests that PER bridges between CRY and CLOCK:BMAL1, to allow CRY to initiate transcriptional repression (Kume et al. 1999). *Per2* has also been shown to oscillate even in the absence of a tissue specific functional clock. Kornmann et al. (2007) over expressed REV-ERBα, in the liver, which suppressed *Bmal1* expression, resulting in an arrhythmic

liver clock and the loss of *Cry*, *Rev-erb* and *D site of albumin promoter binding protein* (*Dbp*) rhythms, although the rest of the animal was rhythmic. However, a subset of 31 transcripts, in the 'arrhythmic' liver, still oscillated including *Per2* and other heat shock proteins (Hsps). Promoter analysis suggested that *Per2* has 5 heat shock elements within 1700bp of the promoter region conserved in mouse, human, rat and dog (Kornmann et al. 2007) indicating a much simpler independent oscillator aside from the more complex core loop (Fig 1.1). Other data though has shown that the *Per2* transcript does not oscillate in the SCN of *Bmal1* knockout, arrhythmic mice, as seen by in-situ hybridisation, (Bunger et al. 2000) and neither was it shown to oscillate in liver explants from *Bmal1* knock out mice (Liu et al. 2008) or liver from SCN ablated mice, (Reddy et al. 2007), using qPCR. Together these data suggest that *Per2* can oscillate in the absence of a local clock but the central clock must be intact to relay systemic signals. Furthermore, the loss of *Rev-erb α* resulted in constitutive levels of *Bmal1*and *Clock* transcript and protein but unaltered *Per2* expression at transcript or protein level (Preitner et al. 2002), leading to further evidence to support this independent *Per2* peripheral oscillator.

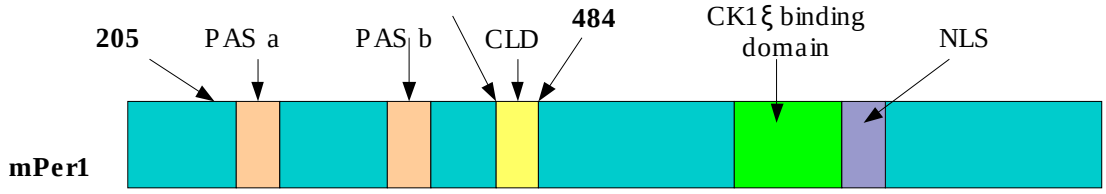

Figure 1.4: Schematic representation of the various domains of *mPer1*: Per ARNT Simple minded (PAS) dimerisation domain, cytoplasmic localisation domain (CLD), nuclear localisation domain (NLS), numbers refer to amino acid position. Adapted from Vielhaber (2001).

Per1 is involved in phase resetting. Mutations, in the rodent, can shorten the period length (Zheng et al. 2001) or result in an arrhythmic phenotype after a few days in constant darkness (DD) (Bae et al. 2001). Mutations in *Per2*, can also lead to an altered behaviour rhythm resulting in the shortening, then an arrhythmic behaviour after a few days in DD (Bae et al. 2001). Furthermore, a *Per2* -/- mouse has been shown to have reduced activity i.e. reduced wheel running activity and the loss of *Per2* results in the loss of the CORT rhythm, but the CORT increase in response to stress remains the same as the WT (Bae et al. 2006; Yang et al. 2009). In the most severe human form, a *Per2* mutation can result in Familial advanced sleep phase syndrome (fASPS) (Toh et al. 2001), a genetic condition in which residue 662 of the hPER2 protein is mutated from a serine to glycine. This single

mutation results in the carrier waking up and going to sleep earlier each day, leading to a severely disrupted life, out of phase with the rest of society. However, mutations in the PER3 protein cause only a subtle effect on the rhythmicity in rodents, with a slight shortening of the period length in constant conditions (Shearman et al. 2000). However, allelic differences in PER3 in humans can have dramatic effects on sleep, but in this case a sleep delay. PER3 contains a region called variable-number tandem-repeats (VNTR) and dependent on the number of repeats, either 5/5 or 4/4, determines the endogenous clock phase, a 5/5 repeat determines a more morning type (people who prefer to wake up early but also go to bed early) whereas the 4/4 determines and evening type (people who prefer to sleep late in to the morning/afternoon but stay up all night), which can ultimately lead to delayed sleep phase syndrome, opposite to that of fASPS (Viola et al. 2007).

Double mutant mice with either *Per1/3* or *Per2/3* disrupted show a phenotype no more severe than the single *Per1* or *Per2* mutant, suggesting *Per* redundancy and that *Per3* is not essential in the core mechanism driving locomotor activity (Bae et al. 2001). However, if both *Per1* and *2* are mutated then the animal shows an immediate arrhythmic phenotype in DD, suggesting that both these homologues are essential (Bae et al. 2001; Zheng et al. 2001). Furthermore, evidence suggests that PER1 and PER2 proteins independently control output pathways. For example, the gene *cellular retinal binding protein 1* (*CRBP1*) (essential for vitamin A synthesis) will continue to oscillate in a *Per2* mutant mouse but not in a *Per1* mutant. Conversely, the gene *NG27* (identified as part of the major histocompatibility complex II locus - involved in antigen presentation on the surface of specific cells e.g. macrophages - but with unknown function) remains rhythmic in a *Per1* mutant but not *Per2* mutant mouse. There are also examples where *Per1* and *Per2* have combined control of other circadian pathways, such as heme biosynthesis (Zheng et al. 2001).

1.6.2.1 The Cryptochrome genes

The CRY protein was first characterised in 1996 by Hsu et al, with a further homologue being discovered when trying to purify CRY1, termed CRY2. These two CRY proteins share a 73% identity, with each other at the amino acid level and CRY1 and 2 share 48% and 51% sequence identity respectively, with the *Drosophila* (6-4) photolyase (Hsu et al. 1996). CRY proteins are found to oscillate in most tissues of the body studied, in phase with PER and in anti-phase to BMAL1. CRY proteins have also been found in certain

retinal neurons, and mice lacking the *Cry* genes present a reduced pupillary light response i.e. a reduced pupil contraction in the presence of bright light (Van Gelder et al. 2003).
On mRNA export to the cytoplasm and protein production the two CRY proteins will bind to the C-terminus of PER1 and 2, with an unknown stoichiometry (Gachon et al. 2004b). This interaction stabilises the PER proteins, which was proposed to be the rate limiting factor for the CRY:PER interaction in the cytoplasm with nuclear translocation then taking place (Kume et al. 1999). However, endogenous protein phosphorylation studies by Lee et al. (2002) suggested otherwise and that the CRY proteins were not indeed the rate limiting step but rather the PER proteins were. Kume et al. (1999) also suggested that the CRY proteins were responsible for the PER:CRY complex nuclear translocation and that they were primarily nuclear proteins, but mutational studies have shown that the nuclear localisation domain (NLS) in PER2 is essential for nuclear translocation of the PER:CRY complex (Miyazaki et al. 2001), which backed up the evidence by Lee et al. (2002) showing that CRY remained cytoplasmic in *Per1/2* double knock out mice. This suggests that the PER proteins are required for feedback repression and that they are far more important in nuclear translocation than the CRY proteins. However, CRY1 has been shown to interact with histone deacetylases (see 1.3.6.3) in order to repress *Per1* transcription in a rhythmic manner (Naruse et al. 2004) and is potentially the reason for elevated *Per1* levels in *Cry1* and *2* knock out mice, suggesting that the CRYs are a vital part of the feedback mechanism of a functional clock. There is considerable redundancy in this heteromeric complex, as the *Cry* genes can be mutated individually without it resulting in complete arrhythmia in DD (van der Horst et al. 1999; Vitaterna et al. 1999). A double knock-out phenotype is different though, the *Cry1/2* double knockout mouse is immediately arrhythmic, as is the *Per1/2* double knockout, when run under constant darkness (Spoelstra et al. 2004; Zheng et al. 2001).

There is conflicting evidence, however, as to the oscillations of *mCry1* and *mCry2* mRNA. One laboratory has reported that both *mCry*1 and *2* are expressed in the central oscillator, the SCN (section 1.4), but only *mCry*1 mRNA cycles (Thresher et al. 1998) with peak expression about CT10-12. However, others have reported that both *mCry*1 and 2 mRNA oscillate in the SCN, yet with a less defined *mCry2* rhythm with a peak stretching from CT8 to CT16 (Okamura et al. 1999). Both the respective proteins oscillate in phase, in the SCN (Kume et al. 1999). The CRY proteins are also important in cell cycle progression,

from S to M phase, and mice with an impaired *Cry* gene show impaired cell cycle progression in hepatocytes, but not in the SCN (Matsuo et al. 2003).

1.6.3 CLOCK, NAPAS2 and BMAL1

1.6.3.1 CLOCK

Clock is a large gene spanning 24 exons comprising of about 10,000 bp of DNA (King et al. 1997). Both the mRNA and protein are constitutively expressed in the central oscillator, the SCN (Gekakis et al. 1998), yet show strong oscillations in the majority of peripheral tissues (such as the retina, heart, liver, kidneys, testis and ovaries) (Reppert and Weaver 1997), suggesting a more complex role for CLOCK than purely a core clock gene.

Using a 'forward genetic' approach, that is a 'treat and see' approach similar to that seen with the early *Drosophila* work, mice were treated with N-ethyl-N-nitrosourea (ENU), which randomly mutates DNA. One mutation led to a 3-4 hour period extension in the activity rhythm, with a loss of rhythm in constant darkness (Vitaterna et al. 1994). It was later discovered that there had been an adenine to thymidine transversion at the third base position of the 5' splice donor site of intron 19, which resulted in the deletion of the entire exon 19, comprising 51 amino acids (King et al. 1997). This mutation was termed $CLOCK^{\Delta 19}$ and suggested that CLOCK was an essential component of the circadian clock. A decade later Debruyne et al., showed that CLOCK null mice i.e. there is no CLOCK present in the mouse still had a behavioural rhythm, albeit shorter than the WT approximately 23.3h compared to 23.8h (DeBruyne et al. 2007b)**,** which suggested that the $CLOCK^{\Delta 19}$ was a dominant negative protein and that CLOCK was not essential for a rhythmic mouse. However, peripheral clocks such as the liver and lung do require CLOCK to be present for an oscillation as *Clock* -/- liver and lung are arrhythmic (DeBruyne et al. 2007c).

CLOCK protein has recently become of particular interest as it was found to have histone acetyltransferase (HAT) activity (Doi et al. 2006). Thus CLOCK is potentially involved in chromatin remodelling as it can acetylate lysine residues in histone proteins (in particular histone H3). In work following that of Doi (2006) it was found that PER2 was acetylated and that SIRT1, a NAD^{+} dependent histone deacetylase (HDAC), binds to the

CLOCK:BMAL1 heterodimer in a time dependent manner and deacetylates PER2 (Asher et al. 2008), HATs and HDACs are further discussed in section 1.7.3.

1.6.3.2 Neuronal PAS domain protein 2 (NAPS2)

Neuronal PAS domain protein 2 (NPAS2) was identified as a paralogue of CLOCK, in the forebrain (Reick et al. 2001), which could functional substitute and maintain a rhythmic mouse (Debruyne et al. 2006). Further work by Debruyne et al. (2006) knocked-out *Clock* using a Cre-LoxP system which targeted the removal of 2 exons encoding the bHLH domain, required for BMAL1 dimerisation and therefore the CLOCK protein was non-functional. This system was used for whole animal knock-out and for tissue specific studies, where Cre recombinase was expressed (Debruyne et al. 2006). Once *Clock* was knocked-out, the adult continued to show rhythmic locomotor activity with a period only 20 minutes shorter than in the wild type, but with an altered response to light, becoming active 2 hours before lights off. Furthermore, the maintenance of the circadian activity cycles in the absence of CLOCK protein suggested that CLOCK is not an essential protein for the rhythmic expression of clock genes, such as *mPer1/2,* and their respective proteins, yet the amplitude of their expression was reduced in the knock-out genotype (Debruyne et al. 2006) with respect to *Rev-erbα*, *Per1* and *Bmal1* mRNA; although *Per2* expression was maintained. This then suggested an alternative binding partner for BMAL1, hypothesised to be NPAS2, (a known paralogue of mCLOCK and had been shown to co-immunoprecipitate with BMAL1, in whole brain extracts from both wild type and CLOCK deficient mice) (Debruyne et al. 2006). However, this hypothesis was not verified as *Npas2* mRNA was undetectable in the SCN of either wild type or CLOCK deficient mice, by *in-situ* hybridisation (Shearman et al. 1999), yet *Npas2* mRNA and protein levels were increased in the CLOCK deficient liver (Debruyne et al. 2006). However, more recent work has shown that NPAS2 can functionally substitute for CLOCK in the SCN using CLOCK deficient mice, which were still rhythmic in constant darkness but with a shorter period than the WT. Also *NPAS2* -/- mice, with *CLOCK* present were also rhythmic in constant darkness, but this time with a period almost equal to the WT. In the CLOCK and NPAS2 double knock out, the mouse is immediately arrhythmic when placed into constant darkness (DeBruyne et al. 2007a). Together these data strongly suggest that NPAS2 can functionally replace CLOCK in the SCN and at a behavioural level, yet in the periphery this is not the case as NPAS2 cannot functionally replace CLOCK (DeBruyne et al. 2007c). It could also be possible that BMAL1 forms a homodimer, as revealed by

electorphoretic mobility shift (EMS) assay *in vitro* (Rutter et al. 2001), yet probably unlikely, as BMAL1:BMAL1 DNA binding was inhibited by NAD^+/NADH co-factors (Rutter et al. 2001).

1.6.3.3 BMAL1

BMAL1 (MOP3) is another bHLH PAS domain containing protein, first identified through a yeast-two-hybrid screen as a binding partner for CLOCK and PER1 (Gekakis et al. 1998). BMAL1 is found in all the major clock cells in the brain and periphery. *Bmal1* mRNA, unlike *clock* mRNA, is rhythmic and peak expression is in the middle of the circadian night, about CT18, in anti-phase to *Per2* (Hastings et al. 2003; Honma et al. 1998). Expression in the SCN exhibits a much lower amplitude than in the periphery, with about a 3 fold change from peak to trough in the SCN and retina, compared to 50 fold change in the heart and liver (Peirson et al. 2006). However some report that the BMAL1 protein (as well as CLOCK) is constitutively expressed in the SCN and that light does not affect BMAL1 degradation (von Gall et al. 2003). There is evidence to suggest that the binding of the CLOCK:BMAL1 heterodimer is linked to the energy state of the organism by the ratio of NAD(P)H:$NAD(P)^+$, which complements the work by Doi et al. (2006) showing the NAD^+ dependent SIRT1 being directly involved in the central oscillator, and shown by Asher et al. (2008) to bind to the CLOCK:BMAL1 complex in a circadian manner and to deacetylate PER2. During a high NAD(P)H:$NAD(P)^+$ ratio the equilibrium is shifted towards E-box binding whereas the opposite is the case for low levels. This was reported by Rutter et al. (2001) who used EMS to assess the DNA binding ability of the NPAS2:BMAL1 heterodimer in the presence of NADH and NADPH, both in the reduced and oxidised state. Optimal NPAS2:BMALl1 DNA binding was observed when the per cent of reduced to oxidised was greater that 75%, with co-factor levels at physiologically relevant concentrations.

Bmal1 genetic knock out mice exhibits an arrhythmic phenotype (Bunger et al. 2000) and present an age related phenotype such as cataracts, reduced subcutaneous fat and organ shrinkage (Kondratov et al. 2006). *Bmal1* -/- mice also show tendon calcification with decreased activity, body weight and longevity (McDearmon et al. 2006). When *Bmal1* expression was restored in the SCN circadian wheel-running was restored but the mice still showed reduced activity and body weight. Yet when *Bmal1* was restored in the muscle, but not the SCN, normal activity levels and body weight were seen but not circadian

rhythmicity, suggesting tissue specific functions for BMAL1 (McDearmon et al. 2006). Furthermore, a genetic screen on mutant BMAL1 proteins found that the C-terminal 43 residues are essential for transcriptional activation and binding to its repressor CRY1, however, this mutation did not impair PER2 binding (Kiyohara et al. 2006). The dual role in the C-terminal suggests the need for a molecular switch to convert between 'on' and 'off'. This is perhaps achieved through cyclic phosphorylation, as the C-terminus is a putative site for CK1ε binding and a mitogen-activated protein kinase (MAPK) binding site (Hirayama and Sassone-Corsi 2005). This then leads to the idea that mammalian oscillations could be controlled through the interaction of post-translational events rather than purely transcriptional oscillations (Kiyohara et al. 2006) (further discussed in section 1.7).

BMAL1 has a homologue in BMAL2, which is expressed in up to 9 tissues specific isoforms and can heterodimerise with CLOCK to induce downstream E-box containing genes (Hastings et al. 2003). *Bmal1* and *2* are very closely associated and if *Bmal1* is knock out then *Bmal2* is also knocked out, a 2 for 1 approach (Bunger et al. 2000; Shi et al. 2010). BMAL2 can functionally replace BMAL1 in a transgenic model to restore a rhythmic behavioural phenotype. However BMAL2 cannot restore a rhythmic oscillation, as seen by PER2::luc, in either the lung or SCN but rhythmic oscillations were restored after dexamethasone (DEX) treatment in the liver (Shi et al. 2010). Interestingly, although *Bmal1* is the only clock gene that when knocked out results in an arrhythmic phenotype (Bunger et al. 2000), it is itself not required to cycle to induce a rhythmic sustained oscillator (Liu et al. 2008), although the reporter used for this observation was PER2::luc and as stated in section 1.6.2.1, *Per2* itself has very specific components to its regulation.

1.6.4 Rev-erbs and Rors

1.6.4.1 Rev-erb alpha and beta

There are two known *Rev-erbs*, termed alpha (α) and beta (β), although classed as orphan nuclear receptors, however, heme has recently been shown to act as a ligand (Raghuram et al. 2007). The *Rev-erbα* gene locus is found on the opposite strand to the α-thyroid hormone receptor (c-erb α) and is expressed in many cell types, predominantly in the brain; with the highest levels in the SCN (Onishi et al. 2002), muscle and brown fat (Harding and Lazar 1993). *Rev-erbβ* is located on a different gene, yet they are both widely expressed

throughout the mammalian system (Liu et al. 2008). Other members of the orphan nuclear receptor family are ROR a, b and c, which all activate transcription where REV-ERB α/β represses it. Both RORs and REV-ERBs have a DNA binding domain and makes contact with a 5 bp A/T rich sequence adjacent to an AGGTCA half site and the half site itself. Unlike the other components discussed, REV-ERBs do not form a hetero/homo-dimer with itself or other nuclear receptors (Adelmant et al. 1996; Harding and Lazar 1993), but do bind with heme. REV-ERBs are a major regulator of the core clock gene *Bmal1*, which has ROR/REV-ERB binding site, or RORE. REV-ERBs are not always regarded as essential clock components, since the clock is still functional in their absence. However, significant differences in period length and light induced phase shifts are seen when *Rev-erbα* is knocked out (Preitner et al. 2002) and there are dramatic effects on other clock components such as *Cry1*, *Clock* and *Bmal1*. It is interesting to point out that even though the amplitude of *Cry1* mRNA is dampened in the *Rev-erbα* knock out mice, the cyclic nuclear accumulation of its protein CRY1 does not alter. It is therefore suggested that *Cry*1 mRNA is not the rate limiting step in nuclear accumulation, similar to that postulated by Chen (2009), and posttranslational modifications play a vital role (Preitner et al. 2002), with all 7 clock proteins (PER1,2, CRY1,2, BMAL1, CLOCK, CK1ε) associating in time dependent, multimeric complexes and all being phosphorylated, (Lee et al. 2001). Reports by Liu (2008) have shown that the two *Rev-erb* genes are functionally redundant, where one can compensate for the loss of the other, but if both are removed then the *Bmal1* rhythm is lost also, as seen in fibroblast cell lines; however the *Per2::luc* rhythm is still present.

1.6.4.2 The family of Rors

RORa is the main isoform, of the ROR family, associated with the clock, with the mouse having four further isoforms RORa 1–4 (Sato et al. 2004b). The RORs bind to the same sequence as REV-ERBα (AGGTCA), which leads to competition between the two receptors (Delerive et al. 2002). RORa is ubiquitously expressed in central and peripheral tissues but cycling seems restricted to the SCN (Liu et al. 2008). RORb is expressed in many brain regions like the SCN but not the periphery, whereas RORc is rhythmically expressed in the liver but not expressed at all in the brain (Akashi and Takumi 2005; Liu et al. 2008). This does not rule out any potential role for RORb and c in regulation of *Bmal1*, as double RORb knock out mice show an extended activity rhythm (Akashi and Takumi 2005). Together these data suggest that, although RORa is the major inducer of *Bmal1*

transcription, RORb and c may aid this in some way, in various central and peripheral tissues.

1.6.5.3 *Dec1* and *Dec2*

Dec1 and *Dec2* are bHLH, E-box containing genes that, like *Per1* and *2, Cry1* and *2* and *Rev-erbα,* are up regulated by CLOCK:BMAL1 mediated transcription (Nakashima et al. 2008). They also serve as transcriptional repressors of CLOCK:BMAL1 mediated transcription either through interaction with BMAL1 (Sato et al. 2004a) or through direct interaction with E-boxes, via their bHLH domain (Kawamoto et al. 2004). However, it is unknown whether DEC1 and 2 can bind directly to E' boxes (CACGTT). This is in direct contrast to how PER and CRY repress CLOCK:BMAL1 transcription, as neither PER nor CRY bind directly to DNA and therefore repress transcription by binding to CLOCK:BMAL1 heterodimer itself (Chen et al., 2009). DEC1 and 2 were able to suppress CLOCK:BMAL1 induced transcription of a E54-TK reporter (made with a region of the *mPer1* promoter containing 3 functional E-boxes upstream of a luciferase gene), from 125% to 13.3% and 5.9% respectively (Honma et al. 2002). Regarding DEC2 this repression was far greater than the repressive effects of PER1 or PER2 and CRY1 or CRY2, yet the repressive effects of DEC1 were comparable to those of CRY1 and 2 (Honma et al. 2002). *Dec1* and *Dec2* are both expressed in the SCN in a circadian manner under DD and LD conditions. *Dec1* peaks in the early subjective day where *Dec2* peaks in the middle of the subjective day. The *in-situ* hybridisation signal for *Dec1,* in the SCN, was weak compared to other brain regions (parietal and piriform cortices) yet the *Dec2* signal was strong (Honma et al. 2002). Interestingly, *Dec1* and *2* respond differently to a 30 min light pulse given in constant darkness. A light pulse given at 20:00, 24:00 and 4:00 (clock hours not circadian hours) resulted in a significantly induction of *Dec1* but not *Dec2,* and a pulse at 24:00 decreased *Dec2* (Honma et al. 2002). *Dec1* -/- mice have behavioural rhythms slightly longer than WT mice in DD (24.1 ± 0.05h and 23.9 ± 0.03h h respectively) and in *Dec1-/-* fibroblasts *Dec2*, *Per1*, and *Dbp* are all phase advanced by 2.8, 2.0, and 2.5h respectively, when comparing the first peak relative to the WT cells. However, this advance is lost after 4 cycles as the period of each gene (*Dec2*, *Per1*, and *Dbp)* in the *Dec1-/-* fibroblasts (24.7, 25.2, and 25.0 respectively) is longer than its WT counterpart (23.6, 24.4, and 24h respectively). In contrast the phases of *Per2* and *Cry1* were unaffected (Nakashima et al. 2008). Knock down of *Dec2* by siRNA had less of an effect as it resulted in a phase advance of *Dec1* and *Dbp* for 3.9 and 1.0 h, respectively, at

the first peak and a slightly increased the expression level of *Dec1, yet* it did not change the expression of *Per1* and the second peak of *Dbp* was in phase with the WT (Nakashima et al. 2008). Furthermore, knock down of *Dec2* in *Dec1* -/- cells reduced the amplitude of *Dbp* and *Per1*, again advancing their phase by 1.3 and 3.3h respectively yet had little effect on *Per2* and *Cry1* expression and phase. The knock down of both *Dec1* and *Dec2* had a much greater effect on the expression of *Dbp* or *Per1* than either *Dec1* or *2* alone (Nakashima et al. 2008). Furthermore, expression of *Rev-erbα, Bmal1, Clock*, and *Npas2* were all decreased in the absence of both *Dec1 and 2* (Nakashima et al. 2008) suggesting that although not essential clock components they contribute to the robustness of the clock.

1.6.5.4 *E4bp4, Dbp, Hlf* and *Tef* as clock output loops

Albumin gene D-site binding protein (DBP) is a proline and acidic amino acid rich (PAR) basic leucine zipper (bZIP) transcription factor, which is linked to the circadian clock in mammals (Yamaguchi et al. 2000). *Dbp* mRNA has been shown to oscillate in the SCN (in animals housed in DD) (Lopez-Molina et al. 1997; Yan et al. 2000), and behavioural rhythm of the *Dec-1 -/-* mouse is 30 min shorter than the WT (23.26h and 23.76h respectively) (Lopez-Molina et al. 1997). The loss of *Dbp* also results in diminished circadian amplitude of slow wave sleep, an augmented sleep fragmentation and a reduction of spontaneous locomotor activity (Franken et al. 2000; Lopez-Molina et al. 1997). It has been suggested that the circadian transcription of *Dbp* is regulated by CLOCK:BMAL1 activation and PER:CRY mediated suppression, through E-boxes in the intronic region of the gene (Ripperger et al. 2000; Yamaguchi et al. 2000). Furthermore, DBP can induce the *mPer1* promoter in a transcriptional assay and is expressed in the correct phase to induce *mPer1* in the SCN (Yamaguchi et al. 2000). Other members of this family, which share the PAR bZip domins are hepatic leukemia factor (HLF) and thyrotroph embryonic factor (TEF), and both *Hlf* and *Tef* transcript levels have been shown to by rhythmic in the SCN, and in phase with *Dbp* (Mitsui et al. 2001). It has been suggested that the functions of DBP are redundant and that TEF and/or HLF compensate for its loss in the *Dbp* null mutant mouse (Gachon et al. 2004a). Interestingly, in adrenalectomised mice 100 gene transcripts were arrhythmic, compared to WT counterparts, as assayed in the liver but *Dbp* (along with *Per2*) were still rhythmic suggesting an important role in the liver, perhaps associated with metabolism (Oishi et al. 2005).

Gachon et al., (2004a) produced different genetic knock out mice consisting of a combination of the loss of two of the following: *Dbp*, *Hlf* and *Tef* or a triple knock out mouse. Interestingly, a mouse harbouring the loss of *Tef* resulted in the sudden death of the animal, with the death rate highest in the triple knock out (Gachon et al. 2004a). More detailed observations revealed that the triple knock out mouse mortality was due to tonic/clonic convulsions with symptoms that were typical for audiogenic seizures; however, routine histopathology of brain sections did not reveal any major differences between WT and knock out brains. Furthermore, at nine months old the triple knock out mice exhibit signs for early aging, such as cachexia (general weakness and fatigue and muscle atrophy), lordokyphosis (curvature of the spinal column) and an absence of vigor (Gachon et al. 2004a). In a gene array experiment, which compared transcripts from the liver and kidney of WT and triple knock out mice (*Dbp-/-*, *Hlf*-/-and *Tef-/-)* 14 of the 77 transcripts down regulated in the kidney were also down regulated in the liver (Gachon et al. 2006), with many of the genes being involved in lipid and xenobiotic metabolism. In the array, known target genes of the Constitutive Androstane Receptor (CAR), such as members of the *Cyp2b* and *Cyp3a* family were down regulated (Gachon et al. 2006). CAR is expressed in hepatocytes and epithelium villi of the small intestine (Handschin and Meyer 2005; Qatanani and Moore 2005). Upon activation with xenobiotic compounds, such as barbiturates, CAR dissociates from its chaperones and stimulates target gene transcription such as *Cyp2b10* (similar to action of the steroid receptors) as a heterodimer with retinoid-X receptor (RXR) (Ueda et al. 2002). In the triple knock out mouse, *CAR* transcript expression was constitutively low, as was one of its target genes *Cyp2b10.* Intraperitoneal injection of phenobarbital significantly induced expression of *Cyp2b10* in both WT and triple knock out liver and small intestine, but in the knock out the expression was greatly attenuated (Gachon et al. 2006). Furthermore, in the triple knock out mouse the liver, of both male and female mice, was increased in size (a marker of xenobiotic stress) and the mice had increased plasma levels of alanine aminotransferase (a marker for liver damage) (Gachon et al. 2006). Further work on the *Dbp-/-*, *Hlf*-/-and *Tef-/-* triple knock out mouse revealed that it has a heart pathology too, developing cardiac hypertrophy and left ventricular dysfunction (associated with low blood pressure) (Wang et al. 2010). Blood pressure was also significantly lower and heart rate was significantly higher in the triple knock out compared to the WT mouse (Wang et al. 2010). The triple knock out mouse also had increased activity of the sympathetic nervous system. In terms of relevance to humans this is somewhat significant as there are certain mutations in the

human *Bmal1* gene which have been associated with hypertension and type II diabetes (Woon et al. 2007), and there is considerable conservation between *Bmal1* regulated and PAR bzip transcription factors (Gachon 2007).

The protein adenovirus E4 promoter ATF site binding protein (E4BP4) is a bZip transcription factor with a very similar DNA binding domain to DBP, HLF or TEF but which lacks the PAR domain (Cowell and Hurst 1994; Cowell et al. 1992). E4BP4 has been studied mainly in relation to the hematopoietic system (Ikushima et al. 1997; Kuribara et al. 1999) but due to its homology to DBP, HLF and TEF and its homology (in the bZip domain) to VRILLE (Mitsui et al. 2001) (a transcription factor required for a functional *Drosophila* clock, that is structurally related to DBP (Blau and Young 1999), E4BP4 has, therefore, been studied in a circadian context. Mitsui et al., 2001 showed that *E4BP4* mRNA was expressed in many brain regions including the SCN, DMH, hippocampus and dentate gyrus and was rhythmically expressed in the SCN, with low expression during the subjective day and high levels during the subjective night. The respective protein also showed increased expression at CT16 relative to CT4. However, this temporal profile was opposite to that of *Dbp, Hlf* and *Tef* in both the SCN and liver, which was also replicated at the protein level. Furthermore, the PAR containing proteins DBP, HLF and TEF were all shown to induce transcription, whereas E4BP4 suppressed it (Mitsui et al. 2001). *E4bp4* is also a Gc responsive gene with a 4-fold induction, over DMSO, 4h after 1µM DEX treatment in mouse embryonic fibroblasts. This DEX induction was similar to that of *Per1*, yet surprisingly the circadian oscillation, observed in the presence of the vehicle control, was lost after DEX treatment (So et al., 2009).

1.7 Post-translational control of the core clock proteins

1.7.1 Global and multilayered modifications

There is currently a wealth of literature on the vast array of post-translational modifications of clock proteins including phosphorylation, dephosphorylation, acetylation, sumolyation and ubiquitination (Fig 1.5). There are many proteins involved in this modification process including the core oscillator protein CLOCK, recently identified as having HAT activity (Doi et al. 2006). These modifications result in various changes to the clock proteins, and subsequently the cell/organism, For example: pace setting and fine tuning of the oscillator; changing the subcellular localisation and promoting degradation of

individual proteins; and perhaps, more importantly, linking the clock with other internal processes (such as the metabolic state of the organism), proposed to be mediated through the histone deacetylase containing SIRT1 (Duguay and Cermakian 2009). All these processes add increasing degrees of complexity to the core oscillator, which need to be fully dissected before a true understanding of the control mechanisms can be gained.

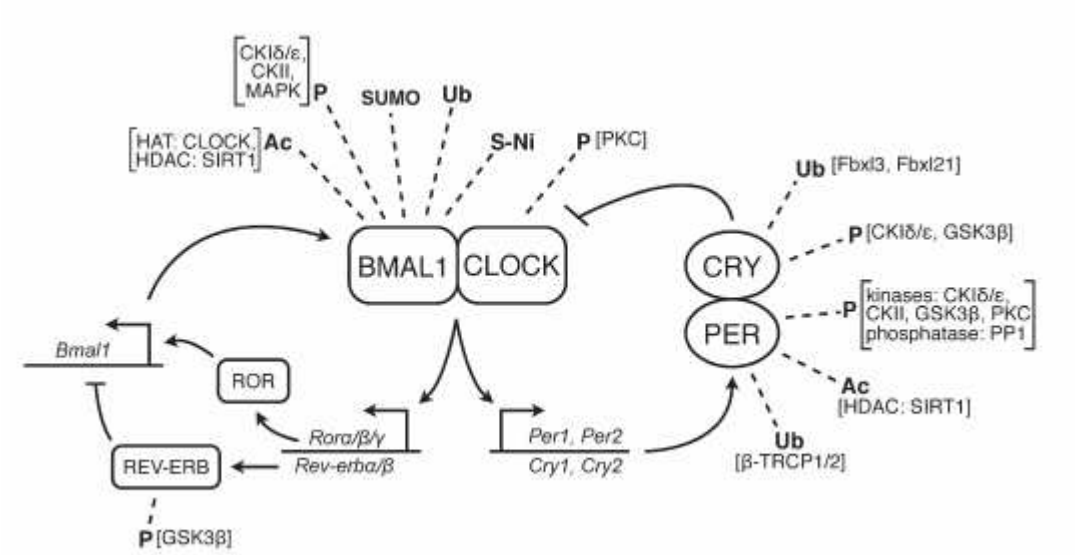

Figure 1.5: Large scale post-translational modification of core clock proteins: Histone acetyl transferase (HAT), histone deacetylase (HDAC), sumoylation (SUMO), ubiquitination (Ub), phosphorylation (P), acetylation (Ac), S-nitrosylation (S-Ni). Taken from Duguay and Cermakian (2009).

1.7.2 Phosphorylation events

There are a number of kinases that are known to phosphorylate the clock proteins, with the most well studied being CK1ε and δ. These two kinases are known to phosphorylate both PER and CRY proteins as well as BMAL1 (Akashi and Nishida 2000; Eide et al. 2002). CK1ε is the more intensively studied due to the gain of function tau mutation, originally discovered in the hamster (Ralph and Menaker 1988) but subsequently shown to be the same in the mouse. This mutation, which was the first mammalian clock mutant, is the result of a C to T transversion at base 178, of which causes a 4h acceleration in the activity of the animal, from 24h to 20h in the homozygous mutant, which is conserved in both hamster and mouse (Lowrey et al. 2000; Meng et al. 2008a). However, a mouse harbouring a deletion for the ε gene, a CK1ε null mutant, exhibits very little differences in its activity cycle (Meng et al. 2008a), suggesting that ε has a limited role in its WT state, and that perhaps δ is able to compensate for the loss of ε. In fact, more recent data has suggested that δ is the major isoform in clock function, as global knock out is lethal and a

conditional knock out will increase the half life of PER2 by 20% and lengthen the period by 2h, as seen in the liver (Etchegaray et al. 2009).

GSK3β is another kinase, well studied in other areas of biology such as Wnt signalling and glycogen synthase activation, which has been shown to phosphorylate core clock proteins. GSK3β is quite an unusual kinase as it requires a priming phosphorylation to occur on the substrate prior to it being able to phosphorylate the protein itself, this usually occurs about 4 residues away from the target site (Frame and Cohen 2001). GSK3β phosphorylates the C-terminal end of PER2 on Ser9, as shown in the rat, at a site distinct from that of CRY or CK1ε/δ binding. This results in increased translocation to the nucleus, where it can repress CLOCK:BMAL1 mediated transcription (Iitaka et al. 2005), in association with the CRY proteins. The phosphorylation status of GSK3β is itself under circadian control, with peak phosphorylation at CT12 (Iitaka et al. 2005), which is in phase with PER1/2 expression and their phosphorylation status (Lee et al. 2001). However, the phosphorylated form of GSK3β is inactive, which is phosphorylated by Akt, downstream of phosphoinositide 3-kinase (PI3) kinase pathway (Frame and Cohen 2001).

GSK3β has also been shown to phosphorylate CRY2, although not CRY1, and REV-ERBα (Harada et al. 2005; Yin et al. 2006). Phosphorylation of CRY2 results in increased nuclear localisation, like that of PER2, but also degradation, whereas phosphorylation of REV-ERBα promotes protein stabilisation and, therefore, increased *Bmal1* repression. Most recently BMAL1 was shown to be phosphorylated by GSK3β on two residues, Ser 17 and Thr21, priming it for ubiquitination and degradation via the proteasome (Sahar et al. 2010). In summary, GSK3β is able to phosphorylate many of the clock proteins, which together opens a very complex phosphorylation system, all with just one kinase. The use of small molecule inhibitors of GSK3β has resulted in the period shortening, by approximately 2h, in an *hBMAL1::luc* reporter cell line, with a similar outcome observed with siRNA (Hirota et al. 2008). However, inhibition with lithium, another inhibitor or GSK3β, resulted in period lengthening. The reason behind this difference is unresolved, but as the siRNA knock down agrees with small molecule inhibitor data then this seems more likely.

There are other kinases involved in the oscillatory loop, including MAPKp42/44, which play a role in light response in the SCN (Coogan and Piggins 2004) and which oscillate in

the SCN too (Obrietan et al. 1998). Many other kinases have been shown to play a role, albeit minor, through mass kinase inhibitor screening projects, kinases such as casein kinase II (CKII), Akt, PI3 kinase (mentioned above), MAPK p38 and c-Jun N-terminal kinase and protein kinase C (PKC) have all been found to alter period length (Isojima et al. 2009), suggesting a very complex hierarchical system of modification, and just through phosphorylation events.

1.7.3 Acetylation and deacetylation

Acetylation is usually associated with histone modification and the promotion of gene transcription. Whereas deacetylation is associated with the opposite, gene silencing, although genes are not totally dormant after deacetylation as this occurs in association with demethylation. Acetylation is carried out by histone acetlytransferases (HATs) which modify lysine residues on histone proteins that are associated with DNA supercoiling. Deacetylation is the removal of acetylation by histone deacetlyases (HDACs). Transcription of the clock and histone modification has been documented with a light pulse in the night inducing histone H3 phosphorylation and the transcription of *cFos*, an immediate early gene and *Per1*, in the SCN (Crosio et al. 2000). Furthermore, areas of chromatin associated with clock genes *Per1*, *Per2* and *Cry1* are acetylated on histones H3 and H4 during transcription (Etchegaray et al. 2003). HAT activity is often associated with transcriptional activator proteins, that induce gene transcription, such as p300 (Etchegaray et al. 2003), but HAT activity has also been discovered in the clock activator protein CLOCK, which acetylates lysine residues on histones H3 and H4 and is necessary for CLOCK induced gene transcription (Doi et al. 2006). More surprisingly, however, CLOCK is able to acetylate non-histone proteins, in particular BMAL1 (section 1.6.3.3) on lysine 537 (Hirayama et al. 2007). This acetylation does not alter subcellular localisation, its degradation or even its association with the *Per2* promoter. It does, however, enhance the CLOCK:BMAL1-CRY1 association, which then promotes CRY1 mediated repression in association with the PER2 bridge (section 1.6.2.1; (Chen et al. 2009) (Hirayama et al. 2007). The CLOCK:BMAL1 heterodimer (more specifically CLOCK) has been shown recently to acetylate a cluster of 4 lysine residues (480, 492, 494, 495) on the hinge region of the glucocorticoid receptor (GR) (section 1.10.3), which is sandwiched between the DNA binding domain (DBD) and the ligand binding domain (LBD) towards the C-terminal of the protein (Nader et al. 2009). This multiple acetylation pattern represses GR induced

transcription by reducing its association with the glucocorticoid response element (GRE) in GR target genes.

Deacetylation, as stated, is the removal of acetyl groups from both histone and non-histone proteins, in order to repress gene transcription. There are 4 classes of HDACs, termed 1 to 4, and two of these classes have members associated with circadian protein interaction and regulation. Class 1 HDACs have three members involved in circadian regulation, HDAC1/2 have been associated with CRY1 mediated repression of CLOCK/BMAL1 induced *Per1* transcription, after a light pulse (Naruse et al. 2004) and NCoR associates with HDAC3 before complexing with REV-ERBα, to repress *Bmal1* transcription (Yin and Lazar 2005). The second class of HDACs involved in circadian regulation is class 3, which all share homology to the yeast Sir2 protein and are NAD^{*} dependent in their activity (Duguay and Cermakian 2009). SIRT1 is a key member of this class and is known to regulate many biological processes such as cell survival, gluconeogenesis, insulin secretion and neuronal survival (Wojcik et al. 2009). SIRT1 has rhythmic protein expression, in phase with PER2, but there is no rhythm at the mRNA level, suggesting that it is under post-translational modification control (Asher et al. 2008). In SIRT1 knock out MEFs, the levels of *Bmal1*, *Per2* and *Cry1* mRNA were all decreased, yet at the protein level CLOCK was absent and BMAL1 was barely detected, but both PER2 and CRY1 showed increased expression, although there was no circadian rhythm detected (Asher et al. 2008). Together these data suggest that SIRT1 activity is required for a robust and functional clock. It is currently unknown which HAT protein acetylates PER2, although it is tempting to suggest that CLOCK is involved. Interestingly other groups have suggested that SIRT1 also targets BMAL1 for deacetylation (Nakahata et al. 2008) but this was not repeated by Asher et al. (2008), perhaps as Nakahata et al. (2008) had used a transfected cell line whereas Asher et al. (2008) had used MEFs, suggesting that the model of investigation is critical to the outcome of the experiment and that there is differential regulation in different tissues and cell types.

1.8 Peripheral Oscillators

1.8.1 Overview

Even though the central clock (often referred to as the master clock), is located in the SCN there are known to be clocks in many other peripheral tissues and cells. Tissues such as

the liver, lungs and heart each have their own clocks (often referred to as the slave clocks) (Yamazaki et al. 2000; Yoo et al. 2004), but which rely on the SCN to synchronise them to exogenous solar time. This has been shown in SCN lesioned mice where peripheral clocks are no longer co-ordinated, neither between the same organ in different animals or between different organs in the same animal (Yoo et al. 2004), although prolonged rhythms still persist in explanted peripheral tissues in SCN lesioned animals. In a series of interesting experiments Guo et al. (2006) SCN lesioned hamsters and measured endogenous peripheral clock transcripts. In this study, rhythms were lost in all tissues analysed, which was slightly different to that seen with Yoo et al. (2004). However, replacement of the SCN restored these rhythms in some (liver, kidney) but not all (heart, skeletal muscle) tissues. These data confirm that peripheral organs differ in their response to SCN born signals, and that the liver and kidney must be taking their timing cues from another source such as blood born signals or perhaps feeding signals. However, results such as these mean care must be taken when using a 'peripheral' model to investigate the clock, it is more likely that individual tissues act individually rather than as one population (Guo et al. 2006). Furthermore, it is this internal synchronisation, between the SCN and periphery, which is the source of a lot of interest in circadian biology at the moment and will form the basis of much research over the coming years, including within this thesis. The principal questions asked are, 'How does the SCN communicate with the periphery', and 'What are the outputs and consequences from peripheral clocks?'

Peripheral oscillators are entrained to the external environment by the central oscillator, the SCN (Stratmann and Schibler 2006), (Fig 1.6). This is via hormonal signals (such as Gcs), or neuronal signals, but also by other external signals such as feeding (referred to as zeitgebers), where daytime restricted feeding of a nocturnal rodent will invert the phase of the peripheral clocks relative to that of the SCN, which is largely unaffected (Damiola et al. 2000). The ambient temperature cycle is another possible zeitgeber as temperature rhythms can induce oscillation in cultured Rat-1 fibroblasts (Brown et al. 2002). This entrainment process is necessary as mammalian tissues are not directly light-sensitive, though peripheral tissues in lower eukaryotic animals are, such as Drosophila and Zebra Fish (Hastings et al. 2003) and as such do not necessarily require such entrainment. Peripheral clocks, in common with the SCN, are temperature compensated over the normal mammalian temperature range, 33^{o}c to 42^{o}c. The reason for this remains unclear but many animals can lower their body temperature, known as torpor, under certain conditions (i.e.

on calorific restriction, prolonged exposure to short days and low glucose intake). Furthermore, the major temperature decrease is hibernation, yet the animal still needs to wake at an appropriate time, such as the Spring, so must maintain a functional clock (Stratmann and Schibler 2006). This may offer a partial clue as to the reason, yet the method requires further investigation.

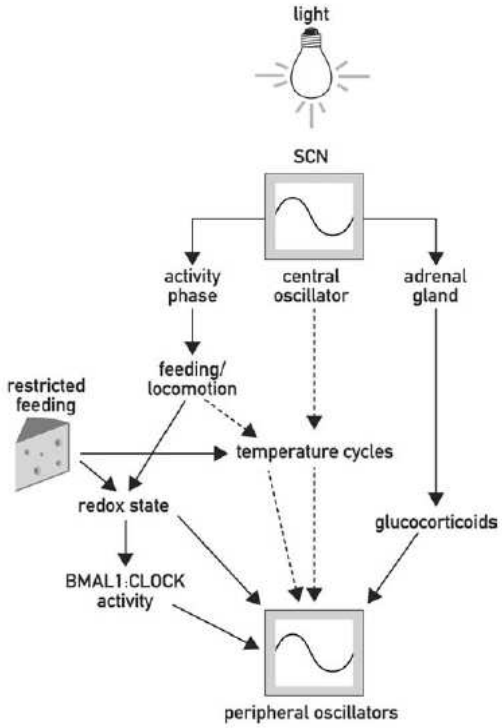

Figure 1.6: **The entrainment of peripheral clocks by the SCN** (Stratmann and Schibler, 2006).

1.8.2 The lung as a peripheral model

Moving away from the central molecular oscillator, in the SCN, this thesis aims to look at the clock in the periphery, with particular interest in the lung, using the mouse as a model. The reasons are relatively simple and two fold. First there has been little work carried out on the lung as a circadian organ compared with other organs such as the liver and heart, even though expression of clock genes in the periphery (Tei et al. 1997) and oscillations specifically in explanted lungs (Yamazaki et al. 2000; Yoo et al. 2004) have been known for many years. Secondly, is that respiratory diseases, such as asthma and chronic obstructive pulmonary disease, (COPD), are generally thought to have a circadian component, for example airways are narrowest in the early hours of the morning (Brenner 2003). In addition Gcs are known to be potent regulators of the peripheral clock

(Balsalobre et al. 2000a) (discussed more in section 1.10), are used widely in the treatment of inflammatory diseases such as arthritis and inflammatory bowel disease but also in the treatment of asthma to alleviate the symptoms of inflammation (Barnes and Adcock 2003), yet surprisingly this treatment does not work too well in COPD patients (Barnes et al. 2004).

1.8.3 Lung structure: Clara Cells and Type II Pneumocytes

The mammalian lung is structured with the trachea leading from the nasal cavity which splits into the bronchi (one each for the left and right lobes), each bronchus then splits into the bronchioles and they terminate in the alveoli. Many cells make up the various sections of the lung but key cell types are the Clara cells and Type II pneumocytes. Clara cells are a well differentiated cell type with a high secretory activity, located in the bronchial epithelium of the mammalian lung (Fig 1.7). Clara cells are non-ciliated and form a major cell type of the bronchioles along with some other ciliated cells (Cassel et al. 2000). Numerous proteins are known to be secreted from the Clara cells but the major protein is the Clara Cell Secretory Protein, CCSP (Cassel et al. 2000), which is specific to the Clara cell and, therefore, can lead to easy identification (Gibbs et al. 2009). CCSP itself is a small 10kDa homodimeric protein, and has been shown to co-express with both CLOCK and PER2, in the lung. Isolated Clara cells have a sustained oscillation with a period of about 23.8 hr +/- 0.4, and selective disruption of these cells with naphthalene abolishes the oscillation in lung explants (Gibbs et al. 2009); leading to the conclusion that these cells are of vital importance to the lung oscillation. Furthermore, IL-6, an oscillating cytokine involved in the inflammatory response (discussed further in 1.8.), has been shown to be released from bronchial epithelial cells and although Clara cells were not specifically cited, there is a potential link between this cell type and the circadian aspects to pulmonary inflammation (Khair et al. 1996).

Type II pneumocytes are a further specialised cell type within the alveoli of the lungs (Fig 1.8), which potentially express CLOCK and BMAL1, however, evidence is still circumstantial (Gibbs et al. 2009). They are sometimes referred to as the ‘corner cells’ of the alveolus due to their location in the corners of the alveoli in healthy lungs (Ulich et al. 1994). Type II pneumocytes have a multifunctional role within the lung, which includes secretory, synthetic and progenitor capabilities (Bhaskaran et al. 2007b). The Type II cells act as progenitors for the Type I cells, also within the alveolar epithelium in the event of

injury (Fig 1.8). Type II cells are also metabolically active, with their major role being to produce and secrete surfactant proteins. In contrast, the Type I cells are relatively inactive, and their main function is to provide a suitable interface for oxygen exchange between air and the capillaries (Ulich et al. 1994). Surfactant is comprised of phospholipids, especially phosphatidylcholine and phophphatidylglycerol, and the surfactant proteins released/produced by the pneumocytes are, SP-A, B, C and D (Mason et al. 2003). They are secreted to reduce surface tension at the air liquid interface in the lung, preventing alveolar flooding, alveolar instability and small-airway closure (Mason et al. 2003). There have also been limited reports that SP-C has a circadian expression pattern (Kim et al. 2003) and that transcript expression of *SP-A*, *B* and *C* is up-regulated by exogenous Gcs administration, yet no change is seen after adrenalectomy (Fisher et al. 1991), suggesting a minor but significant role in administered doses like that seen with asthma. The Type II cells contain a unique organelle called a lamellar body, in which surfactant is stored before secretion, and the differentiation of Type II cells occurs relatively late in foetal development as the surfactant system needs to mature before birth (Li et al. 1995). It is believed that Type II cells play a prominent role in the morphogenesis of foetal lungs as well as producing the surfactant proteins necessary for the transition from the amniotic fluid to the air (Ulich et al. 1994). It is understood that Gcs, estrogen, thyroid hormones and epidermal growth factor may enhance foetal lung development and promote surfactant production *in vivo* (Ulich et al. 1994). Both Clara and Type II cells have been identified as the putative epithelial lung stem cells. Clara cells are associated with the regeneration following tissue damage, whereas the re-modelling of alveolar structure following damage or increased metabolic demand is attributed to Type II cell conversion to Type I, (Bhaskaran et al. 2007).

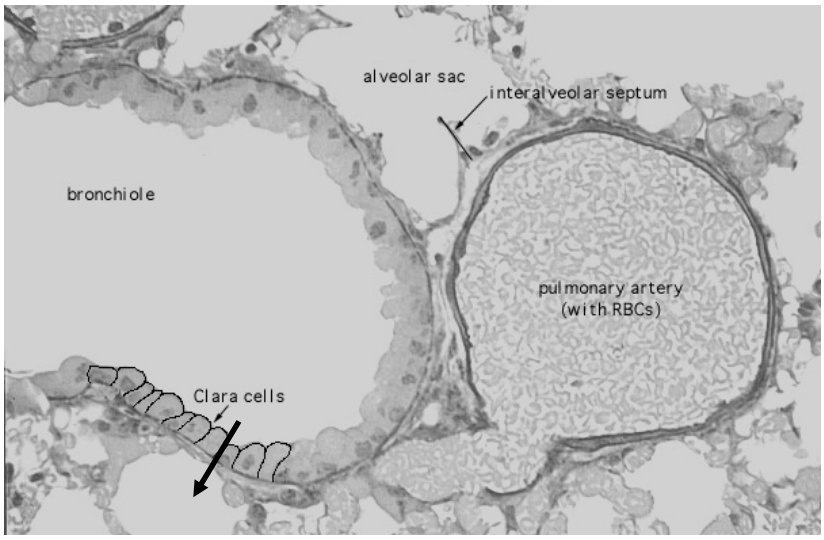

Figure 1.7: Clara cell location in the lung bronchioles of the human lung: This image shows the position of the Clara cells which line the bronchioles in the lung. Clara cells are outlined and marked (image taken from www.bu.edu/histology/i/13805loa.jpg).

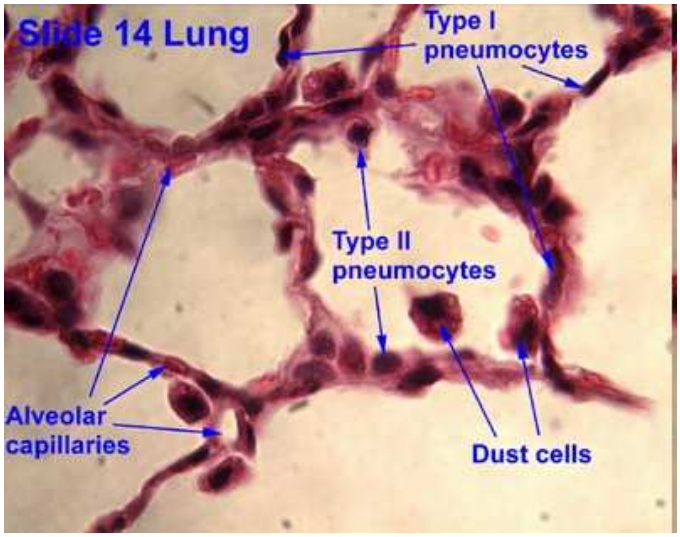

Figure 1.8: Position and shape of Type I and II pneumocytes in the lung: This image shows the position and shape of the type I and II pneumocytes in the lung (image taken from http://www.ouhsc.edu/histology/Glass%20slides/14_14.jpg).

1.9 C/EBP as clock controlled output genes

1.9.1 The family of C/EBPs

CCAAT/Enhancer binding proteins (C/EBPs) are a family of transcription factors, which belong to the bZip family of proteins. The first C/EBP protein was identified by McKnight and co-workers which bound to a CCAAT element in the promoter of target genes. Further work also identified the bZIP DNA binding and dimerisation domain, which led to

the discovery of many more proteins with this structure (Landschulz et al. 1988). There are six known isoforms of C/EBPs, which are classified as: α, β, γ, δ, ε and ζ; they all contain the C-terminal bZip domain but the N-termini differ in length and composition. Furthermore, α, β and ε have multiple protein products, resulting from a secondary downstream ATG start site, a leaky ribosome or selective proteolysis, and most probably a mixture of the three. The α, β, γ, δ isoforms are all intronless, that is they only have one coding exon whereas the ε and ζ isoforms have 2 and 4 respectively (Ramji and Foka 2002). C/EBPs have been identified, over the last decade, as having a pivotal role in many cellular processes, such as controlling growth and differentiation and immune and inflammatory responses (Ramji and Foka 2002). C/EBP α and β are particularly difficult to study as knock out mice lead to death with regard to α (Wang et al. 1995), with conditional knock outs also proving problematic (Lee et al. 1997) and either death or serious liver problems with regard to β knock out mice (Roesler 2001). C/EBP α, β and δ are expressed in high levels in many peripheral tissues such as the liver, lungs and adipose tissue; whereas ζ and γ are ubiquitously expressed and ε is restricted to myeloid and lymphoid cells. C/EBPs have been implicated in the differentiation of many cell types including: adipocytes, hepatocytes, ovarian luteal cell, neuronal cells and others. The mRNA of C/EBP β and δ is induced during mitosis of the undifferentiated cell in response to an increase in cyclic adenosine mono phosphate (cAMP) and Gcs respectively, with C/EBP α being expressed after exit from the cell cycle and with *C/ebp α* being induced by β and δ (Ramji and Foka 2002).

Using microarray transcriptional profiling, with a NIH 3T3 cell line Gery et al. (2005) provided a link between the circadian clock and C/EBPs, providing evidence to show that both *Per2* and *Rev-erba* were altered by C/EBPs, as well as the clock output gene, *Dbp*. The greatest induction, however, was with C/EBPε, giving an 11 fold increase in *Per2* transcription. In a confirmation assay, a *Per2* promoter-luciferase construct was co-transfected with C/EBPα and ε resulting in a 7 and 5 fold transcriptional increase, showing that *Per*2 was induced by both proteins (Gery et al. 2005). Other roles for C/EBPs include control of metabolism, cellular proliferation, a role in inflammation and a novel role in long-term synaptic plasticity underlying memory (Alberini et al. 1994) in the Sea slug *Aplysia*, in which *ApC/EBP* was later shown to be under circadian control (Lyons et al. 2006).

There are very strong links between the C/EBP family and the inflammatory response. C/EBP β was first identified as it regulated gene transcription in response to interleukin-6 (IL-6) and IL-1 (Akira et al. 1990; Poli et al. 1990) and now it has emerged that a great many proteins in the inflammatory response are indeed regulated, or at least partially, by the C/EBP family (Ramji and Foka 2002). There is a C/EBP binding site within the promoter of the tumour necrosis factor receptor 1 (TNFα), which is required for both C/EBP α and β induction, and activation of this gene is significantly inhibited if either C/EBP α or β are knocked down by small interfering RNA (siRNA) (Bristol et al. 2009). C/EBP β is also very important for the inflammatory response and macrophage differentiation (Pham et al. 2007; Poli 1998). More recently, it has been demonstrated that both C/EBP α and β can activate expression of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), an enzyme used to convert inactive Gcs to the active form (Ignatova et al. 2009), which act as potent anti-inflammatory agents. In addition to this, C/EBP β specifically binds to the 11β-HSD1 promoter, to up regulate transcription in the presence of TNF α, (discussed further in section 1.12) during an inflammatory response and forming part of the feedback loop to suppress inflammation (Ignatova et al. 2009).

1.9.2 C/EBPs, Type II Pneumocytes and Clara Cells

It has been recognised for a long time that C/EBPs are expressed in many cell types including the lung, in particular the α, β and δ isoforms (Cao et al. 1991) (discussed previously in section 1.9.1). However, little was known about cellular localisation until Li et al. (1995) demonstrated a correlation between C/EBPα and expression of surfactant protein in Clara cells, as well as Type II pneumocytes, although with far greater C/EBPα expression in the latter. Further work has shown a correlation between C/EBP α and CCSP in *in-vitro* cell culture models, (Nord et al. 1998), with further studies showing CCSP gene activation through C/EBPα and delta binding to the C/EBP promoter elements (Cassel et al. 2000). However, C/EBPδ has far more effect on activating transcription than the α isoform.

C/EBPα is known to target genes associated with lipid metabolism and it has been shown in hepatocytes that C/EBPα activates expression of apolipoprotein B (Li et al. 1995), which is a component of low density lipoproteins. C/EBP α mRNA was shown, more that a decade ago, to be present in the Type II pneumocytes in the lung and in a paper produced by Li et al. (1995) C/EBPα expression also correlated with the expression of surfactant

protein genes. It was further shown by Li et al. (1995) that there is more C/EBPα mRNA in the lung than in either the spleen or kidney, using the rat as a model, with a large portion of this mRNA resulting from the Type II cells. Furthermore, in the C/EBPα knock out mouse part of the reason for death shortly after birth is the hyperproliferation of the Type II pneumocytes, which results in the reduction of the alveolar air space and an inability to breath efficiently (Flodby et al. 1996).

In summary, there is good evidence for clock gene localisation in both Clara cells and Type II pneumocytes and Clara cells are known to oscillate. C/EBPs are known to be present in Type II pneumocytes and are known to regulate CCSP gene activation, as well as Type II cell surfactant protein expression. These data form a good basis to a hypothesis stating that the Clara cells and Type II cells are clock regulated components of the lung and that C/EBPs play a prominent role in their physiology as a clock mediated gene/protein.

1.10 Glucocorticoids and circadian regulation

1.10.1 Corticosterone: hormonal production and secretion

The majority of peripheral tissues and cell types studied contain functional clocks, yet they cannot be entrained directly by light and must, therefore, be entrained via the SCN through an alternate route such as neuronal or hormonal, so that the peripheral clocks are in phase with the environment (Oster et al. 2006)**.** It has been suggested that Gcs are suitable blood born factors which could relay the phase of the clock between the SCN and the periphery (Balsalobre et al. 2000)**,** as the Gc analogue dexamethasone (DEX) can both induce *Per1* and *Per2* transcript expression and induce sustained circadian rhythms (Balsalobre et al. 2000a; So et al. 2009). Alternative factors, proposed to transfer the phase signal to the periphery include; cAMP, protein kinase C (PKC), receptor tyrosine kinases, which are intrinsic factors (Balsalobre et al. 2000b) and retinoic acids and the epidermal growth factor receptor, which are extrinsic factors (Koyanagi et al. 2006b), but Gcs are regarded as the predominant circulating factor**.** These alternative factors must be in use in order to relay the phase of the central clock and therefore external time, as the removal of the adrenal glands (thus loss of the Gc signal) does not automatically result in the loss of a circadian rhythm. In adrenalectomised mice over 100 genes lost their circadian rhythm in the liver but *Per2* and *Dbp* did not (Oishi et al. 2005). This is further supplemented by work on tumours where there was no significant difference seen in the circadian expression

of *Bmal1, Clock, Per2 or Cry1* in tumours implanted in to adrenalectomised or sham operated mice, yet the circadian expression of Topoisomerase I was lost (Kuramoto et al. 2006). The rat *Per2* rhythm in the central region of the amygdala and the oval nucleus of the bed nucleus of the stria terminalis is lost when the adrenals are removed, and regained when CORT was supplemented in the drinking water (Segall et al. 2006). Together, these data suggest regional specificities of CORT regulation and alternative synchronising factors other than glucocorticoids.

Gcs are derived from a cholesterol precursor and are synthesised in the adrenal cortex, in regions called the zona glomerulosa (ZG) and zona fasciculata (ZF) (Oster et al. 2006). Gc release, from the adrenal, is under both hormonal and neuronal control, which results in both circadian and ultradian secretion (Chrousos 1998; Windle et al. 1998a; Windle et al. 1998b) (Fig 1.10). Although relatively little work has been carried out on the ultradian rhythm, in relation to the clock, it does appear to be circadian modulated with a less frequent pulse release in the short period tau mutant hamster compared to the WT (Loudon et al. 1994).

In terms of hormonal regulation, the SCN activates the rhythmic release of corticotrophin-releasing hormone (CRH) from the paraventricular nucleus (PVN), which is released into the median eminence that subsequently induces the release of adrenocorticotropin hormone (ACTH) from hypophysial adrenocorticotroph cells in the anterior pituitary (Simpson and Waterman 1988). However, ACTH secretion can also be stimulated by arginine vasopressin (AVP) and oxytocin, both released into the median eminence from nerve terminals leading from the PVN, which can then induce ACTH release from the posterior pituitary (Dickmeis 2009). ACTH is then released into the peripheral blood stream and can induce the release of CORT from the ZG and ZF in the adrenal cortex (Simpson and Waterman 1988). This is known as the hypothalamic pituitary adrenal axis (HPA). Interestingly though, there are few direct projections between the SCN and the medioparvocellular (mp) division of the PVN (mpPVN) (Fig 1.9), where CRH and AVP neurons originate from (Vrang et al. 1995), although the SCN does show direct connections with the subparaventricular zone (subPVZ) and the dorsomedial nucleus of the hypothalamus (DMH), which then connect with the mpPVN (Dickmeis 2009), so perhaps an indirect SCN connection via the DMH and mpPVN. In Syrian hamsters where the SCN had been lesioned, but a fetal SCN had restored the behavioural rhythm the

CORT rhythm was not restored. Equally there was no melatonin rhythm in SCN lesioned animals where a graft had restored the behavioural rhythm, nor was testis regression observed (Meyer-Bernstein et al. 1999). Together these data suggest that endocrine rhythms require greater neural circuits than those required for activity rhythms. ACTH release is itself under both circadian and ultradian regulation, similar to that of CORT (Carnes et al. 1989; Chrousos 1998); in addition to this *CRH* mRNA also has a circadian component to its synthesis (Watts et al. 2004), resulting in the tight circadian control of CORT release. However, ACTH release has been shown to precede *CRH* transcription and as a result their interaction may only be slight.

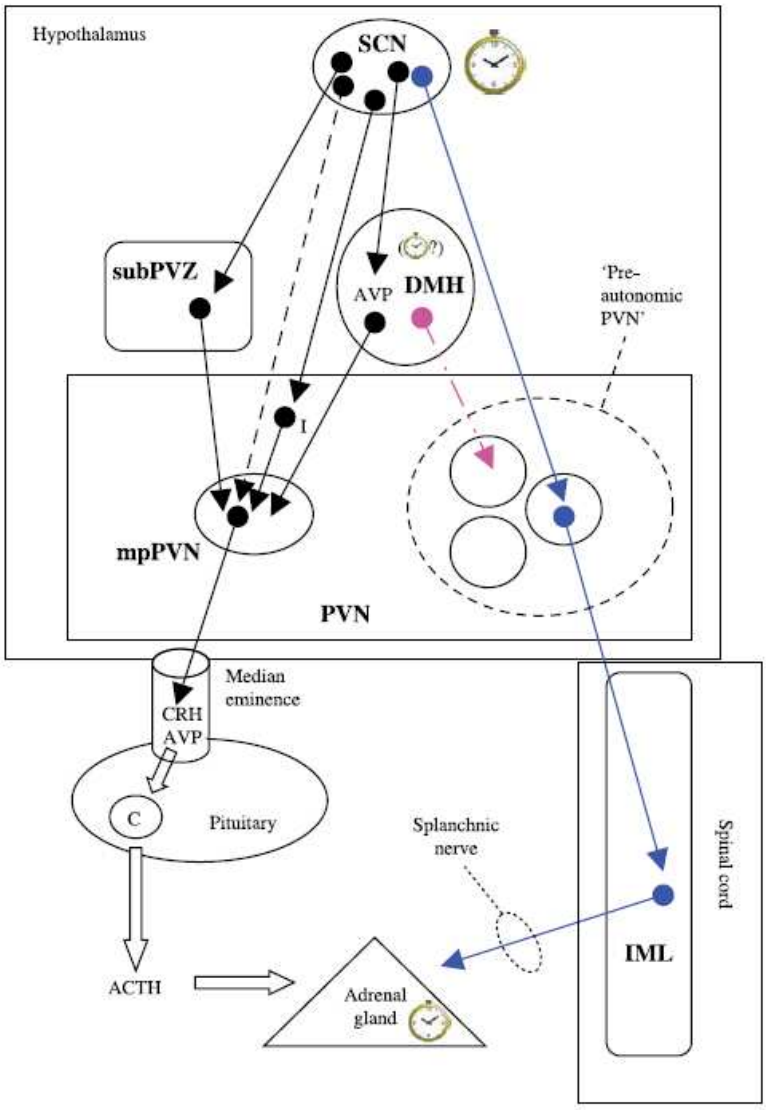

Figure 1.9: Hormonal and nerve connections between the SCN and adrenal gland: Suprachiasmatic nucleus (SCN); subparaventricular zone (subPVZ); dorsomedial hypothalamus (DMH); paraventricular nucleus (PVN); medioparvocellular division of the PVN (mpPVN); interneuron of the PVN (I); corticotrophe cell of the pituitary (C); intermediolateral column of the spinal cord (IML); arginine vasopressin (AVP);

corticotrophin releasing hormone (CRH); adrenocorticotropic hormone (ACTH), (Dickmeis 2009).

Furthermore, under various experimental conditions, reports have been made that the release of ACTH and CORT do not align, and as such the regulatory process of CORT by ACTH, may be uncoupled (Dickmeis 2009), yet other studies suggest that ACTH release peaks approximately 2h prior to the CORT peak (Oster et al. 2006). These observations may be due, in part, to the ultradian pulses of both CORT (Fig 1.10) and ACTH coupled to the acute stress response of the animal (where CORT release dramatically increases the animal's survival response). It has been hypothesised that this stress response is the reason, or at least part of the reason, behind the observed ultradian rhythm (Lightman et al. 2008; Windle et al. 1998b).

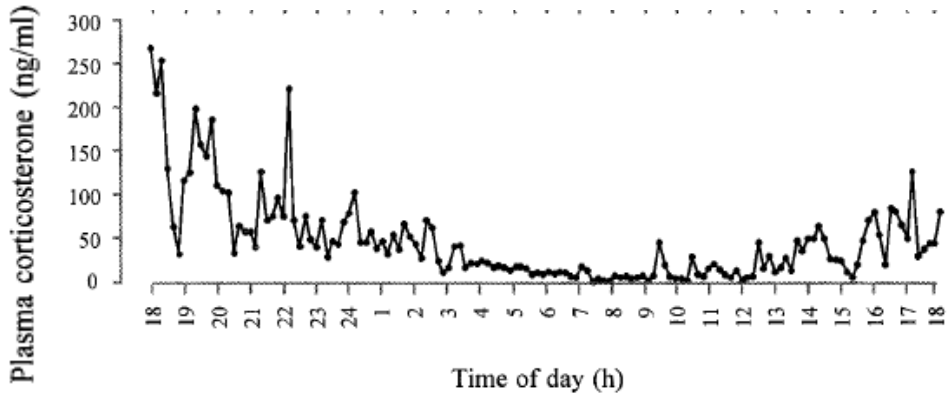

Figure 1.10: The circadian and ultradian rhythm of corticosterone in female rat over 24 hours taken in ten minute intervals: This trace shows approximately 27 pulses in 24h which equates to one ever 51 min (Windle et al., 1998).

1.10.2 Corticosterone: neuronal production and secretion

The above pathway states the generally accepted route through which Gcs in the adrenal gland are synthesised, but it is also known that there are alternative routes of production leading from the SCN to the adrenal. In both gulf killifish and rats, surgical removal of the pituitary gland did not result in the loss of the Gc rhythm even in the absence of ACTH; yet the Gc rhythm was lost in rats when their adrenal glands were denervated (Dickmeis 2009), suggesting neuronal control. Virus tracing has shown a neuronal connection between the SCN and adrenal gland involving multiple synapses. Using Fig 1.9 as a guide, the SCN connects to the pre-autonomic PVN neurons and then to the preganglionic neurons of the intermediolateral column (IML) in the spinal cord. This connection then innervates the adrenal gland via the splanchnic nerve (Buijs et al. 1999). Interestingly this

pathway does not overlap with the HPA axis outlined in section 1.10.1 and Fig 1.9 as the two areas of the PVN involved are distinct regions, suggesting a divergence in regulation at the PVN level.

In a study using light pulses in the rat Buijs et al. (1999) demonstrated that a 5 min light pulse in the early part of the subjective night (CT14) resulted in a repression of CORT release but not when light pulsed in the late subjective night or day, as seen in intact animals, incidentally no suppression was observed in SCN lesioned mice. However, a similar study using a 30 min light pulse in the early subjective night failed to detect an increase in CORT up to 60 min after the pulse, then there was a large sustained induction of CORT, which had returned to baseline conditions by 180 min. This induction was independent of an ACTH increase, and showed different temporal events to the immediate change induced by a stress response (Ishida et al. 2005). The induction was also lost after denervation of the adrenal and after SCN lesion. The 30 min light pulse resulted in increased expression of *Per1* in the adrenal, when administered in the subjective night but not the day, also there was increased phosphorylation of ERK1/2, 60 min after the light pulse but not CREB phosphorylation (Ishida et al. 2005). This is different to what is seen in the SCN after a light pulse in the early night, where CREB is phosphorylated in response to light and binds to it response element (CRE) in the promoter of *Per1* (Albrecht et al., 1997) (section 1.5.2.1). These data show that there is a different regulatory mechanism in central and peripheral tissues, with regard to light induction.

1.10.3 The nuclear hormone receptors: glucocorticoid and mineralocorticoid

The GR is part of a family of receptors which includes the mineralocorticoid receptor (MR), vitamin D receptors and retinoic acid receptors, which all act as ligand dependent transcription factors and share a similar gross structure and conservation. The GR is highly conserved throughout vertebrate species and is made up of: a N-terminal region; DBD in which there are two zinc finger motifs to allow DNA binding; a hinge region; a LBD, and a C-terminal region (Stolte et al. 2006). There are 2 GR splice variants termed GRβ and GRγ, in addition to the more widely known ligand binding receptor GRα, mentioned above. GRβ does not bind ligand, due to differences in the C-terminal region, and as a result was largely ignored for many years, however, over recent years interest has been resurrected in this isoform due to its wide tissue specificity and the idea that it may act as a dominant negative mutant to the GRα isoforms (Lu and Cidlowski 2004; Yudt et

al. 2003). A further splice variant GRγ has been isolated, which has an in-frame insertion mutation between the two zinc finger motifs in the DNA binding domain (Rivers et al. 1999) and makes up about 4-8% of the total GR, although it has much less transcriptional activity than GRα and has been associated with human cancers, with a similar insertion found in membrane GR (mGR) and the human MR (Lu and Cidlowski 2004).

In its inactive state the GR is located in the cytoplasm of the cell, bound to chaperone proteins, which are made up of Hsp and are essential for the opening of the cleft to allow the ligand to bind. Hsp70 binds first to the GRα LBD and induces a conformational change in the ligand binding cleft; Hsp90 is then able to bind to this complex and fully open the binding cleft and allow the ligand, such as CORT, to bind (Stolte et al. 2006). The chaperones will then dissociate and the GR can be activated by phosphorylation and translocate to the nucleus, where it will bind to the palindromic response element in the target genes (GRE) (Fig 1.11).

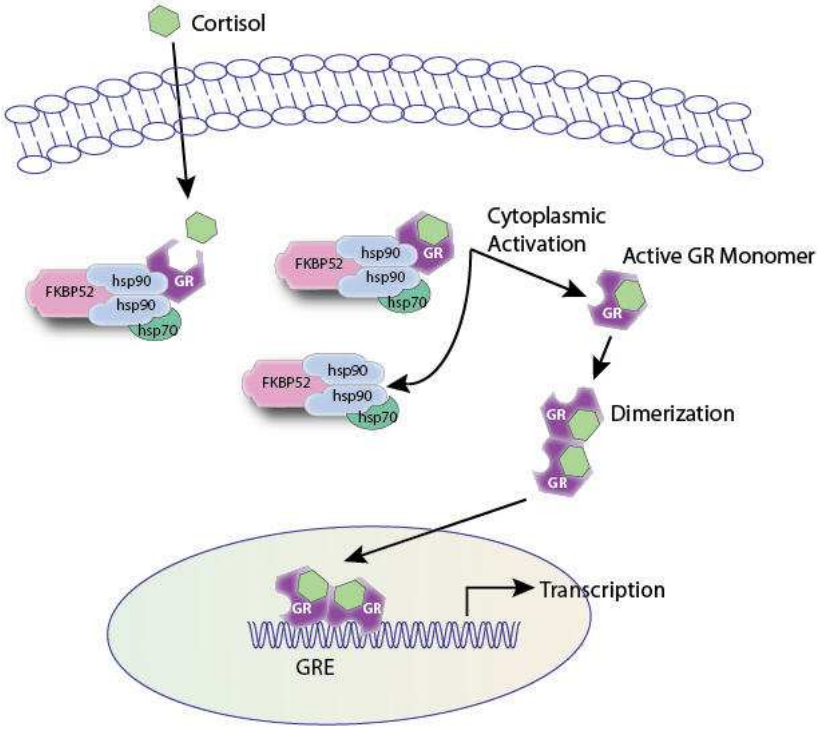

Figure 1.11: Nuclear receptor signalling: Here the GR is shown as a homodimer yet the same pathway is used by the MR homodimer and the GR:MR heterodimer. Image taken from http://www.panomics.com/images/94_2_GRHOW_1_V1.jpg.

Despite its name, the MR shows a 10 fold higher binding affinity for Gc than the GR (De Kloet et al. 1998). While GR appears to be ubiquitously expressed throughout the brain and periphery, at both mRNA and protein level (Arriza et al. 1988; Morimoto et al. 1996)

the MR seems to be far more restricted with its expression in peripheral tissues such as the kidney and in brain regions (like the hippocampus and pituitary) (Funder and Sheppard 1987). The MR cannot distinguish between its main ligand aldosterone and CORT, so local enzymatic conversion of active to inactive CORT is used to allow aldosterone to bind in target tissues such as the kidney. Hydroxysteroid 11-β dehydrogenase (HSD11β) type 1 and 2 mediate the conversion between active and inactive CORT. The type 2 enzyme, expressed in MR target tissues removes the hydroxyl group on the 11th carbon rendering CORT inactive, whereas the type 1 enzyme, more widely expressed replaces it (Dickmeis 2009). The type 1 enzyme has been shown, in the rat, to be expressed in a circadian manner at the mRNA level, with increased expression in the morning relative to the evening, although the timing of this is out of synchronisation as peak CORT expression is prior to the onset of activity (in the night) and peak mRNA of the HSD11β type 1 enzyme is 12h earlier. However, this has been reported in the hippocampus and has not been observed in the periphery (Buren et al. 2007).

1.10.4 GR, Gcs and circadian regulation

Although the circadian and ultradian regulation of glucocorticoids has been well documented, there have been mixed reports on the circadian regulation of GR itself, mainly characterised at the transcript level. In HeLa cells, the GR transcript showed no circadian oscillation, either in the presence or absence of the Gc analogue DEX (Nader et al. 2009). However, in the same study the highly responsive GR induced gene, the glucocorticoid induced leucine zipper (GILZ) did show circadian transcript regulation. A further study characterising the expression of all 49 mouse nuclear receptors in metabolically active tissues, white and brown adipose tissue (WAT and BAT respectively), liver and muscle revealed that 25 were rhythmically active, including GR. However, the GR was only rhythmic in WAT and BAT and peak expression was in anti- phase to each other, with WAT peaking at zeitgeber time 12 (ZT12) and BAT at ZT0. Furthermore, MR was not found to be rhythmic in any of the tissues studied (Yang et al. 2006), although the tissues studied were not classical MR target tissues. The GR protein has not been shown to be under direct circadian regulation, as seen in the lung (Gibbs et al. 2009), but a day/night difference was seen, in cytosolic expression, in the hippocampus, but not in the hypothalamus (Furay et al. 2006). Furthermore, many heat shock proteins are under circadian control, which complex with the GR in the cytoplasm (Kornmann et al. 2007; Reinke et al. 2008). The GR/heat shock complex has been shown to be under region

specific circadian regulation. Furay et al. (2006) reported that there was no time of day difference in the cytosolic expression of Hsp70 or 90 in the hippocampus, but circadian variation was observed in the GR/Hsp90 complex, with a greater association in the evening relative to the morning.

In a detailed study of the systemic regulation by glucocorticoids in SCN ablated mice Reddy et al. (2007) demonstrated that a single dose of DEX could re-synchronise 20% of the liver circadian transcriptome and almost 60% of circadian genes were either up or down regulated by DEX. Interestingly, of the genes re-synchronised by DEX, two-thirds carried a putative GRE in their promoter or first intron, and of those, the majority were enriched for other regulatory transcription factors, suggesting dual regulation (Reddy et al. 2007). Further investigation revealed that hepatocyte nuclear factor 4 α (HNF4α) was a key element in co-regulation both with GRE and in the absence of the GRE. *HNF4α* was shown to be highly rhythmic in this study, peaking in phase with *Per2*, and rhythmicity lost in the *Clock -/-* mouse, but interestingly no *HNF4α* rhythm was detected in the liver in the Yang et al. (2006) study. However, in the *HNF4α* gene locus there are three canonical E-boxes and three putative GREs and the gene is induced in response to DEX. Together these data suggest that HNF4α is a true Gc signal relay, working both in partnership and individually with the GR to synchronise the liver clock, however, regulation of other peripheral tissues is undetermined.

1.10.5 Other nuclear receptors and circadian timing

This thesis has focused largely on CORT resetting of the circadian clock, mediated through the GR, with a contribution from the MR. Even though CORT release is known to be under circadian control (section 1.10.1) and the GR almost ubiquitously expressed, the GR does not appear to be rhythmic in all tissues where it is expressed. The GR has been shown to be rhythmically expressed, at the transcript level in WAT and BAT peaking at ZT0 and ZT12 respectively (Yang et al. 2006), but not other metabolically active tissue such as the liver and muscle. However, at the protein level in the murine lung the GR was shown to be constitutively expressed (Gibbs et al. 2009). Furthermore, in Zebrafish larvae the expression of GR transcript and protein is constitutive over the circadian day, even under a LD cycle (Dickmeis et al. 2007). Also the MR is not thought to be under circadian control, as it was arrhythmic in the tissues studies in the Yang et al. (2006) study. However, there are 49 nuclear hormone receptors in the mouse and in the study of Yang et

al. (2006), which examined WAT, BAT, liver and muscle expression, 25 of these nuclear receptors were expressed in a circadian manner. In all, six nuclear receptors were rhythmically expressed in all four tissues studied: *estrogen related receptor β* (*ERRβ*), *Rev-erbα* and *β*, *germ cell nuclear factor* (*GCNF*) and *testicular receptor 4* (*TR4*). Yet 14 others were rhythmically expressed in two or three of the tissues, such as *TR2*, *retinoic acid receptor α* and *γ (RARα* and *γ)*, *thyroid hormone α* (*THα*) and *PPAR α*, *γ*, *δ* (peroxisome proliferator-activated receptor α) . The vitamin D receptor, RORα and β and thyroid hormone receptor β only cycled in one tissue. Interestingly, when the time of peak expression was characterised, ZT4 was the time when most nuclear receptor showed peak expression, and of the receptors that were rhythmic in more than two tissues, eight maintained the same phase between tissues (such as ERRα, ERRγ, Rev-erbα, Rev-erbβ, RORγ and TRa). These data suggested that there is some importance surrounding ZT4, but the reason behind it remains unknown, although it is the early part of the subjective day (rest period for nocturnal animals) so perhaps it is to do with metabolising food and fat storage. However, other receptors were uncoupled between tissues (such as PPARγ and δ, GR and GCNF). Uncoupling is unusual and suggests that some nuclear receptors may be entrained by different zeitgebers in different tissues (Yang et al. 2006).

In the discussion above, of the latter group of nuclear receptors, i.e. uncoupled between differed tissues, PPARγ is of particular interest to circadian transcriptional expression (Schmutz et al. 2010). There are three members of the PPAR family (α, γ and δ), which have been shown to regulate lipid metabolism and energy homeostasis, in muscle, liver and adipose tissue (Evans et al. 2004; Lee et al. 2003). Yang et al. (2006) showed that *PPARα*, which is involved in hepatic fatty acid oxidation (Kersten et al. 1999), cycled in WAT, BAT but not liver, whereas *PPARγ*, involved in lipid storage and lipogenesis in the liver (He et al. 2003), cycled selectively in WAT and liver. *PPARδ*, which is ubiquitously expressed, is rhythmic in BAT and liver and oscillates in phase with *uncoupling protein -1* (*UCP-1*), which suggested a link between diurnal variations in body temperature (section 1.5.3.5) and the circadian clock via *PPARδ* (Yang et al. 2006). This link is further enhanced as *PPARα* is also expressed in phase with *PCG 1α*, known to be involved in cold and diet-induced thermogenesis (Lowell and Spiegelman 2000) and *UCP-1*, together suggesting how endogenous temperature may be linked to the circadian clock and vice-versa. Also the PPARγ agonist, fenofibrate, has been shown to induce circadian clock gene

expression (*mCry1*, *mRev-erbα* and *mBMAL1*) in Rat-1 cells, similar to that induced by serum shock, although *mPer2* was not mentioned in this study (Canaple et al. 2006).

PER2, but not PER1, has recently been shown to coimmunoprecipitate with PPARγ via an LXXLL motif located at the N-terminal of the PER2 protein (Schmutz et al. 2010). PPARγ is known to bind to the m*BMAL1* promoter and induce circadian expression in the liver (Canaple et al. 2006). The interaction between PPARγ and PER2 was greatest at ZT20, a time when *mBMAL1* expression was highest (Schmutz et al. 2010). PER2 was also shown to co-precipitate with REV-ERBα, PPARα, HNF4α, and TRα, all of which have been shown to express in a circadian pattern (Reddy et al. 2007; Yang et al. 2006). Interestingly, Schmutz et al. (2010) showed that PER2 bound to the *mBMAL1* promoter, which coincided with the repression of this gene. PER2 binding overlapped the binding region of REV-ERBα, and PER2 binding was REV-ERBα dependent, as PER2 binding was lost in *Rev-erbα* -/- mice. PER1 binding was not observed at the BMAL1 promoter. Furthermore, PER2 was shown to bind to the *mBMAL1* promoter at a PPARγ binding site, at a time which correlated with *mBMAL1* activation. In conclusion, Schmutz et al. (2010) showed that BMAL1 regulation (in the liver) is controlled by two circadian systems: rhythmic REV-ERBα repression (with the addition of PER2) and rhythmic activation by nuclear receptors such as PPARγ, which use PER2 as a co-activator.

The estrogen receptor α (ERα) is another nuclear receptor that has reported links to circadian timing. PER2 suppresses ER transcriptional activation and induces its degradation through direct interaction. Estrogen has also been shown to induce *mPer2* transcription (Gery et al. 2007). Furthermore, estrogen added to tissue explants from the uterus of PER2::luc ovariectomised mice resulted in a period shortening effect (Nakamura et al. 2008). However, there is little evidence to support any circadian oscillation of *ERα*, whereas *ERβ* mRNA oscillates in phase with *Per2* via a conserved E-box element (Cai et al. 2008). The *ERRα* oscillates in the liver, uterus and bone. Interestingly, *BMAL1* and *Rev-erbα* are not expressed in bone, however, *Per2* is expressed and rhythmic (Horard et al. 2004). This suggests an independent oscillator in this tissue, perhaps similar to the independent *Per2* oscillator observed in the arrhythmic mouse liver in the Kornmann et al. (2007) study, where it was proposed that systemic signals were inducing this secular oscillation.

The androgen receptor (AR), although not directly shown to be under circadian control (unlike that of the ER and ERR above) has been localised to the SCN (Karatsoreos et al. 2007). In male and female mice the AR is localised to the 'core' SCN (Fig 1.3), yet with greater expression in male mice, which is in contrast to the GR, which is ubiquitously expressed apart from in the SCN (Balsalobre et al. 2000a). Following gonadectomy, Karatsoreos et al. (2007) reported a lengthening of free-running period, a reduction in duration of activity and a decrease in the total daily activity (wheel revolutions). The authors also noted a loss in activity onset precision (i.e. the difference between the calculated onset of activity from the previous day and the actual onset of activity). Further integration between the circadian clock and the AR comes from the biphasic expression of testosterone release, as seen in the rat. Testosterone peaked at 12.59h (light phase) and 04.04h (dark phase) with the peak expression of CORT falling in between the two testosterone peaks. Interestingly, the administration of the Gc analogue methylprednisalone in their drinking water abolished the biphasic testosterone rhythm, but after adrenalectomy the biphasic pattern was still observed, yet with a greater degree of variation (Waite et al. 2009). Together, these data described above suggest that the GR and MR are not the only nuclear receptors involved in resetting the circadian clockwork and that many more of the 49 receptors in the mouse are either under direct circadian control themselves or are regulated by clock proteins (PER2 and BMAL1).

1.10.6 Gcs and the stress response

As well as synchronising to the daily light dark schedule the animal must also deal with environmental stressors such as a compromised immune system, restricted food availability, aggressive behaviour and excessive heat or cold. The animal may also have to deal with internal stressors such as abnormal growths and nerve injuries (e.g. splanchnic nerve leading to the adrenal gland) (Nader et al. 2010). Induced stress experiments by ether or a novel environment has been shown to produce an increase CORT levels over a 24h cycle but plasma concentrations remained rhythmic, in female rats but not as pronounced in male rats. Furthermore, a significant increase in adrenal CORT levels were observed in the stressed female rat at the start of the dark phase but not during the rest period (Dunn et al. 1972). In addition to this, restraint stress has been shown to induce larger stress responses in the early light phase compared to the dark phase (Kant et al. 1986) and lesioning the SCN results in the loss of the CORT rhythm but not in the ability to respond to stress (Sage et al. 2001).

The stress response is modulated by the HPA axis in a similar manner to that of the circadian response outlined in section 1.10. CRH and arginine vasopressin (AVP) are released from the PVN and act on the corticotrophe cells of the pituitary to release ACTH, which acts on the adrenal cortex to release Gcs. Gcs then feedback to inhibit their own production, thought to be mediated via the inhibition of glutamate release on to CRH-containing cells in the PVN, via membrane GR (a proposed non-genomic action) (Di et al. 2003). However, other brain regions and effectors are interconnected with the stress response. α-melanocyte stimulating hormone and β-endorphin (both derived from pro-opiomelanocortin i.e. POMC) and adrenaline/noradrenaline, which are produced in the A1/A2 centres of the brainstem's locus ceruleus and in the central autonomic nervous system (Charmandari et al. 2005; Chrousos and Gold 1992). Furthermore, the serotonergeic pathway located in the raphe nuclei of the mid-brain and the histaminergic system of the hypothalamus are also stimulated in the stress response (Chrousos 2009). Although the main effectors of the stress response are Gcs and adrenaline/noradrenalin, IL-6 is released from immune cells and other peripheral cells, in a non-inflammatory stress response, to adrenaline via β-adrenergic receptors (Chrousos 2000). The end result of the stress response is to stimulate the fear/anger and reward systems; the brain centres involved in sleep-wake as well as the metabolic, immune and cardio-respiratory systems (Chrousos 2009). It is also very important for the animals' survival to maintain effective homeostasis of the stress response as fearless individuals have increased risk of mortality due to underestimating danger, whereas overly fearful individuals can result in reduced social interaction (Chrousos 2009).

Early work on the phase shifting effects of mild stressors (such as cage or litter changes, novel wheel confinement and social interactions) suggested that locomotor activity was vital for phase shifting (Mrosovsky et al. 1996). However, other work demonstrated that circadian phase shifts could be induced by gentle handling combined with sleep deprivation, which was not a result of wheel running or increased activity (Antle and Mistlberger 2000). Interestingly, in the hamster a stress response induced by forced restraint, during the subjective day, does not induce a circadian phase shift, nor does it sustain behavioural arousal. Furthermore, restraint combined with noxious air blasts did induce both sustained behavioural arousal and an increased cortisol response but no phase shift was observed (Mistlberger et al. 2003). Also sleep deprivation by keeping the

hamsters on a pedestal over water induced a small, yet significant, phase shift with 9 of the 12 hamsters shifting less than 30 min. In the same study by Mistlberger et al (2003) a 3h 'resident-intruder' stress test induced a phase shift of over 1h (which correlated with an increase in activity) in the smaller (resident) animals but no difference was observed in the larger (intruder) ones (cortisol levels were not measured). The results from this study suggest that arousal induced phase shifts do not correlate with an increase in Gc through the stress response, but are dependent on some degree of activity.

Taking the behavioural and cortisol observations of Mistleberger et al. (2003) a bit further, it has been demonstrated that the circadian and stress response of CORT, in the mouse, can be uncoupled using a vasoactive intestinal peptide (VIP) KO mouse model (Loh et al. 2008). A large number of the SCN neurons that project to the PVN use VIP as the neurotransmitter and VIP injection into the PVN can induce ACTH and CORT release (Alexander and Sander 1994; Teclemariam-Mesbah et al. 1997), suggesting that VIP signalling is an integral part of the HPA axis. However, in the VIP -/- mouse the animals' locomotor activity is severely disrupted in DD, with the onset of activity occurring 8h earlier than predicted, from the previous LD cycle, coupled with a shorter free-running period and many mice becoming arrhythmic after a few weeks in constant darkness (Colwell et al. 2003). In the VIP -/- mouse the circadian expression of ACTH and CORT was lost in both LD and DD and ACTH expression was constitutively higher in the VIP -/- mouse (Loh et al. 2008). However, in response to stress by foot-shock the CORT response was the same in WT and VIP -/- mice, with plasma CORT showing a significant increase, whereas plasma CORT levels in response to light were absent in the VIP -/- animal. Similarly, adrenal gland *mPer1* mRNA levels were significantly increased in response to foot shock stress in WT and VIP -/- animals, but no *mPer1* mRNA induction was observed in the adrenal gland in the knockout mouse in response to light (Loh et al. 2008). These data suggest that the loss of VIP, and a disrupted behavioural circadian rhythm, results in the loss of the endogenous ACTH and CORT rhythm and in the acute response to light, but does not affect the acute response to stress and that the circadian and stress response can be uncoupled.

Taken together the data presented in this thesis and discussion suggest that although Gcs are potent signalling molecules, which can stimulate both rhythmic gene expression and reset the clock through direct interaction *Per1* and *Per2,* they are just one of many re-

setting stimuli. These resetting stimuli include light (and dark), food, other hormones e.g. melatonin and serotonin, temperature and some environmental stressors. In the research environment each of these stimuli are looked at individually and conclusions about their relevance made on that basis. However, in practice and outside the confines of the research environment, it is likely that all stimuli are used in order to maintain internal synchrony with the external environment in order to integrate time, food availability and potential danger for the individuals' ultimate survival.

1.11 Non-genomic signalling

1.11.1 Genomic versus non-genomic mechanisms

The classical model of steroid hormone action (Fig 1.11) states that the steroid hormone, such as CORT, passes through the lipid bilayer binds to the receptor, which dissociates from its chaperones, and can then translocate to the nucleus where it can induce gene transcription. This whole process is relatively slow taking in the region of hours and is sensitive to transcriptional and translational inhibitors, actinomycin D and cycloheximide respectively (Losel and Wehling 2003). However, there are also very rapid changes observed when steroids are added to both cell line and human/animal models, that occur within minutes or even seconds; such as oogenesis, response to stress and vasoregulation, which are insensitive to transcriptional and translational inhibitors (Stellato 2004). Furthermore, these rapid signals have been shown to have effects on enucleated cells, such as red blood cells (Losel and Wehling 2003) and involves the generation of intracellular second messengers and signal transduction cascades such as phosphatidylinositol 3-kinase (PI3 kinase) and MAPK (Hafezi-Moghadam et al. 2002; Solito et al. 2003). A definition of non-genomic action has been given by Losel and Wehling (2003) and states that it is, 'Any action that does not directly or initially influence gene expression, but rather drives more rapid effects such as the activation of signalling cascades.' Non-genomic effects have been reported with the estrogen and progesterone receptors, but more importantly in for this thesis, the MR (Joels et al. 2008; Karst et al. 2005) and GR (Croxtall et al. 2000; Croxtall JD 2002), have been shown to have non-genomic effects too. Interestingly, studies have revealed that the Gc analogue DEX and the MR ligand aldosterone had rapid, non-genomic, effects on intracellular Ca^{2+} levels and localisation, that were insensitive not only to translational inhibition but to receptor antagonists RU486 (GR) and spironolactone (MR) as well (Urbach et al. 2002). In addition, other Gc analogues (budesonide,

triamcilone and hydrocortisone) had a much reduced effect on Ca^{2+} levels, with the authors concluding that this was due to DEX being the least lipophilic and, therefore, would not readily pass through the membrane at the low concentrations used (nM), interacting more with membrane proteins, perhaps a membrane bound GR presumed to be located on the cell surface rather than transmembrane. Indeed, membrane associated GRs were discovered in amphibian and human cancer cells (Gametchu et al. 1993; Orchinik et al. 1991) and a number of years later discovered through immunofluorescence studies to be in human peripheral blood mononuclear cells as well and located on the cell surface (Bartholome et al. 2004). Similarly a membrane associated ER was discovered, which was shown to associate with the protein caveolin (CAV) (section 1.11.2), found in a subset of specialised cholesterol rich lipid rafts called caveolae, that are involved in intracellular signalling (Razandi et al. 2002; Russell et al. 2000). Leading on from this direct association between membrane ER and CAV coupled with down stream signalling events, the membrane GR was shown to associate with the protein CAV-1 and DEX induced rapid signalling events that were CAV-1 dependent (Matthews et al. 2008).

1.11.2 Caveolae and caveolin

Caveolae form part of a rich tapestry of membrane domains, which accumulate to create macromolecular structures that include lipid rafts signalling molecules, cell-cell junctions and membrane invaginations (clathrin coated pits and caveolae) (Lajoie et al. 2009). Caveolae were first described in the early 1950's but their functions were unknown until the advent of knock out and transgenic mouse models, which characterised the involvement of caveolae in various biological events such as lipid uptake and regulation and tumour suppression (Parton et al. 2006). Each caveolae is approximately 65µm at the bulb end and 45µm at the opening end (Fig 1.12) and results in the invagination of the membrane bilayer (Parton et al. 2006). Each caveolae consists of approximately 100-200 caveolin molecules of which there are 3 in the family, termed CAV-1, 2 and 3. CAV-1 is expressed in non-muscle and smooth muscle tissue and CAV-3 is expressed in skeletal, cardiac and some smooth muscle. These two isoforms appear to be the most important for the formation of caveolae, as separate knock out mice do not form the caveolae complex (Drab et al. 2001; Galbiati et al. 2001). In contrast, CAV-2 is generally regarded as the dispensable member of the CAV family regarding the formation of Caveolae, yet *Cav-2* -/- mice have been shown to have an individual phenotype concerning lung function. CAV-2, often co-expressed with CAV-1 and able to form hetero-oligomers in most cell types, does

not disrupt the formation of caveolae when knocked-out, although there is a slight reduction of CAV-1 expression in some cell types (Razani et al. 2002). The loss of *Cav-2* results in hypercellularity, exercise intolerance and an increased number of endothelial cells and probably accounts for this phenotype in *Cav-1* -/- mice, as CAV-2 expression is dramatically reduced in CAV-1 -/- mice (Razani et al. 2002). The caveolae was initially thought to assemble directly in the membrane bilayer, requiring a threshold of caveolin to be present prior to the assembly of the caveolae structure (Fra et al. 1995). However, more recently this hypothesis has been disproven, as green fluorescent protein (GFP) tagged CAV-1 accumulates in the Golgi body and forms the caveolae structure there, before it trafficks to the membrane (Tagawa et al. 2005).

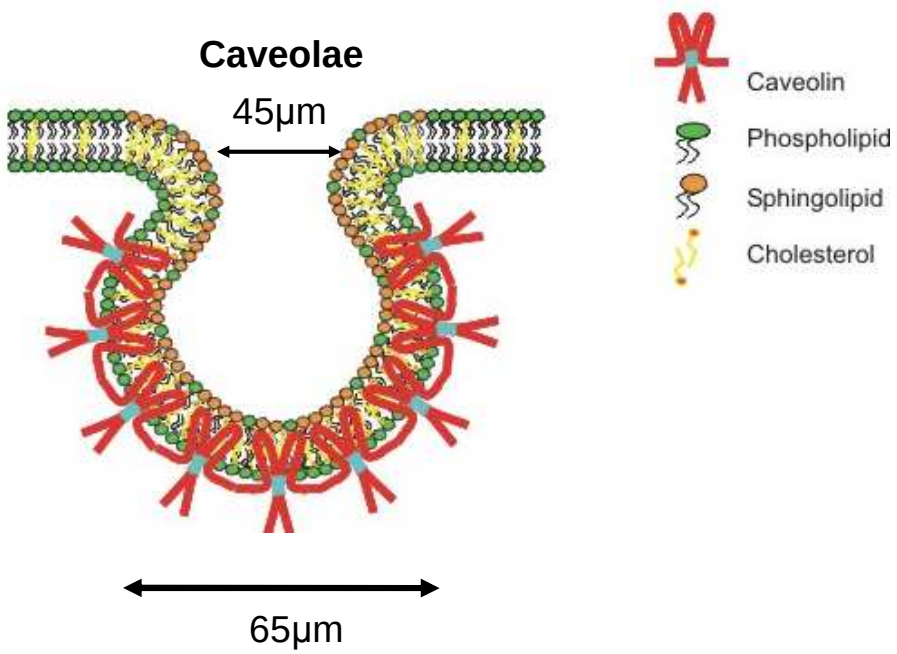

Figure 1.12: A caveolin and cholesterol rich caveolae: Adapted from (www.ruf.rice.edu/~rur/issue1_files/razani.html) with the dimensions from Parton (2006).

Caveolae, in particular CAV-1, have been implicated in a number of inflammatory diseases. The loss of CAV-1 in mice resulted in a reduced inflammatory response in an experimental model of inflammatory bowel disease (Chidlow et al. 2009) and CAV-1 proteins levels were seen to increase in the WT mice on infection. The loss of CAV-1 has also been shown to protect against atherosclerosis through the reduced uptake of low density lipoprotein (LDL) in the endothelium, an effect that is lost when CAV-1 is replaced (Fernandez-Hernando et al. 2009). To further substantiate these anti-inflammatory effects, a direct association has been made between CAV-1 and the main

lipopolysaccharide (LPS) receptor; toll like receptor 4 (TLR4) to significantly reduce the release of key inflammatory mediators IL-6, TNFα and CCL5/RANTES and this CAV-1/TLR4 association is enhanced with heme oxygenase, known to have anti-inflammatory properties (Wang et al. 2009). This definite link between the inflammatory response and CAV-1 signalling will no doubt prove a great topic of interest; particularly in the circadian field as both IL-6 and TNFα are know to be under circadian control (section 1.12).

1.12 Circadian clocks and the inflammatory response

1.12.1 The innate inflammatory response

The immuno-inflammatory response is a vital part of an organism's first line of defence against attack by a foreign body; be it bacterial, chemical or animal based, where proteins are released from cells, in particular those differentiated from mononuclear phagocyte lineage, such as macrophages. This is referred to as the innate immune system. The proteins are known as chemokines and cytokines and induce the classic inflammatory response; redness, swelling, pain and heat (Pickup 2004), which protects the infected area, begins to attack the antigen and signals for more immune cell recruitment, such as B cells, which are part of the humoral, or adaptive, response where specific antibodies are made. The cells which form the innate immune system, collectively known as white blood cells, circulate in a circadian manner. Neutrophils, lymphocytes, monocytes, platelets and eosinophils all show a circadian variation in their circulating cell numbers in healthy humans (Haus and Smolensky 1999), with peak values during the night and trough values in the early morning, which is in anti-phase to circulating Gc levels (Petrovsky et al. 1998). When the circulating white blood cells detect a foreign body various pro-inflammatory cytokines and chemokines are released from virtually all cells involved in the immune response, such as macrophages and eosinophils, into the local site of infection to trigger the immune response and fight infection (Haus and Smolensky 1999). The release of these cytokines triggers various responses in other cells with cytokine receptor molecules, such as altering the expression of membrane proteins, the secretion of other cytokines to generate a cascade of events and cellular proliferation (O'Shea et al. 2002). The release of cytokines is a non-linear response and very small changes in initial conditions, that is the release of 1 or 2 initial cytokines, can result in a large magnitude response very quickly, referred to as 'chaos' in mathematics (O'Shea et al. 2002). The term cytokine is quite a loose term for a number of different protein groups; interleukins are proteins made by and

which act on leukocytes; lymphokines are cytokines made by lymphocytes; monokines are produced by monocytes and chemokines are cytokines with chemotactic properties. Interestingly cytokines are to some degree a redundant system, just like the clock itself, in that many will have the same or similar action, so as to compensate for any losses (O'Shea et al. 2002), or on the opposite side leading to the large magnitude 'chaos' response. However, more interestingly some cytokines have both an immunosuppressive and immunostimulatory effects, such as TNF α, interferon gamma (IFNγ) and IL-2, which can lead to autoimmune diseases such as rheumatoid arthritis (O'Shea et al. 2002). Cytokines such as IL-6, TNFα and IL-1β have not only been shown to be expressed by macrophages and other inflammatory cell types but other resident cells like Type II alveolar macrophages (section 1.8.3), found in the lung (Thorley et al. 2007), showing that the fight against disease involves many diverse cell types.

1.12.2 Oscillating inflammatory cells and mediators

A number of cytokines have been shown to be under circadian control, in humans, even in the absence of any invading antigenic stimulus, such as IL-6, TNFα and IL-1 (Gudewill et al. 1992) as well as the non-stimulated circadian expression in rats of IL-6, TNFα and IFNγ at both the transcript and protein level (Arjona and Sarkar 2005; Guan et al. 2005). Also in a more recent study by Beynon and Coogan 2010, where they looked at circadian differences between younger and older mice, they reported that the IL-1β receptor IL-1R1 had a temporal regulation in the SCN of young mice, peaking at ZT20, but not older mice, yet IL-1β did not show any temporal variation (Beynon and Coogan 2010). They also examined the difference between overall expression in young and old mice, and showed that SCN expression of IL-1β was much lower in older mice compared to the young. The authors also showed an age related difference in the expression in the PVN. In this area the older mice had increased expression of both IL-1β and its receptor with higher levels at ZT20 and ZT24 (Beynon and Coogan 2010). However, most work has been carried out in the presence of a stimulant, usually LPS, a product of the gram negative bacterial cell wall, which works through TLR4 receptor protein and either through a MyD88 dependent or independent pathway, resulting with the former in cytokine production via NFκB or, with the latter, caspase activation (Hoshino et al. 1999). After LPS stimulation cytokines in both human and animal models still show a circadian rhythm at the transcript and protein level, such as IL-6, TNFα, IL-12 and IFNγ (Hermann et al. 2006; Keller et al. 2009; Petrovsky et al. 1998). This post stimulation oscillation was shown by Keller et al. (2009)

to be independent of a systemic Gc rhythm, as it still persisted in the absence of the adrenal gland. Tissues involved in both the innate and humoral inflammatory response, such as the spleen and lymph nodes, as well as macrophage cells have functional oscillators with high amplitude rhythms as seen in the mouse (Hayashi et al. 2007; Keller et al. 2009) and in human leukocytes (Haimovich et al. 2010), although with a much reduced amplitude in the leukocytes. The LPS stimulation of inflammatory cells or systemic LPS administration has been shown to have a potent effect on the core clock genes, resulting in their suppression, again in both human and rodent models (Haimovich et al. 2010; Yamamura et al. 2010). However, this suppression is not always seen, as shown by the lack of effect after systemic LPS administration in the liver of mice, in either *Per1* or *Per2* (Takahashi et al. 2001) and in opposition to the general literature, LPS induced *Per1* expression after 1h in the PVN neurons, part of the HPA axis (section 1.10.1), in a similar manner to a forced swim test stress response. Interestingly *Per2* expression was not altered nor was expression of *Per1* changed in the SCN (Takahashi et al. 2001); together suggesting a very specific PVN based response. Furthermore, mice carrying a loss of function mutation in the *Per2* gene results in the loss of IFNγ rhythm, both at the transcript and protein level (Arjona and Sarkar 2006). Also a *Per2* -/- mouse is far more resistant to LPS induced death and that in the *Per2* -/- mouse IFNγ and IL1β levels dramatically increase, whereas TNFα, IL6 and IL10 remain the same as in the WT (Liu et al. 2006b). It has also been shown that TNFα, as well as IL1β, can suppress CLOCK:BMAL1 E-box mediated transcription, yet other cytokines such as IFNα or IL6 cannot, and therefore explains why LPS suppresses the clock (Cavadini et al. 2007). The ideas discussed above all lead to the suggestion that the circadian clock and the immune response are very closely linked, and evolution has provided cross talk and feedback mechanisms in order for the organism to successfully defend itself from invading pathogens. However, in the case of autoimmune diseases, such as rheumatoid arthritis and inflammatory bowel disease, a greater understanding of the clock and the inflammatory interaction, could lead to improved therapeutics and possible cures.

1.12.3 Glucocorticoids, receptors and macrophages

Glucocorticoids are still today the most common based therapy to treat both chronic and acute inflammation, ever since the discovery that cortisol could lessen the inflammatory response in rheumatoid arthritis patients, (Hench et al. 1949). This suppression works in a number of ways using both gene activation (transactivation) and gene repression

(transrepression), with the latter working primarily through the dimerisation to, and inhibition of, NFκB and AP1 (Beck et al. 2009). Much research has been undertaken to enhance the anti-inflammatory properties and reduce the unwanted side effects of Gcs (such as osteoporosis, growth retardation in children and skin fragility), (Barnes and Adcock 2003), yet little progress has been made in this regard, even though vast amounts are known about cell and tissue type actions.

It has been shown that 28 of the 49 nuclear receptors, in the mouse, are expressed in macrophages including the majority of the steroid receptors, in particular the GR and MR, as well as the progesterone receptor and the estrogen receptor α (Barish et al. 2005). Furthermore, both GR and MR are known to bind Gc (as discussed section 1.10.3) and Barish et al. (2005) showed that both receptors had a different response to LPS administration in bone marrow derived macrophages. LPS only induced GR transcript expression, over baseline, between 4-8h after treatment, which returned to baseline at 16h. However, MR transcript expression was rapidly suppressed after LPS administration showing no expression at 4h post treatment (Barish et al. 2005). In addition to the expression of both GR and MR in macrophages, the effect of Gc, specifically in differentiated macrophages, is enhanced via the expression of HSD11β type I, which converts inactive Gc to active Gc (section 1.10.3), but the expression of the type II enzyme was not detected (Thieringer et al. 2001). In the same study the authors also showed that HSD11β was induced by LPS in a macrophage like cell line. This suggests that circulating Gcs are able to act on the macrophage gene expression via both GR and MR, which is enhanced in the presence of a pro-inflammatory stimulus. The presence of MR suggests a dual action of Gc, depending on the local concentration and perhaps whether the macrophage is stimulated or not, as MR has a 10 fold higher affinity for CORT than GR does. Indeed a concentration dependent response was seen in cultured peritoneal macrophages (Lim et al. 2007), with lower CORT concentrations (nM and sub nM) inducing the expression of IL-12, IFNγ and IL-6 amongst other inflammatory mediators. However, at higher doses of CORT (high nM and μM) this induction turned into a suppression of inflammatory mediators. This dual role of CORT appears, however, to be mediated primarily through the GR, rather than the MR, as knock down of the GR resulted in the attenuation of both induction and suppression, although not fully, whereas knock down of the MR had no effect on either suppression or activation of gene transcription (Lim et al. 2007). The MR has, however, been implicated in a number of diseases with

inflammatory side effects, such as hypertension and atherosclerosis, as addition of the MR antagonist spironolactone acts to reduce macrophage accumulation and aldosterone treatment enhances inflammatory markers (Rickard and Young 2009). Taken together these findings suggest that although the GR is the main mediator of the Gc effects on the macrophage inflammatory response, the MR may have a minor role in the unstimulated response to background levels of circulating Gcs, more in terms of a preparatory role prior to an antigenic response.

1.13 Chronotherapy

Chronotherapy is the timed administration of drugs to better combat the symptoms of diseases, which are known to be worse at particular times of the day. Many diseases appear to have a circadian component to their symptoms such as chronic pain and dermatoses, which are worse in the late evening, where as congestive heart failure, seems to occur in the few hours after midnight with asthma and rheumatoid arthritis occurring in the early morning. Interestingly epileptic seizures seem to have a biphasic onset surrounding sleep, with the hours prior to the onset of sleep and shortly after wake up being the worse times of onset (Ohdo 2010). There are now a number of diseases for which timed administration of the therapeutics has been developed. Nocturnal asthma appears to be better treated with an evening administration of theophylline and an evening dose of 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase antagonist is better to help hyperlipidemia (Ohdo 2010). The treatment of cancer has been shown to be improved with the timed administration of over 30 anticancer drugs in rodent models, with survival rates increased by more than 50% in some cases (Mormont and Levi 2003). One striking piece of evidence for the effective use of chronotherapeutics is that in timed drug trails in children with acute lymphoblastic leukaemia, 80% who were treated in the evening with 6-mercaptopurine and methotrexate were alive and disease free 5 years after the onset of the disease, compared to only 40% who were treated in the morning, the remainder were either dead or still had the disease (Mormont and Levi 2003). Furthermore, the careful timed administration of cancer therapeutics has suggested that larger doses can be tolerated, which means greater improvements to the patients health and wellbeing (Ohdo 2010). IFNα stimulation of lymphocytes in mice was shown to be higher when dosed at 9am compared to 9pm and the number of IFNα receptors in the lymphocytes was greater at 9am rather than 9pm. Also IFNα clearance, through kidney metabolism was greater at 9pm compared to 9am (Ohdo et al. 2000). IFNα treatment of mice caused an IFNα induced

fever response (high body temperature), which correlated with the unstimulated temperature rhythm, but was much higher (Koyanagi et al. 1997; Ohdo et al. 1997). There was also a circadian rhythm in IFNα after IFNα injection, suggesting that the animal responds differently to IFNα stimulus at different times of the day (Koyanagi et al. 1997). These data lead to the suggestion that dosing of IFNα, which is used as an antiviral drug, at a certain time of day could lead to better clinical results.

Our expanding knowledge of the relationship between the circadian clock and health and disease and the ever increasing knowledge to chronotherapeutics will be a very exciting field over the coming years and will undoubtedly lead to greater freedom from disease symptoms and perhaps even recovery from cancers.

1.14 Overall aims

This thesis will address the following:

- Define the mechanism of action of Gc on the pulmonary murine clock using the mPER2::luc mouse and in Rat-1 fibroblast cell line reporters.
- Characterise the extent to which the inflammatory response is gated by the circadian clock and investigate the mechanism behind this control.
- Investigate the transcription factor C/EBPα with regard to its circadian and Gc regulation.

In this thesis, I initially expected C/EBP (in particular the α and δ isoforms) to act as the interface between Gc regulation and the circadian clock, which could lead to re-setting mechanisms on clock genes. These ideas were formulated by knowledge from previous literature and tested by experiments in both cells lines and primary murine lung tissue, outlined in Chapter 6. These experiments failed to replicate earlier studies (MacDougald et al. 1994) and showed no defined circadian rhythm, and for this reason direct actions of Gc on clock genes (*Per1* and *Per2*) were investigated. These latter experiments are therefore described first (Chapters 3 and 4) and data from experimental manipulations of C/EBPs are described in the last data chapter (Chapter 6). Work set out in Chapter 5 describes the circadian effect on the innate inflammatory response and the effect an inflammatory

stimulus (LPS) has on circadian gene expression. This chapter demonstrates how the clock influences other fundamental biological processes and how they feedback onto the clock.

Chapter 2: Materials and Methods

2.1 Mice and Primary Culture

2.1.1 Animal maintenance

All experimental procedures were within the guidelines of the Animals (Scientific Procedures) Act, 1986. All mice were housed at an ambient temperature of 20–22 C, and maintained in a 12-h light, 12-h dark (L:D, 12:12) lighting schedule, except where stated. Food and water was available *ad libitum*. All mice used in these studies were male and aged between 6 and 24 weeks but due to numbering constraints both sexes were used in the *Rev-erb* -/- studies. In general mice were killed using cervical dislocation, in order to maintain the trachea, unless otherwise stated, (section 2.2.1). All killing was done in accordance with the schedule 1 licensing act 1981. Carcasses were destroyed by incineration.

2.1.2 Mouse lines

All mice, used throughout these studies, had a C57/BL6 genetic background, regardless of other genetic manipulations, and these mice will be referred to as wild type (WT) mice.

2.1.2.1 PERIOD2::luciferase mouse

For the majority of studies the PERIOD2::luciferase (PER2::luc) protein reporter model was used, in order to report the phase and period of the circadian cycle using a photo multiplier tube (PMT) (section 2.1.3). These mice were a kind gift from Joseph Takahashi (University of Virginia) (Yoo et al. 2004). The PER2::luc mice were bred through homologous recombination, which results in the *luciferase* gene, fused to the 3 prime end of the endogenous *Period2* gene (genomic DNA not complementary, cDNA), being recombined into the correct genetic locus for *Period2*. This homologous recombination means that the PER2::luc reporter is driven by the same promoter and subject to the same genetic environment as the WT gene, resulting in a highly representative reporter. However, care must be taken when interpreting the results obtained from the luciferase reporters as what is actually being reported is a luciferase signal and an indirect measurement of the protein or promoter reporter placed upstream, and is really only a substitute for direct protein or transcript analysis, by immuno blotting or qPCR.

2.1.2.2 Rev-erbα -/- mouse

In the following studies a *Rev-erbα* -/- mouse was used (Preitner et al. 2002). Preitner (2002) used targeted disruption of the *Rev-er α* gene replacing part of exon 2, all of exons 3 and 4 and part of exon 5 with a lacZ and neomycin gene. This strategy took out the DBD and as a result meant that no messenger ribonucleic acid (mRNA) or protein should be produced, and if any protein product was produced it would be incapable of DNA binding and having any repressive effects. This mouse line was a kind gift from Ueli Schibler (University of Geneva). The *Rev-erbα* -/- mice were bred from a heterozygous X heterozygous mating pair (each parent had one WT copy of the *Rev-erbα* gene, the other copy was knocked-out), so only approximately 1 in 4 offspring were homozygote for the WT or *Rev-erbα* -/- genotype, this was done due to the difficulty in maintaining a homozygote colony.

2.1.3 Theory behind the PMT and use of luciferase reporter models

The PMTs are a way of converting a photon of light, released as a by product of an enzymatic reaction, into an electronic signal. Throughout this thesis the reaction was the conversion of luciferin to oxyluciferin by the enzyme luciferase with a photon of light released as a by product of adenosine tri-phosphate (ATP) being converted to adenosine mono-phosphate (AMP) (Fig 2.1). This reaction has been taken from nature and adapted for use in the laboratory, as the firefly (*Photinus pyralis)* uses the same principle when it glows. As the photons of light are emitted they hit an electrode coated with a photosensitive material, called a photocathode, positioned at the entry window of the PMT. As a result electrons are released into the PMT; this is called the photoelectric effect (Fig 2.2). The PMT contains a number of dynodes which contain an increasing positive charge, giving the electrons greater energy to hit successive dynodes and cause more electrons to be released. Eventually the electrons reach the terminal anode and create a large electrical charge which is detected by a computer software programme and results in a readable output. The computer collects the signal over a 59 second period, and then stores the value as one value, and thus the readout is counts per minute.

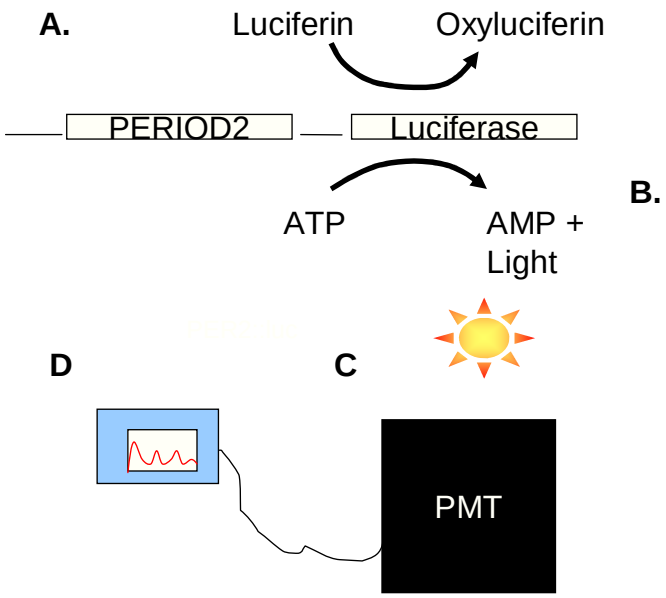

Figure 2.1: Principle behind the PMT: The clock protein (PER2), or promoter region of interest, is fused to the luciferase enzyme, (A). When luciferin, present in the recording medium, binds to the luciferase it is oxidised to oxyluciferin and ATP is converted to AMP with the emission of a photon of light (B). The resulting photons, converted to electrons, are multiplied and collected via the PMT (C) and converted to an electronic signal which can be viewed on a computer (D). Diagram adapted from the internet.

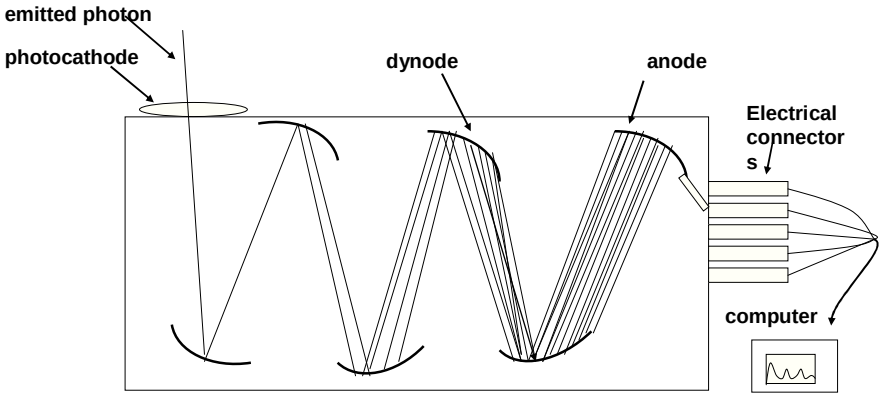

Figure 2.2: Inner workings of the PMT: The emitted photon hits the photocathode and an electron is released. The electron then causes the release of further electrons from a succession of dynodes, which carry an increasing positive charge, until the electrons hit the anode and are converted to an electrical signal and are recorded by a computer. Diagram adapted from http://learn.hamamatsu.com/articles/photomultipliers.html.

2.1.4 PMT data collection and analysis

The PMT data were collected and exported as an excel file, where all data were normalised to make the first full peak equal to 100 (relative units), approximately 24 h after the start. This was done to reduce inter assay PMT variation, in order to better calculate phase change, and to put the lung and cell reporter recordings on the same y-axis scale.

All period and phase analysis was carried out using the rhythm analysing programme (RAP) analysis software, (Okamoto et al. 2005) (for all single unit PMT recordings) or using the Lumicycle analysis software (Actimetrics), for the Lumicycle based recordings. These methods use a Cosinor analysis based on a Fourier analysis. Period and phase were determined starting at least 12h after the time of treatment, to remove any artefacts brought on by the treatment protocol.

2.2 Preparation of primary cells and tissues

2.2.1 Lipopolysaccharide (LPS) treated mice

All mice were maintained as in 2.1.1, with access to food and water *ad libitum*, but were singly housed, for the duration of the procedure. The mice were weighed prior to the study in order to calculate the correct LPS dose. In the first experimental series, the WT mice used were all male. In the second experimental series, the *Rev-erbα* -/- mice, and WT litter mates, were mixed sex but split equally. All mice were in a 12h:12h lighting schedule (lights on 0800, ZT0 lights off 2000, ZT12) for 10 days prior to being released into constant darkness (D:D), for 24h prior to challenge. At projected CT0/CT12, mice were injected, into the peritoneal cavity (i.p. injection), with LPS (usually 1-10 mg/ml), purified from *Escherichia coli* strain 0128:B12 (Sigma, UK), or 0.9% saline control. This was carried out in darkness. The mice were returned to the cages, where they remained in constant darkness, for 4h before sacrifice. In order to collect blood from the animals the mice were first anaesthetised with 5% isofluorane in 200ml/min O_2 and 500ml/min N_2O and then decapitated. The blood was collected into clean 1.5ml tubes and allowed to clot at room temperature (RT) for 2-3 min and then stored on ice. The liver and lungs were then removed and snap frozen on dry ice, wrapped in heat treated tin foil (to denature nucleases) and stored at -80^{o}C. The blood was centrifuged for 10 min at 10000 x g and the resulting serum was removed and stored at -20^{o}C. Sampes were assayed via the BioPlex (section 2.5.4.2).

2.2.2 Agarose perfusion of lungs

Lung slices were prepared, from the PER2::luc mouse. By sectioning the lungs in this manner, the structural integrity of the tissue is maintained so that they could be cultured and subsequently used to accurately report the phase and period of the clock. The PER2::luc mouse was killed, by cervical dislocation, and the trachea and lungs exposed. The base of the trachea, just above where the manubrium would be, was loosely tied with suture. A hole was made towards the upper end of the trachea and a thin tube (Harvard Apparatus) was inserted, internal diameter 0.58mm. A 1ml syringe containing approximately 1ml of 2% low melting agarose, type IXA, (Sigma, UK) was fixed via a 23G (0.25mm) needle (Benton and Dickinson, B and D) to the tube. The agarose, 37^{o}C, was slowly perfused to allow the individual lobes to inflate. Once the agarose was fully administered the tube was removed and the trachea tied off with the suture. The mouse was quickly placed on ice and the lungs covered to allow the agarose to set. After 10-15 mins the mouse was taken off the ice and the inflated lung was removed and placed in ice cold Hanks' balanced buffer solution, without Ca^{2+} or Mg^{2+} (HBSS) (Gibbs et al. 2009).

2.2.3 Slicing and Culture of the Perfused Lungs

The lung, maintained in ice cold HBSS, were separated into individual lobes, 4 right and 1 left lobe, each was sliced separately. The individual lobe was dab dried and then fixed to a metal stage using superglue (Farnell electronics). The lung and stage were placed in a vibratome cutting pool (Campden Instruments), filled with HBSS, and cooled to 4^{o}C. The individual lobes of the lung were then cut, using a stainless steel blade (Campden Instruments), to a thickness of 275µm. Although the thickness of the slice was controlled the resulting area was much harder to maintain, as the lung is not a regular shape, however, an attempt to control this was carried out. The resulting slices were placed in to a 24 well plate, containing pre-warmed 4g/litre glucose Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine, phenol red, sodium pyruvate, sodium bicarbonate and Ca^{2+} and Mg^{2+} (Gibco, Invitrogen). This medium was supplemented with streptomycin and penicillin (Sigma, UK) to give a final concentration of 0.1mg/ml streptomycin and 100 Units of penicillin, but no fetal bovine serum (FBS) was added. The lung slices were maintained at 37^{o}C and supplemented with 5% CO_2 (95% air) in a humidified atmosphere. The slices were subsequently washed 3 times over a period of 3-4h in order to wash out the

agarose. Finally the slices were left for at least 16h before being used experimentally, to show they are free of infection.

2.2.4 Preparation of lung slices for experimental use

Prior to any experimental procedure the lung slices were synchronised to ensure the oscillating cells, within the lung slice and between lung slices, were re-set to the same phase, in a similar manner to a group of people synchronising their watches at the beginning of the day. The slices were treated with 10µM forskolin (Sigma, UK) for 1h. This stimulates an increase in cAMP levels and activates rhythmic gene expression (Balsalobre et al. 2000b), in particular *Per1*. The medium was removed and slices washed with 1x Ca^{2+} and Mg^{2+} free phosphate buffered saline (referred to as 1x PBS) (Sigma, UK) before being transferred to sterile 35mm dishes containing 1ml of recording medium (appendix A), adapted from (Yamazaki and Takahashi 2005). The slices were placed on a Millicell low profile, 0.4µm membrane insert (Millipore, UK) to provide an air-liquid interface and to prevent slice movement. This technique proved essential for robust and reproducible PMT recordings (Gibbs et al. 2009). The dishes and slices were sealed with a 40mm sterile glass cover slip (VWR) and high vacuum grease (Dow Corning) and placed in a PMT recording unit (Hamamatsu, Japan), or Lumicycle (Actimetrics) and recordings taken over a 5 – 7 day period. The temperatures of the incubators was set to 37oC but without additional CO_2 influx, as the dishes were sealed. The addition of sodium bicarbonate in the recording medium (appendix A) keeps the CO_2 at approximately 5%.

2.2.5 Experimental treatment of lung slices

2.2.5.1 The experimental protocol

Preparation

Slices were prepared as stated in 2.2.4 and put on the PMT for approximately 48h, in order to establish an oscillation and an accurate estimate of phase. At the second peak (approximately 48h), referred to as CT12 the slice was removed from the PMT and, in a sterile environment, the cover slip removed and CORT (Sigma, UK) was added to the medium, unless otherwise stated at 100nM, and mixed by swirling. The cover slip was replaced and the slice returned to the PMT and recording continued. In the case where slices were pre-treated with a compound, such as an antagonist, this was carried out first and CORT was administered 60 min later. All reagents were solubilised in 100% dimethyl

sulphoxide (DMSO) (Sigma, UK) and, as such, DMSO was used as the control at no more than 0.1%, except with the methyl experiments where 1% DMSO was used, due to solubility constraints.

Antagonist treatment

The GR and MR antagonists were used at a final concentration of 1µM RU486 (RU), (a kind gift from Dr. Laura Matthews, University of Manchester) and 1µM spironolactone (spir) (Sigma, UK). This concentrations of RU was chosen as it is commonly used in the literature (Balsalobre et al. 2000b), and 1µM spir was chosen as it has GR antagonistic activity at higher concentrations, (Ec50 8µM) (Couette et al. 1992).

Cav-1 disruption

Caveolin signalling was disrupted using 5mM methyl cyclodextrin (methyl) (Sigma, UK), a non-specific compound that can disrupt lipid raft signal transduction via the removal of cholesterol, where caveolae are known to be located (Matthews et al. 2008).

Non-steroidal agonists

Non-steroidal GR agonists, GSK275167A and GSK275169A (referred to throughout as 67A and 69A) (a kind gift from Dr. Stuart Farrow, GlaxoSmithKline, Stevenage) were used in these studies. Although these exact compounds (67a and 69a) have not been published, similar acting compounds accompanied by their structure have (Biggadike et al. 2009). These agonists were found to have EC_{50} values in the very low nM range (Chapter 3) and so were used at 10nM (10 x EC_{50}).

Transcriptional and translational inhibition

Lung slices, treated with either 20µg/ml cycloheximide (translational inhibitor) or 10µg/ml actinomycin D (transcriptional inhibitor) (Sigma, UK), were either pre-treated for 30 min with the above inhibitors and treated with CORT or pre-treated with CORT for 30 min and then treated with the inhibitors, prior to half life ($t_{1/2}$) analysis using a one phase exponential decay algorithm (GraphPad Prism).

LPS treatment

LPS treated slices were prepared as in 2.2.4 but treated with a final concentration of 100ng/ml LPS, at the second peak. The LPS was solubilised in HBSS, so it was used as the control.

2.2.5.2 Lung sections treated for quantitative polymerase chain reaction (qPCR)

The intact lung was removed from the animal and washed in PBS. The lung was cut into small pieces using a sterile blade, roughly 8-12 sections per lung. The sections were placed in DMEM (section 2.2.3) without FBS and incubated at 37^{o}C in 5% $CO_{2.}$ The medium was replaced twice to remove any contaminants. The slices were synchronised for 1h with 10µM forskolin either 24h or 36h prior to treatment, so as to treat at the peak of trough of the PER2::;luc rhythm respectively. At the indicated time the slices were treated with CORT or GR agonist for 4h; or pre-treated for 1h with 1µM RU486 (section 2.2.5.1) and then with CORT or GR agonist for 4h before RNA extraction (section 2.6.2.1).

2.2.5.3 The phase response curve (PRC)

The PRC was carried out in the Lumicycle, as it has the capacity to record a 32 sample experiment. This meant a 12 point PRC for CORT and DMSO could be created at the same time, therefore, reducing assay variation. Slices were set up, as in 2.2.4, and treated with CORT or 0.1% DMSO every 2h, over the circadian cycle, starting at CT12 (second peak). The treated slices were left for 5 days and recordings were analysed, using the Lumicycle software.

2.2.5.4 PRC analysis

Even though there are many PRCs reported in the literature, these are *in vivo* and in response to light. A PRC to both a DEX and forskolin was published by Izumo et al 2006, using *mPer2::luc* cellular reporters. However, the authors used a much more mathematical, and therefore more objective, approach using cosine based algorithms, for phase and period determination (Izumo et al. 2006), which could not be re-created. Therefore, a simplified method was developed. Referring to Fig 2.3, the endogenous period of the slice (x) was taken as the time between peaks 1 and 2, and the slice was treated every 2h after peak 2, (T). However, to exclude Gc induced artefacts this region, T (peak 3), was avoided in the subsequent analysis and the time between peaks 2 and 4, (Y)

was used in the calculation. It was assumed, in the untreated state, that the fourth peak would occur 2 periods after peak 2, and if it did not then this would constitute an advance, if the peak occurred early, or a delay, if it occurred later than predicted. The combined equation 2x-y = n, where n = advance (+) or delay (-), was used as quick analysis method. The pre-treatment period was defined as X and the post-treatment period defined as P. The peaks and periods were defined using the Lumicycle analysis software and all treated slices (DMSO and CORT) were analysed in the same way. This method was used in Gibbs et al. (2009).

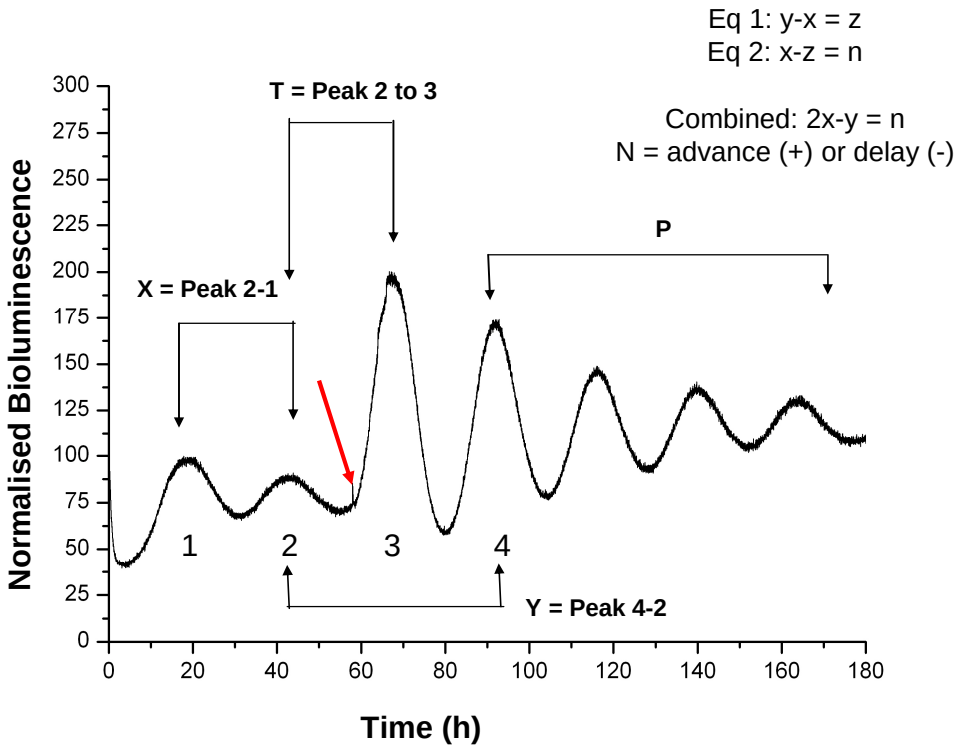

Figure 2.3: Explanation of the PRC analysis. Peaks 1 to 4 are labelled with the time of Gc treatment (CORT) shown by the red arrow. The time between peaks 2 and 3 is not used in the calculations due to CORT induced artefacts. The time between peaks 1 and 2 (X) defines the endogenous period. The difference between X and Y (peaks 2 to 4) gives the advance (+) or delay (-) phase with the post-treatment period defined as P.

2.2.6 Experimental use of Peritoneal Exudate Cells (PECs)

2.2.6.1 Extraction of PECS

The method, described below, is widely used to extract cells from the peritoneal cavity. Somewhere between 60-80% of these cells are macrophages, although other cells such as lymphocytes and plasma cells are present in small numbers. The wash steps following the extraction does remove the majority of non-adherent cells leaving predominantly

macrophages, however, there will always be some impurity. Some protocols suggest pre stimulation with thioglycollate (Bhattacharyya et al. 2007) to enrich the macrophage population, where others use FACS on the exudate (Keller et al. 2009) to select for macrophages. However, here I have opted for a much faster protocol, where there may be some loss of cell purity but the cells maintain a robust inflammatory response.

Mice were killed by cervical dislocation and their abdomens opened (taking care not to pierce the peritoneal cavity). A 21G microbalance needle (B and D) was fixed to a syringe and filled with 5ml of ice cold RPMI 1640 medium without Ca^{2+} or Mg^{2+}, supplemented with 2mM L-glutamine, 5% FBS and a final concentration of 0.1mg/ml streptomycin and 100 Units of penicillin, subsequently referred to as RPMI 1640. The peritoneal cavity was slowly filled with the medium, with care taken as not to rupture any organs. The mouse was agitated for 30 sec and the medium removed from the cavity and placed on ice. This process was repeated. The resulting exudate was centrifuged at 1500 x g for 5 min and the supernatant discarded. The cells were washed with the RPMI 1640 and centrifuged as before. After the wash step, the supernatant was removed and any red blood cells were lysed in 1ml deionised H_2O for 30 sec before lysis being stopped with excess RPMI 1640 (approximately 30ml). Once more, the exudate mix was centrifuged and the supernatant discarded and this process was repeated a further time. Finally the cells were re-suspended in 5ml RPMI 1640 and counted using a CASY cell counter (Innovarits) and cells between 6-20µm in diameter were counted. These parameters discard debris, dead cells and cells too large to be macrophages as they are generally 8-15µm in diameter (Ghosn et al. 2010). Cells were cultured in separate wells in a 24-well plate between 0.4 and 0.5 $x10^6$ cells/ well; these will be referred to as experimental cells. The density was maintained within an experiment but varied between experiments due to overall cell numbers. The PECs were pooled from between 3 and 5 mice, same sex for the PER2::luc cells but mixed sex for the *Rev-erbα* -/- PECs, due to number constraints. The pooled cells were divided into replicates of 3 for each experimental condition. PECs from the PER2::luc mouse were also cultured, into an individual 35mm dish, in order to put them under the PMT, as a representative trace of the phase of the circadian oscillation, roughly about 2.5 - 3 $x10^6$ cells/well were cultured, these will be referred to as reporter cells. All cells were placed at 37^oC in 5% CO_2 for 2h and then washed in 1 x PBS and fresh RPMI 1640 was added and the cells were returned to incubate.

2.2.6.2 LPS Stimulation of PECs

All cells, reporter and experimental, were synchronised 24h prior to the onset of LPS challenge. The reporter cells report the phase of the oscillation and did not receive an LPS challenge, whereas the experimental cells were challenged at the indicated phase. To synchronise, 10µM forskolin was added to the cells for 1h then the medium was removed, cells were washed in 1 x PBS then recording medium was added (see appendix A), 1ml for the experimental and 2ml for the reporter cells. The medium for all cells was the same apart from the reporter cells having luciferin present in order for light to be emitted (section 2.1.3) Experimental cells were placed at 37^{o}C in 5% CO_2 and the reporter cells were placed under the PMT (at 37^{o}C and buffered against CO_2, section 2.2.4) for the oscillation to be reported. At the times indicated, usually the first peak and trough, the experimental cells were treated. Firstly the medium was removed and discarded; fresh, warmed medium was placed on the cells containing 0.8ml of HBSS, 100ng/ml LPS or 100ng/ml LPS mixed with 100nM CORT. The cells were exposed for 4h before the medium was removed and frozen at -20^{o}C ready to be assayed for inflammatory mediator release by enzyme linked immunosorbant assay (ELISA), described in section 2.5.3. The remaining experimental cells were washed in PBS and frozen at -80^{o}C until the cells were lysed for protein quantification, section 2.5.1.

2.2.6.3 Cytospin of LPS treated PECs

Approximately 1 – 2 x10^{5} cells were diluted into 200µl RPMI 1640 and centrifuged at 800 x g for 5 min onto a gelatine coated slide (VWR). The cells were fixed in 4% paraformaldehyde (PFA) for 10 min on ice. The PFA was prepared as follows: 12g PFA (Sigma, UK) was dissolved in 300ml PBS (5 tablets dissolved in 1 litre produce 10mM phosphate buffer, 2.7mM potassium chloride and 137mM sodium chloride, pH 7.4, Sigma, UK), heated to 50^{o}C then 2 – 3 drops of 5M NaOH was added until the solution clarified. The resulting PFA was then filtered through 0.45µm filter paper (Whatman). The fixed cells were washed in H_2O then stained with hematoxylin and eosin (H and E) (section 2.2.6.4).

2.2.6.4 Hematoxylin and Eosin staining (H and E)

The cells were stained with hematoxylin (Sigma, UK), a nuclear stain, for 2 min and washed for 4 min, in tap H_2O. The cells were then stained with eosin (Sigma, UK), a

cytoplasmic stain, for 4 min and then briefly washed. The cells were dehydrated in 50%, 70%, 90% and 100% ethanol (diluted with H_2O) for 2 min each and then the ethanol was cleared in 100% xylene before being cover slipped. Images were collected on an Olympus BX51 upright microscope using a 20x objective magnification.

2.3 Cell Culture

2.3.1 Maintenance of cell lines

All cell lines used throughout this thesis were kept in constant conditions at 37^{o}C in 95% air 5% CO_2, in a humidified atmosphere, furthermore, all cells used were adherent. All luciferase reporter cell lines were created from the Rat-1-R12 (Rat-1) cell line (ATCC), which is an immortal line derived from rat connective tissue fibroblasts. Human embryonic kidney (HEK293), HeLa cells and *Cav-1* WT and -/- cells (all a kind gift from Dr. Laura Matthews) were further cell types used in these studies. The *Cav-1* WT and -/- cells are an immortalised line derived through spontaneous immortalisation from the thymus of *Cav-1* -/- mice and WT litter mates. All cells lines were considered infection free and free of mycoplasma contamination. Cells were maintained in DMEM (section 2.2.3) supplemented with 10% FBS and 0.05mg/ml streptomycin and 50 Units of penicillin. In order to maintain a healthy cell population and to utilise them, the cells were passaged every 7 days. Firstly cells were detached with a 0.25% trypsin, 0.02% ethylenediaminetetraacetic acid (EDTA) solution (Sigma, UK), adding an appropriate amount to cover the base of the dish (1 ml would detach cells in a 75cm^2 dish). When the cells were fully detached the trypsin was inhibited by the serum in fresh DMEM and the cells were collected and aliquoted at an appropriate density in to plates or put back into new flasks to incubate.

2.3.2 Reporter cell lines

A number of circadian reporter lines have been used in this thesis, many of which were cloned in the Loudon laboratory by Dr. Qing-Jun Meng who kindly allowed the free use of all these constructs. These include *mPer2::luc* 600 base pair (bp); *mPer1::luc* 600 bp; *hC/ebp alpha:luc* 1300, 650, 280 and 240bp and the *hC/ebp delta:luc* 1700bp. The constructs were cloned into the pGL4.16[Luc2CP/Hygro] vector (Promega) but with added chicken beta globulin insulator sequences, in tandem copy, surrounding the promoter-driven luciferase expression region (Fig 2.4). This was previously shown to reduce

unwanted silencing and to maintain a robust signal (Meng et al. 2008). The plasmid also carries the gene for ampicillin (Amp) resistance, which allows for selection in a bacterial host, and the gene for hygromycin (Hyg) resistance, which allows for selection in a mammalian host. Hyg (Invitrogen) was continually used to supplement the DMEM (section 2.3.1), at a concentration of 200ug/ml, in order to maintain the inserted plasmid.

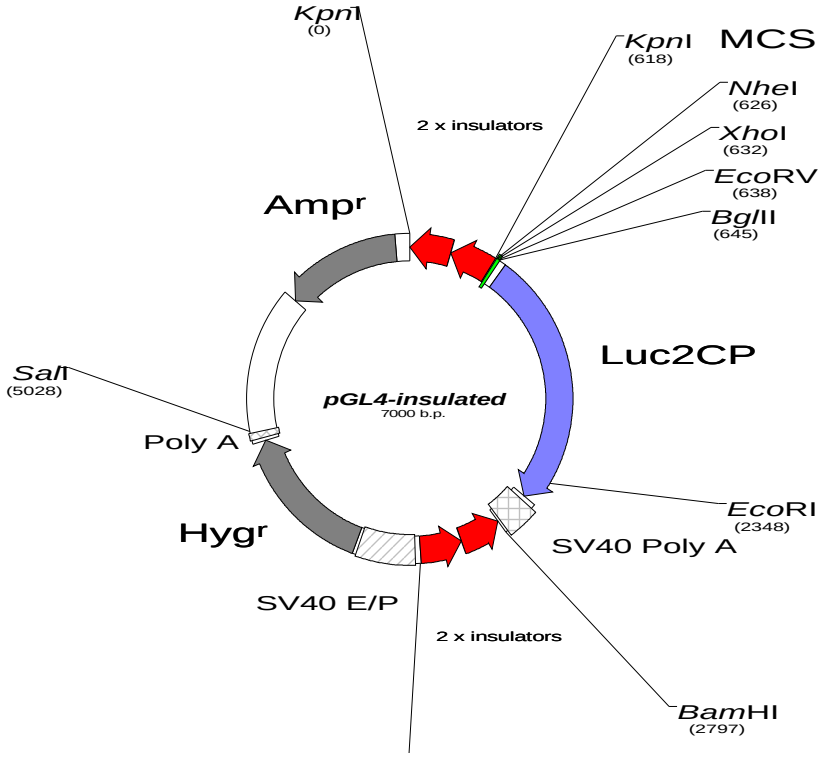

Figure 2.4: Vector map of the pGL4::luc insulated plasmid: The plasmid contains the luciferase gene, shown in blue (Luc2CP), the Amp resistance gene (Amp^r) and the Hyg resistance gene (Hyg^r). The tandem repeat insulated sequences are shown in red.

2.3.2.1 C/EBP alpha protein over expression

HEK293 or HeLa cells were cultured in a sterile 6-well plate until 50%-80% confluent (approximately 5 – 8 x10^5 cells), according to the Fugene6 protocol. A transfection mix of 97µl serum and antibiotic free DMEM and 3µl Fugene6 (Roche) (concentration of 3µl/µg of plasmid) was incubated at RT for 5 min. 1µg of hC/EBP α or control plasmid, pcDNA4/HisMax C (Invitrogen), was added to the transfection mix (the plasmid also contains a 6 x histidine (His) tag, a kind gift from Dr, Qing-Jun Meng) and incubated for a further 20 min at RT, before being added drop wise to the cells. The cells were incubated at 37^{o}C for 48h then lysed for protein extraction, see 2.5.1.

2.3.2.2 Cell preparation for endogenous C/EBP alpha expression studies circadian C/EBP alpha expression

Rat-1 cells were cultured in 35mm dishes, until 100% confluent, and synchronised with 10µM forskolin for 1h, as in 2.2.4. The cells were incubated as in 2.3.1 and 24h later the cells were lysed and protein extracted and stored at -80^{o}C, as 2.5.1. This was considered time 0 and protein was extracted from subsequent cells, every 4h over the 24h period. Protein was also extracted from the lungs of mice synchronised to a 12:12 L:D light cycle then released into constant darkness for 24h prior to schedule 1 and lung extraction. This *in-vivo* work was carried out by Dr Julie Gibbs.

DEX effect on C/EBP alpha expression

Rat-1 cells were cultured in 35mm dishes, until 100% confluent, and synchronised with 10µM forskolin, and returned to the incubator. 24h later the cells were treated with either 100nM DEX or vehicle (0.1% DMSO) for the stated time, lysed and stored at -80^{o}C, as section 2.5.1.

Clock and BMAL1 over expression

Rat-1 cells were cultured in 35mm dishes until 50%-80% confluent, according to the Fugene6 protocol. A transfection mix of 97µl serum and antibiotic free DMEM and 3µl Fugene6 (concentration of 3µl/µg of plasmid) was incubated at RT for 5 min. To separate tubes containing the transfection mix, either 1µg of pcDNA3 (Invitrogen) control, 0.3µg of pcDNA3-Clock or 0.3µg of pcDNA3-BMAL1 (each made up to a total of 1µg of DNA with 0.7µg of pcDNA3) was added and incubated at RT for 20 min. Again, to a further tube containing the transfection mix, 0.3µg pcDNA3-Clock, 0.3µg pcDNA3-BMAL1 and 0.4µg pcDNA3 was added and incubated for 20 min at RT, the transfection mixes were added drop wise to separate dishes. The cells were incubated at 37^{o}C for 48h then lysed for protein extraction, see 2.5.1. In summary, there was a pcDNA3 control, pcDNA3-Clock, pcDNA3-BMAL1 and a pcDNA3-Clock-BMAL1 transfection mix, all which contained 1µg of DNA. All plasmids were a gift from Dr. Qing-Jun Meng.

2.3.3 Cloning of *mPer1::luc* 2500bp construct

Per1 is the only clock gene with a conserved GRE present within the promoter region, located between -1918bp and -1933bp (table 2.3 for sequence), 5' to the ATG transcriptional start site, (Yamamoto et al. 2005), (5' refers to the region left of the transcriptional start site, if transcription occurs in a left to right manner, the region to the right is labelled as 3'). The construct also contains a CRE between -1800bp and -1807bp (Montminy et al. 1986) and three E-box elements at 226bp, 589bp and 1335bp. A 2500bp region of the *mPer1* promoter 5' to the ATG was sub cloned from an existing 7500bp fragment in a pGL3::luc vector (Promega).

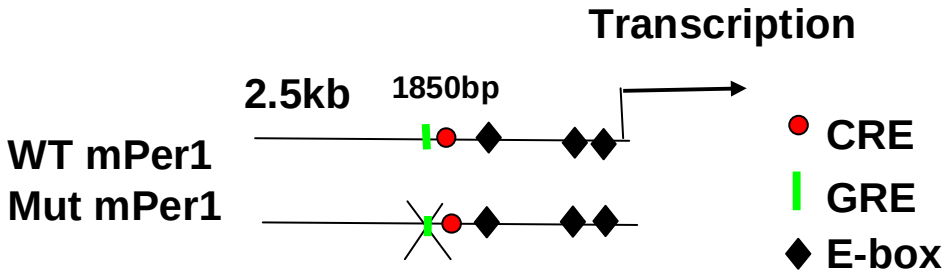

Figure 2.5: Cloned *mPer1* WT and mutant construct: A cartoon depicting the *mPer1* promoter 3kb upstream of the transcriptional start site, showing the relative positions of the CRE (1807bp-red), GRE (1933bp-blue) and E-boxes (226bp, 589bp, 1335bp-black). The black cross represents the mutated GRE construct (section 2.3.7) which leaves the CRE intact.

A NheI (5'-GCTAGC-3') restriction site was added to the 5' end of the forward (F1) primer and an EcoRV (5'GATATC3') site was added to the 5' end of the reverse (R1) primer. The sequences were taken from the genome browser website Ensembl and the 2500bp *mPer1* promoter was analysed to ensure no conflicting restriction sites. Sequences were purchased from MWG biotechnology, Germany (table 2.1). A 4 base sequence of CGGC was added before the restriction sites to allow the enzyme to 'dock'. The melting temperature (T_m) was calculated using Promega BioMath base stacking equation, to ensure both primers were within 2^{o}C of each other.

5' to 3'	**Primer sequence (restriction site is show in bold)**	**T_m (^{o}C)**
Forward (F1)	**CGG CGC TAG C**GG GGA TTT ATG TGC ATG CT	67
Reverse (R1)	**CGG CGA TAT C**AA CCC AGA AGC TCC GCG AG	68

Table 2.1: *mPer1* 2500 primer sequences.

The F1 and R1 primers (MWG, Germany), *mPer1::luc* pGL3 (7500bp) plasmid, deoxy nucleotide tri-phosphates (dNTPs) (Bioline) and HotMaster Taq DNA Polymerase (Eppendorf) were combined and nuclease free H_2O (Ambion) added to make a final volume of 20µl (table 2.2). The mix was placed in a thermal cycler and the 2500bp region of interest was amplified using the principle of the polymerase chain reaction (PCR), using the following sequence: initial denaturing step of 94^oC for 2 mins followed by 30 amplification cycles of 94^oC for 20 sec (denaturing) and 68^oC for 20 sec (annealing) and 70^oC for 3 min (elongation).

Reagent	**Final concentration (per reaction)**
F1 (10µM)	0.4µM
R1 (10µM)	0.4µM
mPer1::luc pGL3 (7.5kb)	10-50ng
dNTP (2.5mM)	0.2mM
HotMaster Buffer x 10	x 1
HotMaster Taq DNA Polymerase	0.75 Units
	Made up to 25µl in nuclease free H_2O

Table 2.2: *mPer1* 2500kb amplification reagent.

The PCR product was run on a 0.8% agarose gel, containing ethidium bromide, (both Sigma, UK) which intercalates into the DNA and allows its visualisation under U.V. light. The resulting band was cut out and purified using QIAgen gel purification kit, according to the manufacturer's protocol. The DNA was digested with 10 Units of NheI and EcoRV together with NEB buffer 2 (New England Biolabs, NEB) at 37^oC for 3h, with a small aliquot run on a gel to establish purity. The digested fragment was ligated into the insulated pGL4::luc vector (Fig 2.4). Ligation was carried out at 16^oC for 16h using T4 DNA ligase (NEB), according to the manufacturers' instructions, using a DNA to vector ratio of 3:1.

2.3.4 Transformation of the plasmid

Transformation is the process by which a plasmid of interest is taken up by host bacteria, in order to take advantage of bacteria's rapid replication machinery and amplify a gene of interest. DNA is a hydrophilic molecule (water loving) and will not readily pass through the hydrophobic (water hating) membrane of the bacteria. In order to allow the DNA to pass through the bacterial membrane cells are made competent with making small holes in the membrane with a high concentration of Ca^{2+}, and DNA can then pass through the membrane after a brief heat shock.

The DH5 alpha supercompetent bacterial cells (Invitrogen) and pGL4-*mPer1* 2500kb (pGL4-*mPer1* 2500bp) plasmid (10 – 50ng DNA) were mixed and incubated on ice for 30 min; the bacteria/DNA mix was heat shocked at 42^{o}C for 45 sec and returned to ice. The heat shocked mix was added to 250µl of SOC medium (Invitrogen) and placed at 37^{o}C for 1h, shaking at 200 revolutions per minute (rpm). The resulting solution was centrifuged at 2000 x g for 1 min and 150µl of supernatant was removed and the mix was re-suspended. The transformation mix was spread onto a Luria-Bertani (LB) agar plate (University of Manchester media stores) supplemented with 100µg/ml Amp. The plates were dried briefly and placed upside down, at 37^{o}C for 16h in a humidified atmosphere, so any potential colonies were pure clones rather than hybrids.

2.3.5 Amplification and purification of the plasmid

Potential colonies were selected and grown for a further 16h in 2 ml of LB medium (University of Manchester media stores) supplemented with 100µg/ml amp, (LB Amp). Aliquots were taken from each colony and kept for storage at -80^{o}C, as a glycerol stock, using a 50:50 mix of bacteria:40% glycerol. The resulting bacteria were lysed and plasmids extracted using QIAgen mini prep kit, following the manufacturers' protocol. Aliquots of extracted plasmids were digested with NheI and EcoRV and run on a gel as previous (section 2.3.3). Plasmids showing a fully digested band at 2500bp were then sequenced (see 2.3.6),

2.3.6 Sequencing and plasmid purification

Potential plasmids containing the *mPer1* insert were sequenced using the in-house sequencing service (University of Manchester). First the region of choice was PCR amplified using BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturers' instructions using the F1 primer (table 2.1), this was used

as primers specific to the pGL4 backbone were not available. 100ng plasmid was added to 4µl of BigDye and 1µl of 10 x reaction buffer together with 10µM of F1 primer and made up to 10µl with nuclease free H_2O. It was placed in a thermal cycler at 96°C for 4 min followed by 30 cycles of 98°C for 30 sec, 50°C for 15 sec and 60°C for 4 min. The amplification reaction was purified by adding 1µl of 3M sodium acetate pH 5.2, 1µl of 10mg/ml linear polyacrylamide (LPA) (both Sigma, UK), 0.5µl of 15mg/ml glycoblue (Ambion, UK) and 25µl (2.5 volumes) 100% ethanol, to the reaction. The resulting mix was left at RT for 20 min then centrifuged at 13000 x g for 10 min. The supernatant was removed and 100µl of 70% ethanol (30% H_2O) was added and mixed. This was centrifuged at 13000 x g for 5 min and the supernatant was removed and the pellet air dried. The pellet was sent to the sequencing facility. Providing the sequence was correct, the bacteria were taken from their respective glycerol stock and grown on an LB Amp plate at 37°C for 16h. Resulting colonies were cultured in 2ml LB Amp medium for 8h at 37°C shaking at 200 rpm. This starter culture was then transferred to a larger 50ml LB Amp culture using a 1 in 1000 dilution (50µl bacteria in 50ml LB Amp) and incubated for 16h at 37°C, shaking at 200 rpm. The resulting bacteria were lysed and plasmids extracted using QIAgen midi prep kit, following the manufacturers' protocol. Nucleotide concentration (ng/µl) and purity (idea ratios for 260:280nm peak absorbances are 1.8 for DNA or 2.0 for ribonucleic acid, RNA) was determined on a NanoDrop 1000 spectrophotometer (Thermo Scientific). Plasmids were stored at -20°C.

2.3.7 Mutagenesis of *mPer1* GRE

Yamamoto et al. (2005) showed that the GRE in the *Per1* promoter was active through a mutagenesis study (Yamamoto et al. 2005). In this study a 3 base pair mutation reduced the response to the Gc agonist, DEX, by 95% in a luciferase assay. Here the mutant clone was re-created in order to study its effects in a real time cellular environment, in response to Gc stimulus. The same mutation as Yamamoto et al. (2005) was introduced to the *pGL4-mPer1* plasmid and PCR amplified using a mutagenesis kit (Stratagene) according to the manufacturers' instructions. The primers used are shown in table 2.3 and the T_m was calculated using the Stratagene T_m calculator. Potential mutant clones were grown up, as in section 2.3.5, sequenced and followed by a large scale culture and purification of the mutant plasmid (section 2.3.6). Plasmids of both WT and mutant clones were used to generate both transient and long term genome integrated (stable) reporter cell lines (section 2.3.8.2).

	Sequence (5' - 3')	**T_{m} (^{o}C)**
WT	cca tca gcc aag **aga ACA cga tgt tcc** cta gtg cgc tg	
F1 mutant	cca tca gcc aag **aga CAG cga tgt tcc** cta gtg cgc tg	79.6
R1 mutant	ca gcg cac tag **gga aca tcg CTG tct** ctt ggc tga tgg	79.6

Table 2.3: *mPer1* GRE sequence. The WT and mutant sequences are as shown with the GRE highlighted in bold; the mutated bases and corresponding WT sequence are in upper case.

2.3.8 Generation of transient reporters and stable cell lines

2.3.8.1 Transient transfection of *mPer1::luc* luciferase reporters

To identify experimentally functional clones for the *mPer1::luc* WT, and mutant reporters, Rat-1 cells were cultured in separate, sterile 10 cm dishes, at a density of 100 x10^4 cells, 24h prior to the transfection. In separate sterile tubes (Eppendorf) 250µl of serum and antibiotic free Opti-MEM (Invitrogen, UK) was combined with 3µg of WT or mutant plasmid (Firefly luciferase) and 0.1µg of Renilla luciferase (from *Renilla reniformis)* as a control plasmid (a kind gift from Dr Midori Kayahara). Fugene6 transfection reagent was finally added, at a concentration of 3µl/µg plasmid. The transfection mix was incubated at RT for 30 min then added to the cells in a drop wise manner, ensuring even distribution. The cells were incubated for 16h at 37^{o}C, 5% CO_2 then split into a 96-well plate, using a 1 in 200 dilution per well. The plate was incubated for 16h, as previously described, and cells were treated with either: 10µM forskolin, 100nM CORT, 10nM GR agonist 67a and 69a, or 0.1% DMSO. After 4h incubation, cells were lysed and luciferase activity recorded using the Dual luciferase reporter assay system (Promega), according the manufacturers' protocol, and a luminometer (Beckman). The pGL4-*Per1* (Firefly) luciferase was read first at 560nm, this reaction was then stopped and the Renilla luciferase was read at 480nm. A ratio of Firefly to Renilla luciferase was taken and normalised relative to the WT control.

2.3.8.2 Generation of stable reporter cell lines

Both WT and mutant *mPer1::luc*, confirmed by a luciferase assay (section 2.3.8.1), were transfected into Rat-1 cells and allowed to stably integrate in the genome, through random

insertion. 12µg of each plasmid was linearised with SalI (NEB) for 3h at 37°C, checking an aliquot on a gel, as section 2.3.3. The linearised plasmid was precipitated, as in 2.3.6, and the resulting plasmid was re-suspended in 50µl of nuclease free H_2O and quantified. Rat-1 cells were cultured in 10 cm dishes and incubated until 30%-50% confluent (approximately 0.2 – 0.5 x 10^6 cells). 18µl of Fugene6 was added to 582µl of DMEM (supplement free) and incubated at RT for 5 min. 6µg of linearised plasmid was added to the transfection mix and incubated for a further 20 min at RT, then added to the cells in a drop wise manner. Transfected cells were incubated at 37°C for 48h then the medium was removed and cells washed in 1x PBS. DMEM supplemented with Hyg was added to the cell and incubated at 37°C. To drive selection of *mPer1::luc* positive cells the DMEM with Hyg medium was changed every 48h until individual colonies of 10 - 30 cells appeared. Successful colonies were removed by sterile, trypsin soaked 3mm cloning discs (Sigma, UK) and cultured in 12 well plates with DMEM and Hyg. The medium was changed every 48h until cells were confluent and transferred to 25 cm^2 flasks (T-25) (Corning, UK) and eventually to a 75 cm^2 flask (T-75) (Corning, UK). Cultured cells, both WT and mutant, were then ready to be used experimentally within the PMTs, (section 2.3.9).

2.3.8.3 Luciferase assays with stably transfected C/EBP alpha and delta cells

Cells were cultured as 2.3.1 and prior to use were grown in sterile 6 well plates until 100% confluent (approximately 1 x 10^6 cells). 24h before experimental treatment, cells were synchronised with 10µM forskolin for 1h then the medium was removed and cells were washed with 1 x PBS and fresh medium was replaced. 24h later cells were treated with either 100nM DEX or 0.1% DMSO for 4 or 24h. After the elapsed time the medium was removed and cells were washed in 1 x PBS then lysed and a luciferase assay was performed using Luciferase Assay Kit (Promega), according to the manufacturers' protocol and read on a luminometer (2.3.8.1). A *Renilla* control was not used as the reporter was integrated into the genome and the cells were grown from a single cell population. The resulting luciferase values were then normalised between 0 and 100 relative luciferase units (RLU) with 100 units equal to the DMSO control.

2.3.8.4 MR transactivation assay

The transactivation assay is a method of exploiting the ability of a liganded nuclear receptor to induce transcription. In the transactivation assay, described below,

transcription induced is a mouse mammary tumour virus (MMTV) luciferase reporter which contained 4 copies of the GRE sequence, (kind gift from Dr. Midori Kayahara, University of Manchester), upstream of a minimal promoter. The transcriptional activation can be used to determine the median effective concentration (Ec50) value for an unknown compound. This is the point that the concentration of agonist induces 50% of the total activation of the system.

HEK293 cells were plated into sterile 10cm dishes, at a density of 100 x 10^4 cells per well. 16h later, a transfection mix of 3µg of hMR expression plasmid (Govindan and Warriar 1998) (kind gift from Dr. M. Govindan, Laval University, Canada), 3µg *MMTV-luc* and 0.1µg *Renilla* were added to 250µl of Opti-MEM. To this 18µl of Fugene6 was added and then incubated at RT for 30 min then added, drop wise, to the cells. The transfected cells were incubated at 37^{o}C for 16h and then split in to a 96 well plate with a 1 to 222 dilution (45µl per well from a 10ml volume) and then incubated for a further 16h. Cells were treated with the respective compound; CORT or GR agonist (67a or 69a), using a serial dilution ranging from 100nM to 3pM with dilutions of 1 to 3, (factoring in a 10 fold dilution in to the cells, therefore, making stocks 10 x greater). Cells were incubated for a further 16h and then the medium was removed, cells were washed in 1 x PBS and then lysed and the luciferase signal read and analysed, as section 2.3.8.1. The Ec50 value was defined by fitting the data points to a one phase exponential decay algorithm (GraphPad Prism). The GR transactivation assay was carried out by Dr Midori Kayahara in the same way as described above but with an hGR expression plasmid, rather than the hMR.

2.3.9 Experimental treatment of cell lines

2.3.9.1 Preparation of reporter cell lines for the PMT

Cells were cultured in to individual 35mm sterile dishes until they were 100% confluent, approximately 1 x 10^6 cells. The cells were then synchronised for 1h with 10µM forskolin (as in section 2.2.4). The medium was removed and the cells were washed in 1 x PBS and 2ml of recording medium was added. The dishes were sealed with sterile cover slips and grease (section 2.2.4) and were place in the PMT units, either single units or in the Lumicycle, and recordings taken. Data points were collected every minute, with the single PMT units, or every 10 min with the Lumicycle, throughout the experiment. All cells were experimentally treated, and analysed, in the same way as the lung slices (section 2.2.5.1).

2.3.9.2 Treatment of cells for quantitative PCR

Rat-1 cells

The cells (Rat-1 or *Cav-1* WT and -/-, section 2.3.1) were cultured in 6-well plates until they were 100% confluent, approximately 1 x 10^6 cells, and synchronised for 1h with 10µM forskolin 24h prior to treatment. The medium was removed and the cells were washed in 1 x PBS and 2ml of DMEM (section 2.3.1) was added and returned to the incubator. 24h later Rat-1-*Per2::luc* cells were treated with either DMSO, CORT or GR agonist for 4h or pre-treated with 1µM RU486 for 1h then CORT or GR agonist was added before RNA extraction 4h later. The RNA was extracted as in 2.6.2.2

Cav-1 WT and -/- cells

Cav-1 cells (WT and -/-) were synchronised as above (2.3.9.2 Rat-1 cells) with CORT or DMSO for 1h, 2h or 4h prior to RNA extraction. *Cav-1* WT and -/- cells were also synchronised as above but remained untreated and RNA extracted at the indicated times 12h apart (Fig 4.8). The PI3K inhibitor, LY294002 (Sigma, UK) was added at 50µM to *Cav-1* WT cells 1h prior which to 100nM CORT administration. The cells were then harvested and RNA extracted at 2h and 4h post treatment for analysis of *mPer2* by qPCR. The RNA was extracted as in 2.6.2.2

2.4 Flow-through culture system

2.4.1 Background

A flow-through culture system was established previously by Dr. Andrew McMaster in order to study the temporal effects of different stimuli on the regulation of the genome, in a system that could mimic the pulsatile nature of endogenous Gc (Windle et al. 1998b). The advantage of this system meant that Gcs could be added and removed, on demand, without disturbing the cellular environment, as there was a constant flow of new medium. The basic principles of this system were taken and modified, in this thesis, to establish a flow-through reporter system linked to the PMT, so that real time recordings could be taken and experiments carried out at specific phases of the circadian cycle.

2.4.2 Linking the Flow-through to the PMT

For all but one flow-through experiment, mPER2::luc lung slices were used as the reporter (section 2.2.3/4), as reporter cell lines would not tolerate the flow of medium. A peristaltic pump (Watson-Marlow, UK) was used to continually pump recording medium (appendix A) through sealed 35mm culture dish at a rate of 6.8ml/h. This rate was based on Dr. McMaster's previous findings where the wash out rate was able to mimic that of an endogenous Gc pulse. Heat shrink (Maplin electronics, UK) was fitted to an appropriate length of marprene tubing (Watson-Marlow, UK) with a 0.5mm internal bore, so as to remain stable in the pump. The tubing was sterilised in an autoclave and the system was assembled in a sterile atmosphere.

Holes were placed in the filter lids of two 225cm^2 (T225) flasks (one for recording medium and one for waste) and a T25 (recording medium with Gc). Roughly 900ml of recording medium was added to the relevant T225 flask, enough for about 5.5 days and a further 20mls, supplemented with Gc, was added to the T25. A 35mm dish had 5 holes drilled into it, it was then washed and sterilised with 70% ethanol and U.V. light. The membrane insert was secured, and the sterile tubing was attached, shown in Fig 2.7, and sealed with grease. The free end of each tube was inserted into the respective flasks; waste, recording medium only and recording medium with CORT, (Fig 2.6). The lung slice was synchronised, 1ml recording medium was added to the well and the dish sealed, (section 2.2.4).

The set up was transferred to a 37^{o}C incubator, which contained the peristaltic pump and a mechanical on/off switch. The switch acted to pinch close one tube (medium with CORT) whilst allowing the other to remain open (medium only), (John Simpson, University of Manchester), then at a given time this could be reversed. A subsequent improvement to this linked the switch motor to a computer-driven timer, thus allowing for pre-set on/off events. The tubes were fitted to the pump (using the heat shrink as a stopper) and inserted to the switch, as shown in Fig 2.6, in a linear manner. The dish containing the lung slice was placed in the PMT unit which was sealed with tin foil. The pump was turned on and the system allowed to equilibrate for 30 min before the PMT recordings began.

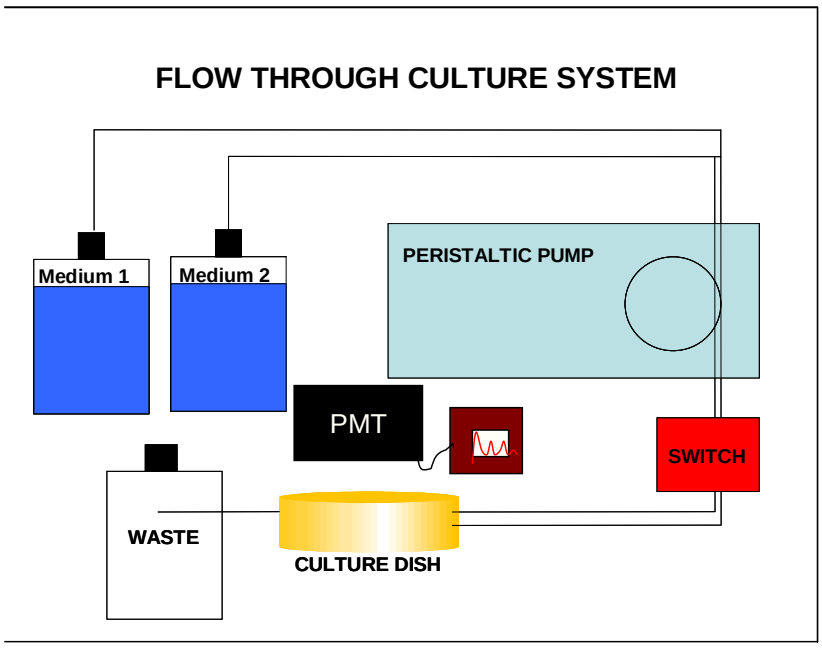

Figure 2.6: Diagram of the flow-through system. Tubes were inserted into two flasks, one containing recording medium and the other recording medium with Gc, fed through the peristaltic pump and into a mechanical on/off switch. The tubes lead into the culture dish, containing the mPER2::luc lung slice, placed in a PMT unit, linked to a computer. A single output tube leads to a waste flask. The flow rate was set to 6.8ml/h.

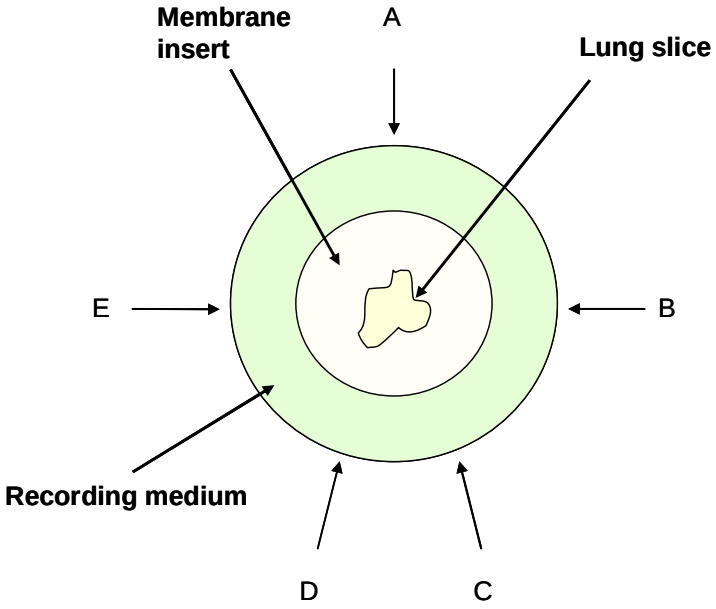

Figure 2.7: Plan view of the 35mm dish modified for flow-through culture. Holes were drilled at points A-E and the three tubes were fixed in position, waste (A), recording medium only (C) and recording medium with Gc (D). The membrane insert was fixed in position with tubing (B and E) and the insert was placed on recording medium.

2.5 Protein quantification

2.5.1 Protein assay

The bicinchoninic acid (BCA) protein assay was used for quantification. Cells were lysed in 400µl of buffer (appendix B) for 30 min at 4°C, collected and centrifuged at 13000 x g for 20 mins. The supernatant was stored at -80°C and allowed to thaw, on ice, before use. A standard curve was made using a known concentration of bovine serum albumin (BSA) (Sigma, UK). A 6 point BSA dilution series was made, 0, 100, 200, 400, 800 and 1000µg/ml, diluted in lysis buffer and the unknown sample was diluted in lysis buffer, 1 in 10, to make a final volume of 50µl. An appropriate volume of the BCA reagent mix was prepared using 1ml BCA solution (Sigma, UK) with 40µl 4%(w/v) $CuSO_4$ (Sigma, UK) per sample. The BCA reagent was mixed and added to the sample and standards. All samples were incubated at 37°C for 30 min and then read, in triplicate, at 560nm on a plate reader (Bio-Rad). A colour change is detected as the protein reduces Cu^{2+} to Cu^{+} in a concentration dependent manner, forming a purple colour. The BCA assay was used here rather than the quicker Bradford assay as the detergent, in the lysis buffer (Triton X-100, appendix B), interfered with the Coomassie Blue stain (active compound in the Bradford assay) resulting in a blue colour which was indistinguishable from a high protein concentration. Therefore, the Bradford protein assay could not be used with this specific lysis buffer (appendix B).

2.5.2 Immuno (Western) blotting

Immuno blotting is a technique used to detect a specific protein in a mixed protein sample, or lysate. The lysates which can come from cells or tissues, and are first denatured, to form a primary chain of amino acids, then coated with a large negative charge, using sodium dodecyl sulphate (SDS), they are separated by mass by passing a voltage through a gel matrix.

The protein was quantified as in 2.5.2 and a total of 50µg was immuno-blotted against the respective antibodies. The protein was denatured at 95°C for 10 min in the presence of 20µl loading dye, (see appendix B for reagents). The samples were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using a 8% gel (appendix B) and passing 120 volts (V) for 1h then 180V for 4h, through the system, using a Protean II gel ready system (Bio Rad). The samples were resolved in parallel with a dual colour protein marker

(Bio Rad), range 15 to 250 kilodalton (kDa), in a Tris SDS running buffer (appendix B). The resolved proteins were transferred to a 0.2µm nitrocellulose membrane (Bio Rad) for 16h using 35V at 4^{o}C, in a methanol based transfer buffer (appendix B). The transferred protein was stained with ponceau S (Sigma, UK), to check for even loading. The membrane was washed with deionised H_2O and blocked for 4 hours in 5% skimmed milk (Marvel) in 20mM Tris–HCl pH 7.6 buffered saline (TBS) (supplemented with 0.1% Tween-20, (TBST) without agitation (both Sigma, UK). The primary antibody was added, diluted in 5% milk TBST, and incubated for 16h at 4^{o}C, shaking gently. Antibodies used were rabbit anti-C / EBP α, C-terminus monoclonal (Epitomics), diluted 1 in 500; alpha tubulin clone DM1A mouse monoclonal (Sigma, UK), diluted 1 in 2000; anti His-tag mouse monoclonal (Serotec) diluted 1 in 200. The membrane was washed with 3 x 15 min TBST washes, to remove unbound antibody and the secondary antibody was added diluted in 5% milk TBST for 1h at RT, shaking gently. The secondary antibodies were horseradish peroxidase (HPR) linked anti mouse or anti rabbit (raised in sheep or donkey respectively) whole antibody immunoglobulin G (IgG) (GE Healthcare). Excess antibody was washed off with 5 x 15 min washes in TBST and then imaged using the enhanced chemiluminescence (ECL) Amersham advanced detection system (GE Healthcare) and a manual developing/fixing protocol (Sigma, Uk) with a Biomax film (Kodak). To develop the exposed film, the developing and fixing reagents were made up according the manufacturers' protocol, and the film was washed in the developer until a dark band appeared with the correct molecular weight. The film was washed with H_2O and placed in the fixing solution until the film was clear. The solution was washed with H_2O and the film left to dry. The resulting images were quantified using densitometry software. The immuno blots regarding the lung slices (section 2.2.5.5) were carried out by Dr Laura Matthews using GR M20 (Santa Cruz), alpha tubulin (Sigma, UK) phosphorylated (P) $GR^{Ser\ 211}$, $pPKB^{ser473}$, P-MAPK $p42/44^{thr202}$ (al NEB). The immuno blots were scanned and analysed by densitometry using SigmaScan software and are represented as expression relative to α- tubulin.

2.5.3 Enzyme-linked immunosorbant assay (ELISA)

The ELISA technique is a way of detecting and quantifying a protein of interest in an unknown sample. It utilises the specificity of antibody detection, similar to an immuno blot, but the detection antibody, linked to the HPR enzyme, undergoes a chemical reaction, resulting in a colour change, which is proportional to the concentration of protein. This

reaction is carried out in parallel with a known concentration of samples and so an accurate determination of protein can be found.

ELISAS for mouse CXCL1 (KC), IL-6, TNFα, CCL5 (RANTES) and CCL2 (MCP1) were carried out using the ELISA DuoSet (R and D systems) according to the manufacturers' protocol, as follows: All incubations were carried out at RT. 50µl of capture antibody was added to a 96-well plate (Nunc, Denmark) in serum free 1 X PBS (10mM phosphate buffer, 2.7mM potassium chloride and 137mM sodium chloride, pH 7.4, Sigma, UK) and left for 16h. The antibody was removed and the plate washed 3 times in 1 x PBS with 0.1% tween-20 (PBST). The plate was blocked with 150µl of 1% low endotoxin BSA (Sigma, UK) dissolved in 1 X PBS (referred to as reagent diluent) for 1h and washed in PBST. Standards and samples were prepared and added to the plate; unknown samples were added neat and with a 1 in 2 and 1 in 10 dilution, to ensure they were in range with the standard curve. All standards and samples were diluted in basic recording medium (appendix A) and 50µl was added, to the wells, and incubated for 2h. The plate was washed, in PBST, and 50µl of biotinylated detection antibody was added, dissolved in reagent diluent, and incubated for 2h. The plate was then washed and 50µl of streptavidin-HRP (R and D systems), diluted 1 in 200 into reagent diluent, was added and incubated for 20 min. This was removed, the plate was washed and 50µl of substrate solution, a 1:1 mix of hydrogen peroxide and tetramethylbenzidine, was added and incubated for 20 min, in the dark. 25µl of 0.2M sulphuric acid was added to the substrate solution to stop the reaction. This results in the solution turning yellow, in proportion to the protein concentration, and is read at 450nm.

2.5.4 Bio-Plex

2.5.4.1 Principle of the Bio-Plex

The principle of the Bio-Plex (Bio Rad) is illustrated in Fig 2.8 and utilises a similar technique to the ELISA, with a capture and detection antibody being used to bind a protein of interest. However, in addition the biotinylated detection antibody is bound to the steptavidin-phycoerythrin (S-PE) conjugate when added, rather than HRP. The S-PE is a fluorescent reporter and is excited at 532nm and the amount of fluorescence is proportional to the amount of protein. The real advantage to this technique is that the capture antibody is fixed to a bead, which also has a specific fluorescent tag called the 'classification tag'

which excites at 635nm. This means that multiple proteins can be analysed at the same time, in the same sample, simultaneously increasing the amount of data that can be obtained and vastly reducing the amount of sample needed.

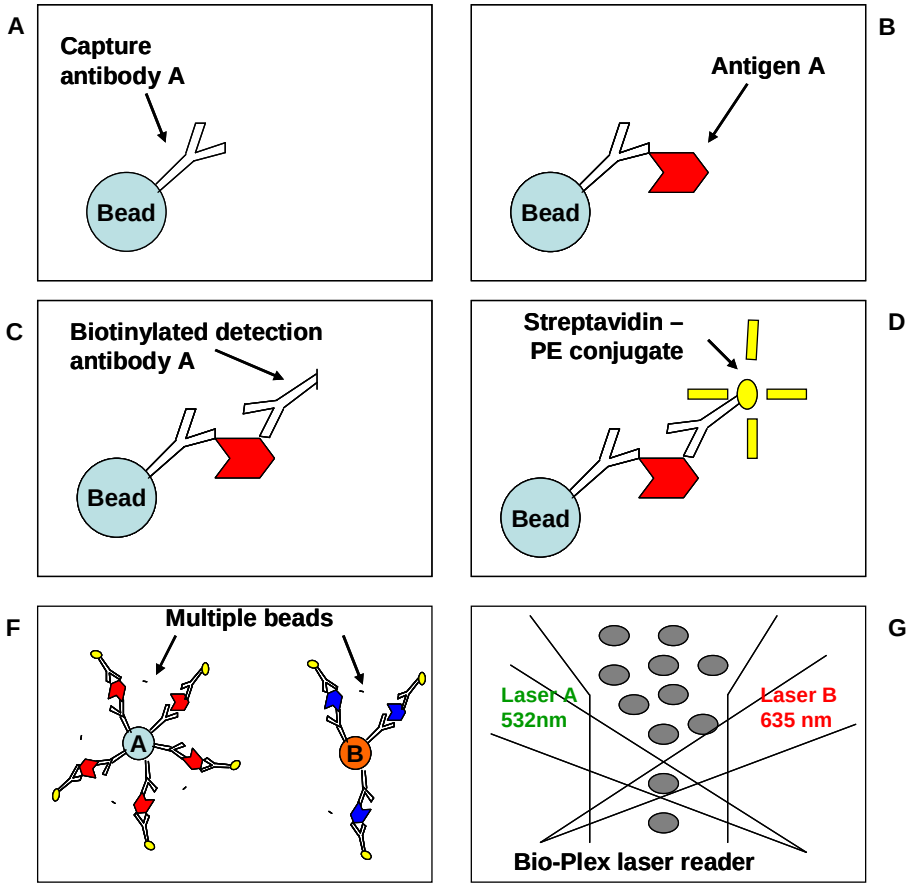

Figure 2.8: Principle of the Bio-Plex: A capture antibody is bound to a bead with a specific fluorescent 'tag' (A). The assay sample is added and the antigen, specific to the antibody, and binds (B). The biotinylated detection antibody is added and it binds to the antigen (C). The streptavidin conjugated PE is finally added to the assay mix and this binds to the biotin (D). The specificity of the bead and antibodies allows multiple beads, therefore, antigens, to be read in the same sample (F) and the Bio-Plex lasers can rapidly detect different beads using laser A and quantify the amount of antigen present using laser B (G).

2.5.4.2 Bio-Plex assay protocol

All reagents and buffers were supplied with the assay kit (Bio-Rad) and all methods were carried out according to the manufacturers' protocol. The assay standards were diluted and the frozen serum samples (section 2.2.1) were thawed to RT, mixed by vortexing and diluted 1 in 4 with mouse serum diluent (Bio Rad). The assay plate was pre-wet with assay buffer and removed using a vacuum manifold filter (Bio-Rad); all subsequent solution

removal and wash steps used this method. The assay beads were added to the plate (50µl/well) and it was washed twice with wash buffer. The standards and samples were added (50µl/well) and incubated at RT for 30 min on a shaker. The plate was washed 3 times in wash buffer and the detection antibody (25µl/well) was added and the plated was incubated for 30 min as previous. The plate was again washed 3 times in wash buffer and S-PE was added (50µl/well) and incubated at RT for 10 min on the shaker. The plate was washed 3 times and assay buffer was added (125µl). The plate was then analysed using the Bio-Plex system and the recommended Bio-Plex manager software.

2.6 Quantitative PCR (qPCR)

2.6.1 Principles of qPCR

There are 3 main ways of quantifying the mRNA transcript of a particular gene of interest. These are real time (RT) PCR, semi-quantitative (or relative) qPCR and quantitative (or absolute) qPCR. The RT-PCR works through the PCR amplification, of a particular mRNA transcript, that has been reverse transcribed to cDNA, (Fig 2.9), but the number of cycles of replication is limited to between 25 and 30, to hit the linear phase of the of exponential growth pattern, allowing the direct comparison between transcripts. However, the PCR products are resolved on an agarose gel, section 2.3.3, which makes it hard to quantify as the reproducibility of the gel can be difficult, and quantification is done by densitometry, which requires computer software and can be inaccurate. Therefore relative and absolute qPCR were developed, which still utilise PCR amplification but coupled to fluorescent tags, (SybrGreen or taqman probes). Throughout these studies I have used semi-quantitative (relative) taqman based qPCR and so will be briefly explained with the aid of Fig 2.9.

Taqman based qPCR works on the principle of PCR but the taqman-probes have a fluorophore 6-carboxyfluorescein (FAM) (peak absorbance 495; emission 516) and quencher, tetramethylrhodamine (TAMRA) (peak absorbance 565; emission 580) attached. The probe binds to the target and on excitation a particular wavelength of light is emitted. In the normal, unbound, state, where the probe is in tact, the fluorophore will emit a wavelength, but this will excite the quencher whose emission spectrum cannot be detected. In the bound state, as the primers extend they will ‘knock’ the FAM fluorophore off the probe, into solution, allowing any emitted light to be detected. The more PCR cycles, the

greater the amount of fluorescence recorded, until it plateaus. The end user can then select the number of cycles (called cycle threshold or Ct) it took to reach a certain value of fluorescence, again within the linear phase of growth. The Ct value is inversely proportional to the amount of transcript and this can be measured relatively or absolutely. If measured relatively the Ct values of the gene of interest are compared to a control gene that does not alter under the experimental conditions, for example beta actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). If measured absolutely then the Ct values are compared to the values of a standard curve, with known concentrations of cDNA.

The region of amplification, however, is vitally important whichever method is being used. Genomic DNA is made up of introns and exons, sequentially arranged, and the exons are what produce the mature mRNA and subsequent protein. The mRNA is reverse transcribed to cDNA and so in order to ensure quantification of cDNA and not genomic DNA the region of amplification must cross an exon boundary; a further control is to pre-treat the RNA with DNase before reverse transcription.

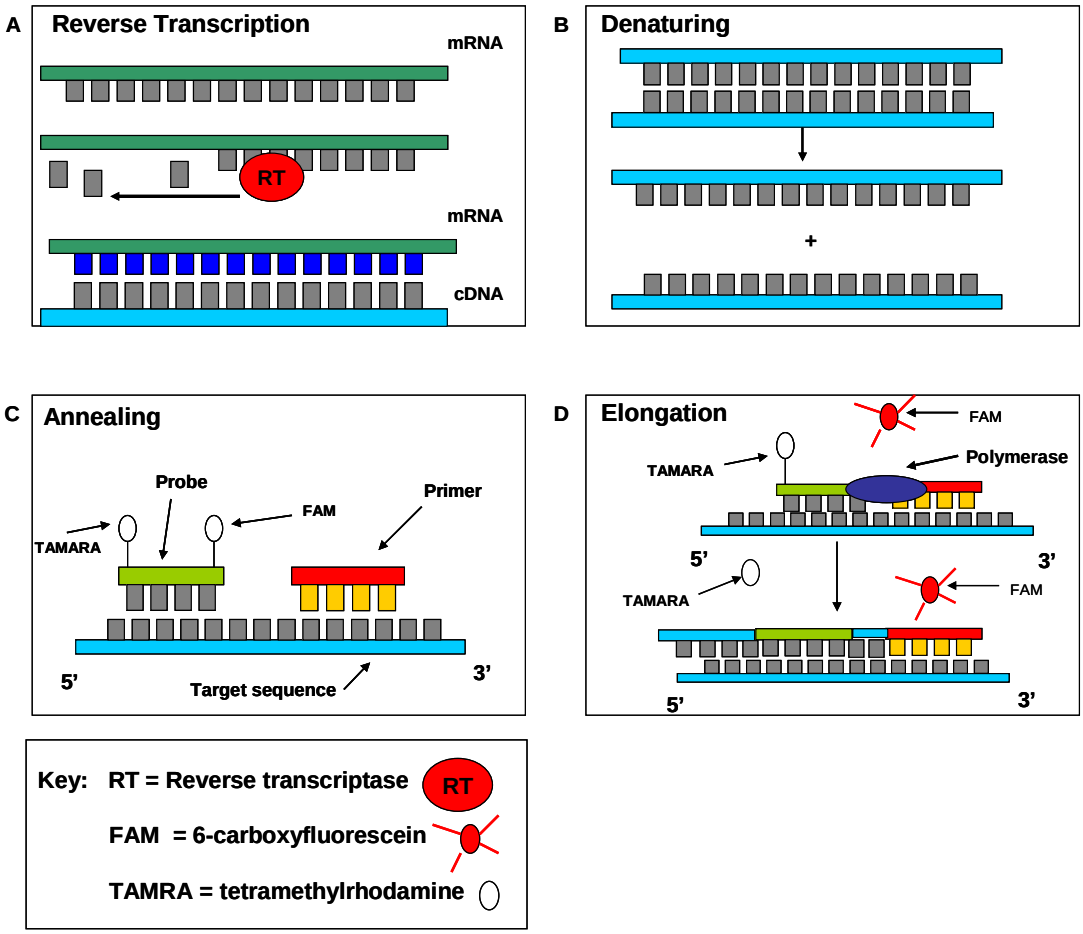

Figure 2.9: The principle behind Taqman qPCR: The mRNA is first reverse transcribed to produce cDNA (A). The cDNA is initially single stranded but shown here as double stranded as it will form in subsequent cycles. The two strands are separated by heat (B). The primers and probe can then anneal to the target sequence (C) and as the polymerase extends from the primer it will hit the fluorophore and release it into the surrounding medium (D). This will remove it from the close proximity to the quencher allowing it to be excited and emit a detectable wavelength of light.

2.6.2 RNA extraction

2.6.2.1 Tissue

Roughly 100mg of frozen tissue was removed from the sample (liver, lung and kidney) using a sterile blade and quickly added to a lysing matrix D tube (MP Biomedicals) containing 1ml TriZol reagent (Invitrogen). TriZol is a phenol based extraction and all work was carried out in the fume cupboard and caution was taken. The tissue was completely homogenised in a FastPrep-24 system (MP Biomedicals) at 4^{o}C. The samples were incubated at RT for 5 min and 200µl of chloroform (Sigma, UK) was added, thoroughly mixed by inversion, and incubated for 2–3 min at RT, then centrifuged at 4^{o}C

at 12000 x g for 15 min. The resulting solution separated in to 3 different phases with the RNA in the upper aqueous phase. 500µl of the aqueous phase was removed and added to 500µl of isopropanol and incubated for 10 min at 4°C, then centrifuged for 10 min at 12000 x g at 4°C. The isopropanol solution was removed as the RNA had precipitated and formed a pellet. The pellet was washed in 70% ethanol (30% nuclease free H_2O) and centrifuged for 5 min at 12000 x g at 4°C. The ethanol was removed and the pellet air dried for 5-10 min and then re-suspended in 100µl nuclease free H_2O. At this stage the RNA from the lung tissue was stored at -80°C due to a low RNA yield of, but the RNA from the liver and kidney was further purified using an RNeasy extraction kit (QIAgen). 10µl β-mercaptoethanol (Sigma, UK) was added to 1ml of RLT buffer and 350µl of this buffer was added to the 100µl RNA (TriZol extraction). This was mixed thoroughly then 250µl of 100% ethanol was added and mixed well. The sample was applied to the purification column and centrifuged at 10000 x g for 15 sec. The flow-through solution was discarded and the column washed twice with 500µl RPE buffer; once at 10000 x g for 15 sec and then at 10000 x g for 2 min, the flow-through was discarded each time as necessary. The RNA was eluted into a sterile RNase free tube, in 50µl of nuclease free H_2O, and was stored at -80°C.

2.6.2.2 Cells

The RNA was extracted from the Rat-1 cells using the RNeasy extraction kit (QIAgen) according to the manufacturers' instructions. The cells were lysed in 350µl RLT (without β –mercaptoethanol) for 2 min on ice. The lysate was added to a QIAshredder (QIAgen) column, and centrifuged at 16000 x g for 2 min, to thoroughly lyse the cells and remove any cell debris. The flow-through was mixed with an equal volume of 70% ethanol, mixed then added to a purification column and centrifuged at 10000 x g for 15 sec. The flow-through was discarded and the column washed with 700µl RW1 buffer. The flow-through was discarded and the column washed twice with 500µl RPE buffer; once at 10000 x g for 15 sec and then at 10000 x g for 2 min, the flow-through was discarded as necessary. The RNA was eluted into a sterile RNase free tube in 30µl of nuclease free H_2O, and was stored at -80°C.

2.6.3 Reverse transcription (RNA to cDNA)

All RNA was reverse transcribed in the same way, regardless of the original source and Fig 2.9 (A) shows a brief description of how it occurs. The RNA was quantified using a Nanodrop-100 (section 2.3.6) and 2µg of RNA was used in the reaction, it was assumed that all RNA was converted to cDNA. All RNA was treated for 30 min at 37°C with 1µl RNase free DNase (Promega), regardless of its purity (section 2.3.6). This reaction was stopped with 1µl DNase stop buffer (Promega), incubated at 65°C for 10 min. A high capacity RNA to cDNA kit (Applied Biosystems) was used for the conversion. The 2µg was converted in a 20µl reaction using 10µl of 2 x buffer and 1µl of 20 x enzyme mix; this meant that the 2µg of RNA had to be dissolved in 7µl, or less. If this was not possible the reaction mix was multiplied appropriately. The reverse transcription reaction was incubated at 37°C for 60 min then the reaction stopped by denaturing the enzyme with 95°C for 5 min. There was a no enzyme and no RNA control produced as well. The cDNA was stored at -80°C.

2.6.4 RT-PCR

The cDNA (section 2.6.3) was thawed on ice and an RT-PCR master mix (table 2.4) was produced, in order to minimise pipetting errors. The enzyme used was the Expand high fidelity PCR Taq polymerase (Roche), which has a 3'-5' exonuclease activity to 'proof read' the new sequence. This enhances reliability of the transcript produced.

Reagent	**Final concentration (per reaction)**
F1 (10µM)	0.4µM
R1 (10µM)	0.4µM
cDNA (2µg)	100ng
Deoxy nucleotide tri-phosphates (dNTP) (2.5mM)	0.2mM
Expand High Fidelity buffer x 10	x 1
Expand High Fidelity Taq DNA Polymerase	2.6 Units
	Made up to 25µl in nuclease free H_2O

Table 2.4: RT-PCR mastermix

The primers MR, GR and GAPDH (MWG, Germany) (table 2.5) were designed by the author ensuring they were between 17 and 30bp long, had 40-80% guanidine/cytosine content and had a T_m between 50°C and 70°C and being no more than 2°C different. The mouse GAPDH primers were a kind gift from Dr .Qing-Jun Meng and the rat MR primers were taken from (Zhou et al. 2000). The primers, dNTPs (Promega), reaction buffer and enzyme were added to the cDNA and mixed. The reaction mix was amplified in a thermal cycler using the following protocol: 94°C for 2 min; then 28 cycles of 94°C for 15 sec, 60°C for 30 sec and 72°C for 45 sec, the final step was 72°C for 7 min. The RT-PCR product was run on a 0.8% ethidium bromide agarose gel, (section 2.3.3) and imaged under U.V light.

Mouse		**Sequence (5' - 3')**
GR 375bp	**F1**	AGGAATTCAGC AAGCCACTG
	R1	GCAAAGCATA GCAGGTTTCC
MR 401bp	**F1**	TTCGGAGAAAG AACTGTCCTG
	F1	AAGTACCTTGG CCCACTTCA
GAPDH 594bp	**F1**	CCTTCATTGAC CTCAACTAC
	R1	GGAAGGCCAT GCCAGTGAGC

A

Rat		**Sequence (5' - 3')**
GR 378bp	**F1**	ATCAAAGGGAT TCAGCAAGCC
	R1	AAAGCAGAGCA GGTTTCCAC
MR 196bp	**F1**	CTTCAAAAGAG CCGTGGAAGGG
	F1	TGCTGTGGTTGC TCCTCGTG
GAPDH 347bp	**F1**	GGTCATCATCTC CGCCCCTTCC
	R1	GGAAGGCCATG CCAGTGAGC

B

Table 2.5: Primers used for RT-PCR: The GR, MR and GAPDH primers used to amplify the mouse transcripts are shown in (A) and the rat primers are shown in (B). F1 and R1 are the forward and reverse primer respectively and the amplified length is shown in the left hand column.

2.6.5 Taqman qPCR

The principle of taqman qPCR is shown in Fig 2.9 and all primers and probes were sequenced by MWG, Germany, except for *Rev-erb α* which was used as a pre-made primer and probe mix (Applied Biosystems, assay number Mm00520708_m1) with an undisclosed sequence but which targeted a 62bp sequence crossing the exon 1 and 2 boundary. There primers and probes were all taken from the literature as the amplified transcripts were common genes in the circadian field. The primers and probes used are shown in table 2.6 along with their respective concentrations. The qPCR enzyme was the fast blue qPCR MasterMix Plus dTTP (Eurogentec). This contained dNTPs, buffer and enzyme at 2 x the concentration required, the buffer was diluted to 1 x and to this the primers, probes and H_2O were added. The PCR reaction was carried out in a 96-well MicroAmp Optical reaction plate (Applied Biosystems) in a 25µl volume with 100ng cDNA and so the volume of enzyme/reagent buffer mix was adjusted accordingly. The amplification and data collection was carried out in an ABI 7300 (Applied Biosystems) using the following cycle: 50^{o}C for 2min, 95^{o}C for 10 min, then 40 cycles of 95^{o}C for 15 sec and 60^{o}C for 1 min. The fluorescence was recorded from cycle 2 with the linear phase being between cycles 12 and 34. Each sample was run in triplicate. The data was then analysed (see 2.6.6).

Gene	**F1 (5'-3')**	**R1 (5'-3')**	**Probe (5'FAM-3'TAMARA)**	**Reference**
mBeta actin	AGGTCATC ACTATTGG CAACGA (200nM)	CACTTCATGA TGGAATTGAA TGTAGTT (200nM)	TGCCACAGGAT TCCATACCCAA GAAGG (200nM)	(Reddy et al. 2007)
mPer2	GCCTTCAG ACTCATGA TGACAGA (200nM)	TTTGTGTGCG TCAGCTTTGG (200nM)	ACTGCTCACTAC TGCAGCCGCTC GT (100nM)	
mCry1	CTGGCGTG GAAGTCAT CGT	CTGTCCGCCA TTGAGTTCTA TG	CGCATTTCACAT ACACTGTATGA CCTGGACA	

	(100nM)	(100nM)	(200nM)	
mBMAL1	CCAAGAA AGTATGGA CACAGAC AAA (200nM)	GCATTCTTGA TCCTTCCTTG GT (200nM)	CTGTCCGCCATT GAGTTCTATG (200nM)	(Reddy et al. 2007)
mPer1	ACCTTGGC CACACTGC AGTA (200nM)	CTCCAGACTC CACTGCTGGT AA (200nM)	ATTCCTGGTTAG CCTGAACCTGCT TGACA (200nM)	
mGILZ	GTGGTGGC CCTAGACA ACAAG (200nM)	TCACAGCGTA CATCAGGTGG TT (200nM)	CACGAGGTCCA TGGCCTGCTCA (200nM)	(Mittelstadt and Ashwell 2003)
rPer2	GCAGGCTC ACTGCCAG AACT (200nM)	CAAGATGATT CTATTCCAGA AGCATT (200nM)	AGCCCCAGCAA GTGATCGAGGA CTAAG (200nM)	(Fahrenkrug et al. 2006)
rGILZ	CTTCTCTT CTCTGCTT GGAGGG (200nM)	CGATCTTGTT GTCTATGGCC AC (200nM)	CACTAGATCCA TGGCCTGCTCA (200nM)	Adapted from (Mittelstadt and Ashwell 2003)
rC/ebp alpha	AGTTGACC AGTGACA ATGACCG (300nM)	TCAGGCAGCT GGCGGAAGAT (300nM)	AGCTGAGCCGT GAACTGGACAC GCT (200nM)	(Sloop K W 1998)
rBeta actin	CGTGAAA AGATGACC CAGATCA (200nM)	CACAGCCTGG ATGGCTACGT (200nM)	TTTGAGACCTTC AACACCCCAGC CA (200nM)	Adapted from (Zeng et al. 2003)

Table 2.6: Primers and probes used for taqman qPCR: All sequences are labelled 5'-3' and the concentrations are shown in parenthesis.

2.6.6 Taqman qPCR analysis

The data from the taqman qPCR experiments were analysed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Each experiment was self contained, which means all controls and comparable treatments were run on the same plate so inter experimental variations could be accepted. An individual threshold value was obtained for each experiment and the corresponding Ct values were used to find the relative transcript abundance compared to a beta actin control. To calculate a relative change the average of the 3 replicate Ct values for each sample was used as follows: This example shows *Per2* as the gene of interest and *beta actin* as the internal control.

1. Average Ct $_{(Per2)}$ – average Ct $_{(actin)}$ = ΔCt

2. ΔCt – the lowest ΔCt = ΔΔCt (so that 1 value = 0 and all the others a greater)

3. Relative change = $2^{-\Delta\Delta Ct}$ (2 is raised to the power – ΔΔCt, meaning that the ΔΔCt value, in 2, is now inverse and the value of 0 is now 1 and all the other are relatively lower)

4. The inverse, in 3, occurs as the Ct value is inversely proportional to transcript abundance and the lowest ΔΔCt value is actually the condition with the greatest amount of *Per2* transcript.

5. The relative change is then converted to a relative fold change by making the smallest value equal to 1 and all other values relative to that.

2.7 Immunohistochemistry

2.7.1 Tissue harvest and fixing in PFA or Bouin's solution

The intact tissue was fixed in order to maintain the structural integrity of the tissue, through the immobilisation of proteolytic enzymes and the cross-linking of cellular molecules. The tissue was removed from the animal and placed directly into 4% PFA (section 2.2.6.3) (all tissues) or Bouin's solution (Sigma, UK) (brain only) and left for a

minimum 48h, at 4^{o}C in order for the fixative to penetrate the tissue. The lung and kidney were then embedded in paraffin/wax (section 2.7.2).

2.7.2 Embedding in paraffin

After fixation with PFA, the kidney and lung tissue was embedded in paraffin wax to preserve the tissue for sectioning. Embedding was carried out using a Microm spin STP-120 processor (UK) according to the following automated protocol: The tissue was placed in an embedding cassette and put through the following solutions to dehydrate the tissue: 70% industrial methlyated spirits (IMS) 3.5h, 90% IMS 1h, 95% IMS 1h, 100% IMS 3 x 1h, xylene 1 x 30 min and 2 x 1h (for clearing) before going into paraffin (Lamb, UK) for 1 x 2h and 1 x 3h. All incubations were carried out at room temperature, except for those in paraffin, which was at 65°C. The tissue was then removed from the processor and placed in the wax holding bath (EC 350-1 wax embedder, Microm, UK). The tissue was then transferred to a molten wax filled base mould and put onto a cold surface to solidify. This was done to hold the tissue in place and protect it once it has been cut. This tissue was cut in continuous 5µm sections using a microtome, which were transferred to a 40°C water bath, where they were mounted onto slides and left to dry.

2.7.3 Free floating brain sections

The PFA and Bouin's fixed brains (section 2.7.1) were cut into 20µm sections on a freezing microtome (Leica) using a non-toxic histological mounting solution to fix the sections to the base. Sections were cut to include the hippocampus. The slices were then placed in 1 x PBS and washed repeatedly until the excess PFA and Bouin's solution was removed and stored at 4°C until stained.

2.7.4 Preparation of cells for immuno fluorescence

Rat-1 cells were cultured on a poly L-lysine coated cover slip, approximately 13mm diameter and 0.17mm thick, and placed in a 6-well plate in DMEM, as section 2.3.1. The poly L-lysine (Sigma, UK) was made up by filter sterilising 25mg lysine dissolved in 25ml deionised H_2O. The cover slip was sterilised in 100% ethanol and dried then coated in the poly lysine for 1h and dried. The medium was removed from the cells, washed in 1 x PBS and fixed in 100% methanol at -20°C for 10 min. The methanol was removed and the cells

re-hydrated in 1 x PBS. This step permeabilises the cells to allow antibody penetration. The cells were stained with the respective antibody.

2.7.5 Immunohistochemical (IHC) staining of tissue

The paraffin embedded sections were de-waxed in a xylene bath for 4 x 5 min and rehydrated in decreasing ethanol washes (100%, 95%, 50%, and 20%) then placed in tap H_2O. An antigen retrieval step was added using fresh citrate buffer (11mM sodium citrate with HCl added to pH 6, Sigma, UK). The sections were covered with the citrate buffer and placed in a microwave, approximately 900W, for 12 min and allowed to cool. This step does improve the quality of staining but is unknown how it works. At this stage the free floating sections were treated as follows, along with the de-waxed paraffin embedded tissue. The cooled sections were washed twice, for 10 min, in a 1 x (10mM) PBS solution (5 x stock of 0.7M NaCl, 13mM KCl, 50mM $NaHPO_4$ and 9mM KH_2PO_4) and once, for 10 min, in PBS supplemented with 0.3% triton-X (Sigma, UK) (PBS-X), while gently shaking. Endogenous peroxidase was blocked with 1% H_2O_2 solution (Sigma, UK) diluted in PBS-X for 30 min, while gently shaking. The 2 x PBS and 1 x PBS-X wash step, as previous, was repeated and the slides were blocked in 5% serum in PBS-X (blocking solution) for 2h. The serum used was the host species of the secondary antibody (table 2.7). The primary antibody was diluted accordingly in blocking solution, added to the sections and incubated at 4^oC for 16h, shaking gently. The primary antibody was removed and the section were washed 5 x in PBS-X for 5 min, then the appropriate biotinylated secondary antibody was added, diluted again in blocking solution, and incubated for 30 min at RT. The slides were washed 5 x, as before, and ABC complex was added (Vector labs), according to the manufacturer, and incubated for 30 min at RT. This step adds the HRP enzyme linked to streptavidin in order to visualise the antibody bound protein. The slides were washed 5 x as before and stained, for up to 12 min, with a 3-3' diaminobenzidine (DAB) kit (Vector labs) according to the manufacturers' protocol. The nucleus was stained with hematoxylin (Sigma, UK) for 10 sec, and then excess was washed off (lung and kidney only). The sections were dehydrated with increasing ethanol baths (20%, 50%, 95%, and 100%) then cleared with 100% xylene for 4 x 1 min. The sections were cover slipped (lung and kidney) or mounted onto slides and cover slipped (brain) and sealed with DEPEC mounting solution, and then imaged using an Olympus BX51 upright microscope with a x20 objective.

Antibody	Primary or Secondary	Raised against	Dilution used	To detect	Source
GR (M20)	primary	Rabbit	1:200 (IF) lung - 1:200 (IHC) kidney - 1:400 (IHC) brain - 1:2500 (IHC)		Santa Cruz, US
MR (c-19)	primary	Goat	1:200 (IF) lung - 1:200 (IHC) kidney - 1:200 (IHC) brain - 1:250 (IHC)		Santa Cruz, US
Biotinylated anti goat IgG	secondary	Horse	1:200 (IHC)	MR	Vector Labs
Biotinylated anti rabbit IgG	secondary	Goat	1:200 (IHC)	GR	Vector Labs
Texax red IgG anti rabbit	secondary	Goat	1:100 (IF)	GR	Vector Labs
Fluorescein (FITC) Anti-Goat IgG	secondary	Donkey	1:100 (IF)	MR	Jackson Labs

Table 2.7: Antibodies used for immunohistochemical and immunofluorescent staining: Immunofluorescence (IF) and immunohistochemical (IHC).

2.7.6 Immunofluorescent (IF) staining of cells

The methanol fixed cells (section 2.7.4) were washed 1 x PBS (section 2.7.5) and blocked for 30 min in 1 x PBS supplemented with 2% serum (host of the secondary antibody). The cells were washed 3 x 2 min in PBS and the primary antibody, diluted in PBS, was added (concentration shown in table 2.7) and incubated for 30 min at RT, shaking gently. The antibody was washed off with 3 x 5 min PBS-X (0.1% tween) and the secondary antibody was added diluted in 1 x PBS and incubated for 30 min at RT. The cells were washed for 3 x 5 min PBS-X to remove excess antibody and mounted onto a slide and sealed with a cover slip, using vector shield mounting medium (Vector labs), which contained the DNA stain 4',6-diamidino-2-phenylindole (DAPI). Images were collected on an Olympus BX51 upright microscope using a x*2*0 objective and captured using a Coolsnap ES camera (Photometrics). Specific filter sets for DAPI (excitation 365nm, emission 397nm); FITC (excitation 450-490, emission 515-565) and Texas red (excitation 546, emission 615) were used to visualise the stain. Images were then processed and analysed using ImageJ.

2.8 Statistical analysis and representation of PMT data

Statistics were carried out using GraphPad Prism software using the stated comparison analysis using either a One-Way or Two-way ANOVA with a Bonferroni post-hoc analysis, student t-test were also used to compare two sets of data, using a paired t-test to compare before and after treatments or unpaired t-test for individual treatments.. Error bars, where shown, are standard error of the mean (SEM). A number of data sets are represented by graphs showing the individual data points as well as the mean of the distribution. This was done to allow the reader to see the spread of data in data sets where n was greater than 3. Other data sets, where n=3 are represented by histograms ± SEM, as the error shown.

Chapter 3: Glucocorticoid regulation of the peripheral clock

3.1 Introduction

It is well known that Gcs affect the circadian clock (Balsalobre et al. 2000a) but little is known about the fundamental mechanisms involved in re-setting. In order to investigate this re-setting process it is important to dissect the role of two closely related receptors (GR and MR) in their action on the clock as CORT binds to both (De Kloet et al. 1998). To investigate the re-setting mechanism I adopted two different model systems, cell lines and primary tissue. The advantage to using a cell line is that one can manipulate the genetic pathways and insert luciferase driven reporters. The disadvantage, as will be seen in this chapter is that such cell lines are not representative of normal physiology, and for this reason I have adopted the use of *ex-vivo* lung slices. In this thesis I have used lung tissue from the PER2::luc mouse, which is a protein reporter but also m*Per2*::luc, m*Per1 2500*::luc, m*Per1 600*::luc and m*Per1 mGRE (2500)*::luc with a mutated GRE, which are all transcriptional reporters. Here and in subsequent chapters, I have used CORT, rather than DEX, as CORT is endogenous to the rodent whereas DEX has been designed to have a long half life (CORT 25 min, DEX 200 min (Cevc and Blume 2004; Sainio et al. 1988)) and therefore does not represent the true endogenous response. In this chapter I have addressed three characteristics of the effect of CORT on the clock: period, phase shifting, amplitude response and sustained high amplitude oscillation, as described in the model below (Fig 3.1). Throughout this thesis I have used the RAP software analysis (section 2.1.4) to define the phase and period changes after treatment (Fig 3.1), where CORT treatment is shown by the arrow (a) and the initial amplitude response (b). RAP calculated the period over 3-4 cycles from half way between the peak and trough of the CORT-induced amplitude rise (c). The phase shifting effects are defined as time of the subsequent peak following CORT treatment (g) compared to the DMSO peak (h). The phase of DMSO was calculated as in Fig 3.4 and section 2.2.5.3. The period is calculated over the 3-4 cycles (f). To calculate an effect on chronic amplitude, the relative change in peak to trough height (d versus e) was measured and compared to DMSO controls (d' versus e').

Gc acts as a potent synchronising factor for peripheral circadian clocks and also induces high amplitude oscillations. A functional GRE which is known to respond to Gc stimulation has been confirmed in the *Per1* promoter (1800bp upstream from the transcriptional start site) and in this model mutation of this GRE has been shown to inhibit 95% of the Gc activation (Yamamoto et al. 2005). This GRE was the only confirmed GRE in the clock gene promoters, but bioinformatic predictions have suggested other potential

candidates, as yet unconfirmed (Reddy et al. 2007). Recently, *Per2* has been shown to contain a functional GRE but in this case not in the promoter region (within the ninth intron) (So et al. 2009), and other reports have suggested that *Rev-erb α* contains a negative GRE (nGRE) whereby the GR (in the presence of Gc) binds to the promoter and down regulates transcription (Torra et al. 2000).

The GR and MR are expressed together in many central neurons, such as the hippocampus (Sarabdjitsingh et al. 2009) and peripheral regions, such as the lung, kidney, liver and heart (Gibbs et al. 2009; Yang et al. 2006). However, little attention has been given to the potential influence that the MR has on circadian regulation, and its interaction with GR on cloned response elements (GRE). Furthermore, hepatic mRNA cycles for the clock output gene *mDbp* appear to be similar after DEX treatment in tissue derived from WT mice and mice bearing a conditional mutation in the hepatic GR (Balsalobre et al. 2000a). DEX treatment resulted in a 3-4h phase delay in other tissues (heart and kidney) in both WT and mutant mice. Also DEX failed to induce *mPer1* mRNA expression in a liver specific GR mutant mouse, where the GR did not functionally express. Together these data suggests that the GR is required both for the induction and phase shifting effects of DEX.

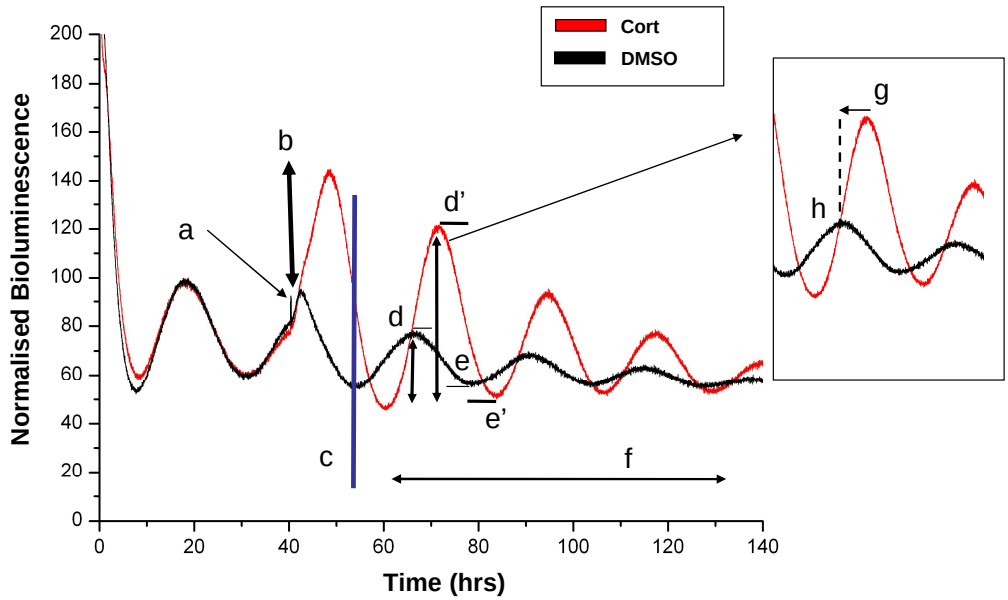

Figure 3.1: Schematic model of PMT analysis: Representative oscillation of PER2::luc lung section and treated with CORT (red) and DMSO (black) to demonstrate where key measurements were taken from. The slices were treated at point (CT12) (a). The initial CORT-induced amplitude rise was measured as (b) with the sustained amplitude measured as the difference between peak and trough bioluminescence for DMSO (d to e) and for CORT (d' to e'). The post-treatment period was taken over the course of 3-4 oscillations (f) originating after any induced amplitude response (c). The phase change is shown inset

as the difference between the CORT peak and the DMSO peak (g-h) taken from the first cycle after the CORT-induced amplitude rise.

3.2 Aims

- To characterise responses of PER2::luc primary lung tissue and Rat1 cell-line reporters to the endogenous Gc, CORT.
- To define the role of the GR and MR in circadian responses.
- To assess the role of the GRE in a clock gene promoter in the re-setting responses to CORT.
- To develop an *in-vitro* model which mimics the endogenous ultradian pulses of CORT, and thereby characterise the effects of physiologic responses to active Gc on the pulmonary clock.

3.3 Results

3.3.1 Vehicle treatment of lung sections

Prior to investigating the action of Gcs on the peripheral oscillator it was important to characterise any effect that the vehicle had on the output, as this could itself affect the phase and period. Treatment of the PER2::luc fusion protein reporter (section 2.1.2.1) with DMSO or recording media induced a 3.5-4.5h advance in the period of the pulmonary oscillation compared to untreated slices (Fig 3.2). There was no difference between either DMSO or recording media alone. The outcome of these experiments is that almost any perturbation of the tissue model causes a phase shift. For this reason all subsequent comparisons are made with reference to the DMSO control.

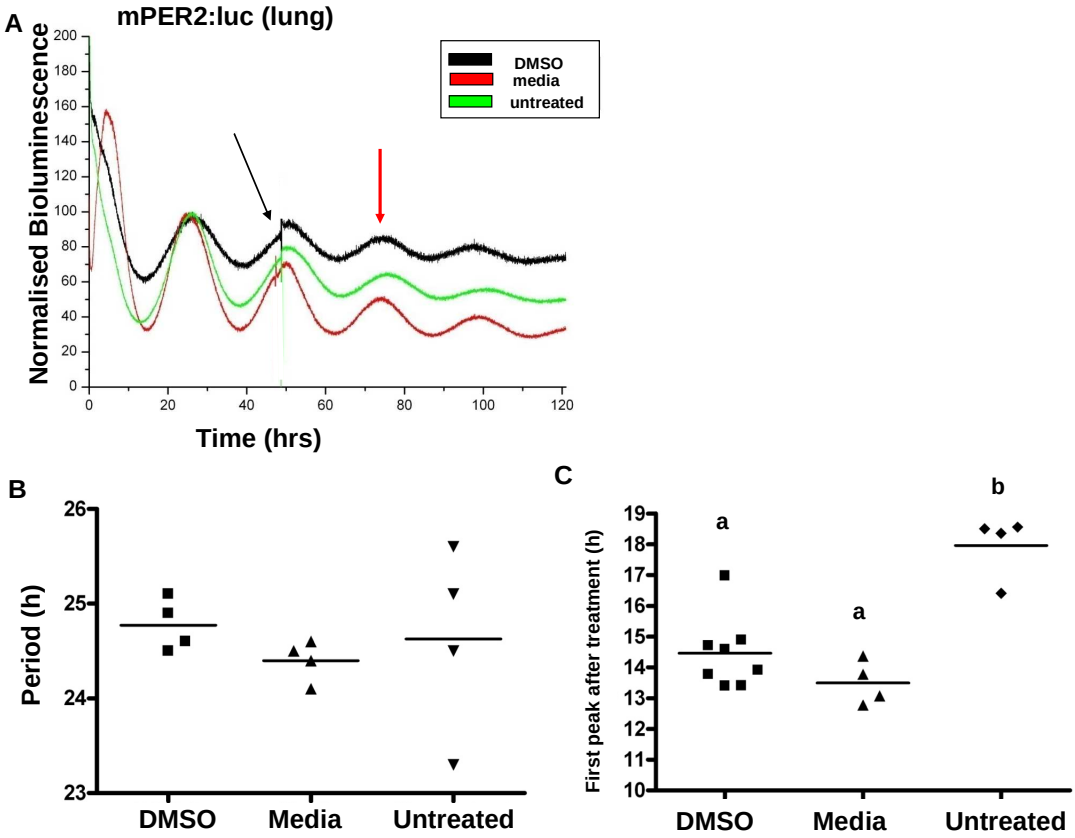

Figure 3.2: DMSO and media effect on the PER2::luc lung oscillation: The representative PER2::luc oscillations are shown in (A) with the black arrow showing the time of treatment (CT12); DMSO (black), recording media (red) and untreated slice (green). The period lengths and time to first peak after treatment (red arrow) are shown in (B) and (C) respectively. Dots represent individual repeats. Statistical significance is shown using a One-Way ANOVA (in B P=0.46, in C P<0.0001) with a Bonferroni post-hoc analysis, (a) versus (b) P< 0.001.

3.3.2 Action of CORT on the pulmonary circadian clock

The action of CORT on the PER2::luc pulmonary clock reporter was first addressed by treating lung slices with different concentrations of CORT (given at the peak of the PER2::luc rhythm, CT12 in Fig 3.3) and recording subsequent phase and amplitude changes. There was a dose dependent delay in phase across 4 log concentrations of CORT (P= 0.0177, Fig 3.3b). A representative example is shown in (Fig 3.3a), but with no increased effect of amplitude above 0.1µM (Fig 3.3c, P= 0). At 0.01µM (10nM) a 4h phase delay was seen without any effect on amplitude (Fig 3.3a, red line). There is a chronic amplitude effect at 0.1µM but not at 0.01µM (P <0.0001, Fig 3.3d). The lower dose (0.01µM) is equal to approximately the Ec50 of CORT on the GR and 10-fold lower for the MR. Concentrations of 1µM and above maybe regarded as supra-physiological

(Windle et al. 1998a). A PRC to 0.1µM CORT was plotted, with a DMSO control, (section 2.2.5.3) over the circadian cycle resulting in large phase advances, with a maximal response from CT12 to CT18 showing an advance between 10h and 13h. Likewise there were large delays, up to 5h, between CT6 and CT10 with the time between CT10 and CT12 being the transition between the advance and delay phase (Fig 3.4). In the DMSO there is a significant interaction between the time of treatment (P = 0.0085, Fig 3.4c) which leads to approximately 3h phase advances and 1h phase delay. Following on from this PRC it would have been a good idea to investigate the time between CT10 and CT12 (i.e. the time that switches between the delay and advance phase) more closely. This could have involved treatments every 15min between these times or at the very least a treatment at CT11 as this was the time all subsequent treatments were made (i.e. CT12, the peak of PER2::luc rhythm).

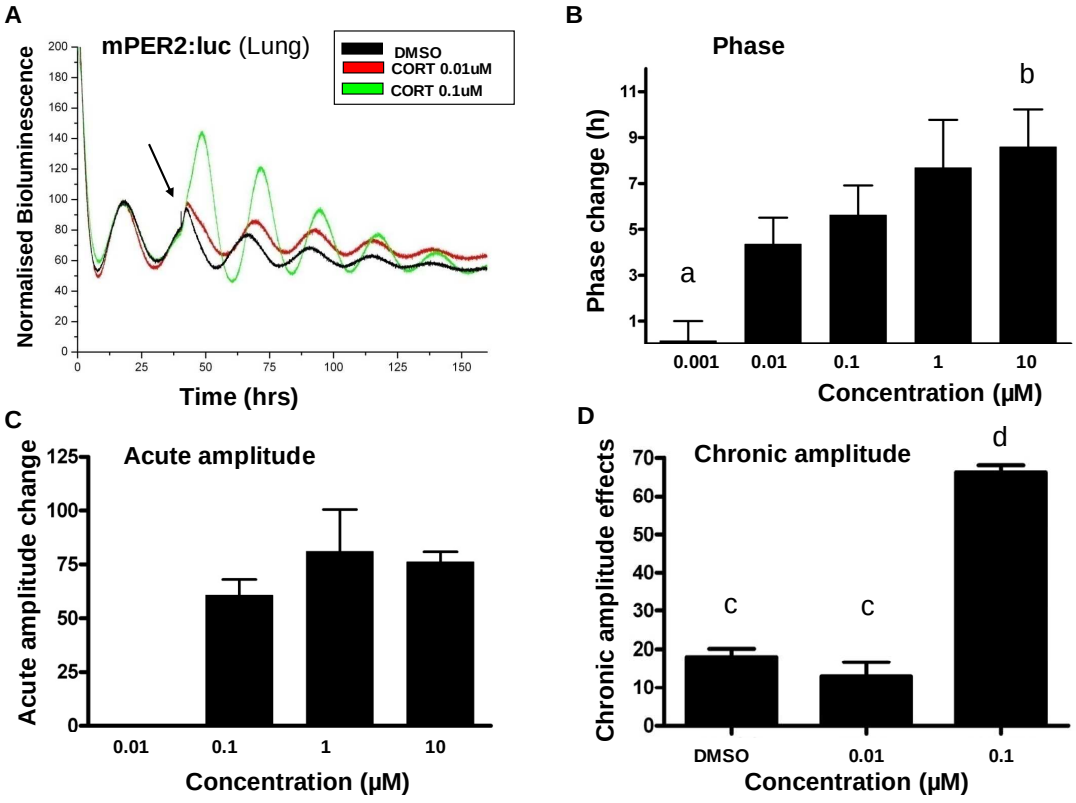

Figure 3.3: Phase and amplitude changes of PER2::luc lung slice in response to CORT: A representative lung oscillation in response to CORT (DMSO in black; 0.1µM CORT green; 0.01µM CORT red) (A) together with phase (B) acute amplitude (C) and sustained (chronic) amplitude changes (D). The phase change is relative to DMSO and the amplitude calculations are as described in Fig 3.1. Treatment time is indicated by the arrow (CT12). Error bars are SEM with n = 3. Statistical analysis is by One-Way ANOVA (in B: P= 0.0177, in C: P=0.0024, in D: P <0.0001) with a Bonferroni post-hoc analysis. In B: (a) versus (b) P<0.05. In C: there is no significant difference between the concentrations which induced an amplitude response, yet there was a difference P<0.05 between 0.1, 1 and 10µM and 0.01µM (as 0.01µM was 0). In D: (c) versus (d) P<0.001.

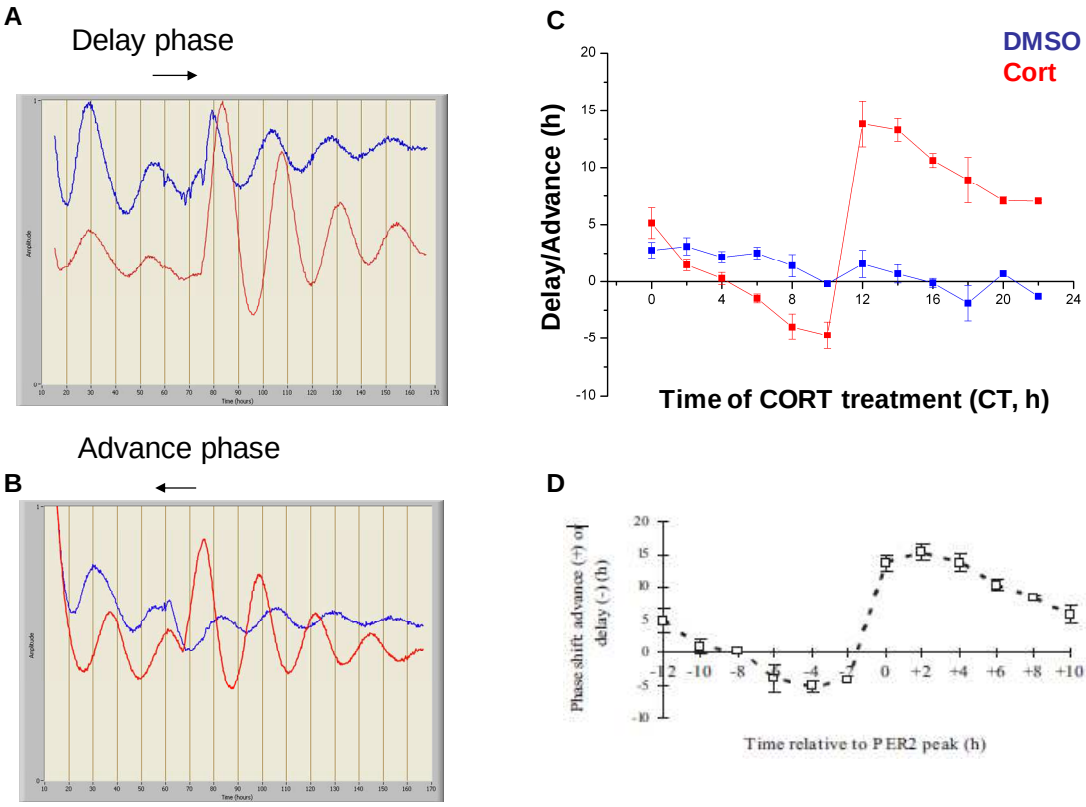

Figure 3.4: CORT induced lung PRC to PER2::luc lung slice: The CORT (0.1µM) induced delay (A) and advance (B) shows the response to CORT (red) and DMSO (blue). The resulting PRC, shown with treatments every 2h, (C) shows DMSO (blue) and CORT (red). A similar PRC to DEX in the pulmonary PER2::luc reporter is shown (D) (Gibbs et al. 2009) depicting both advances and delays of similar magnitude to that in (C). The PRC time points were completed with the tissue from 1 animal. Statistical analysis by One-Way ANOVA revealed an interaction between the time DMSO treatment (P=0.0085) as well as between CORT treatment (P<0.0001). Error bars are SEM, n=3 animals. Lung slices were prepared as in section 2.2.4.

Subsequent analysis revealed that the presence of CORT in the media caused a shortening of period and this did not depend on the phase of the cycle at which it was added, (Fig 3.5). This shortening of period is in direct contrast to the vehicle control where no general pattern emerged following DMSO treatment. Taken together, these data suggest that CORT induces both amplitude effects in the circadian oscillation, phase shifting and period effects, in a dose dependent manner.

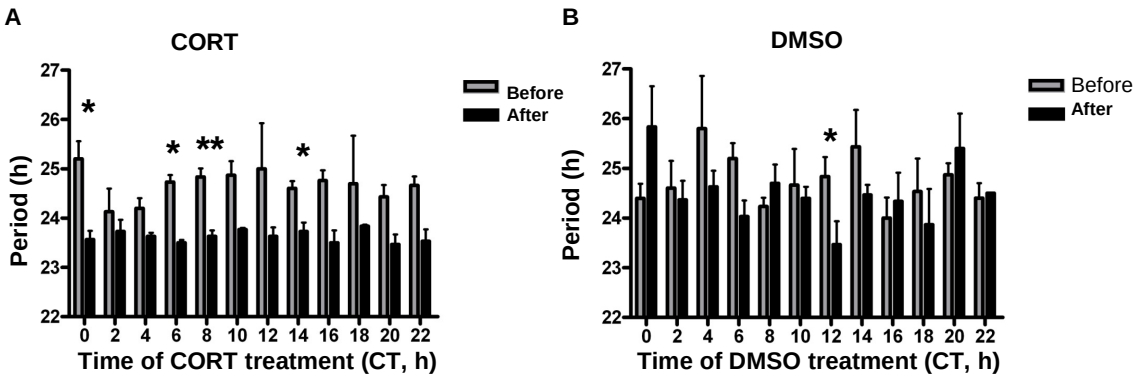

Figure 3.5: CORT induced period change: The period of the mPER2::luc lung slice was compared before (grey bars) and after (black bars) treatment with either 0.1µMCORT (A) or DMSO (B). Error bars are SEM, n=3 animals. Statistical analysis was by a paired student's t-test *P<0.05, **P<0.01, pre versus post treatment.

3.3.3 The steroid receptors GR and MR and their influence on circadian timing

3.3.3.1 Validating of the model systems

In order to define the relative role of the GR and MR I first set out to see if they were expressed in primary pulmonary tissue and Rat-1 cells using RT-PCR. Using GAPDH as an internal control I amplified this in parallel with the GR and MR transcripts. As a comparison I have also used kidney tissue as a control since this organ is one of the main targets for MR action. The data show clearly that both MR and GR are expressed in the lung and in Rat-1 cells (Fig 3.6). The nature of the RT-PCR process means that absolute expression values using different primer sets cannot be directly compared, so the data here are relative. In order to define protein expression I undertook immunohistochemical and immunofluorescence staining of primary tissue and Rat-1 cells (Fig 3.7 and 3.8 respectively). These studies show clearly that GR and MR strongly express in the cells surrounding the bronchial epithelia, presumed to be Clara cells (Gibbs et al. 2009). Furthermore, the GR and MR are expressed in other cell types in the alveolar tissue presumed to be Type II pneumocytes Ulich et al. (1994) (Fig 3.7). Using these antibodies it is clear that both receptors are strongly expressed in the lung. As a positive control for the MR antibody it can also be seen that it is strongly expressed in the renal tubules. The primary negative controls are also shown in Fig 3.7. Further antibody validation was carried out on the hippocampus, an area well characterised in the expression of both GR and MR, (appendix C) as the anti-MR antibody did not work in an immuno blot

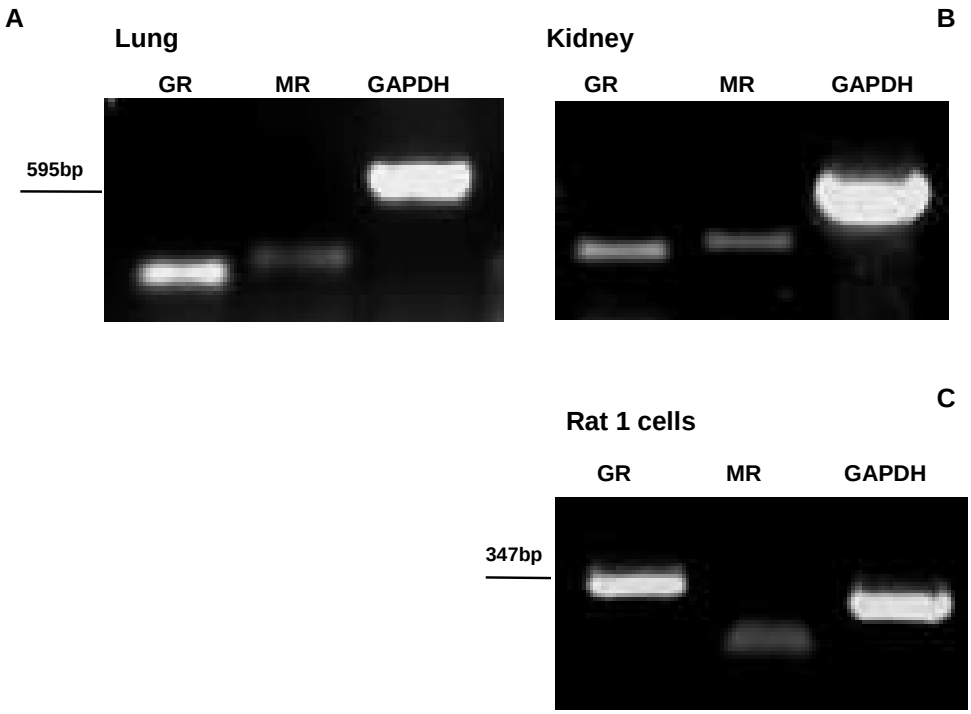

Figure 3.6: mRNA transcript expression of GR and MR: GR, MR and GAPDH transcripts were amplified from the mouse lung (A) and kidney (B) and the Rat-1 cells (C) (different primers sets were used for murine lung and Rat-1 transcript expression). The data are representative expression patterns of n = 3.

In the Rat-1 cells nuclear staining for the GR (red) and MR (green) fluorescence clearly overlays with nuclear DAPI staining (blue) Fig 3.8. In conclusion, the RNA transcript and protein products for GR and MR are expressed in both pulmonary tissue and Rat-1 cells.

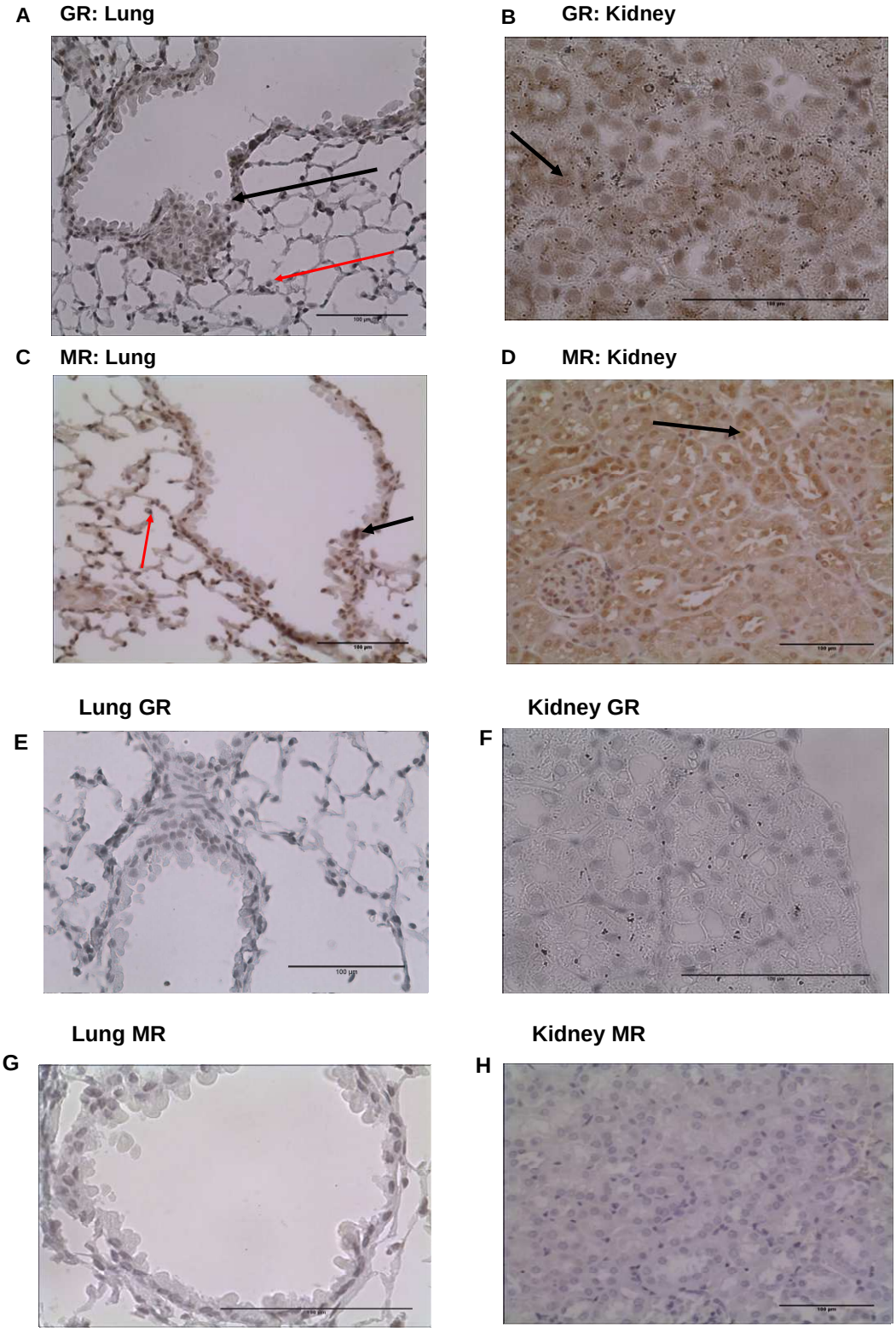

Figure 3.7: Immunohistochemical staining for GR and MR: Both GR (A and B) and MR (C and D) are expressed in the lung (A and C) and kidney (B and D). The black arrows point to areas of high expression; bronchioles in the lung, which include Clara cells, (Gibbs et al. 2009), and tubules in the kidney. The red arrows mark presumptive Type II pneumocytes (Ulich et al. 1994). Primary antibody negative controls are shown for GR and MR in the lung (E and G respectively) and for GR and MR in the kidney (F and H

respectively). The positive cells for GR and MR appear brown and the nuclei appear blue. The scale bars represent 100µm.

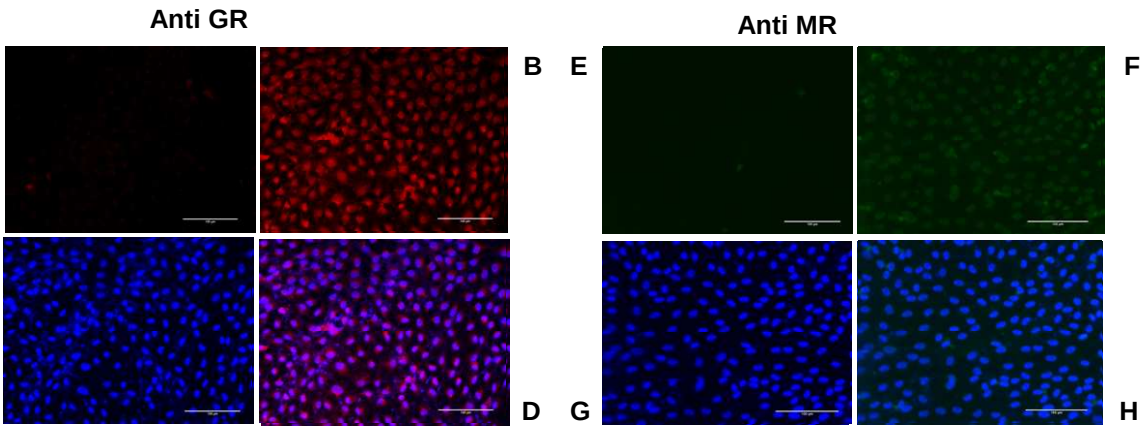

Figure 3.8: Immunofluorescent staining of Rat-1 cells for GR and MR: Panels A-D show GR (red) and panels E-H show MR (green) staining. The GR expression is shown in (B) and the MR expression is in (F). Panels (D) and (H) show the GR and MR primary antibody, respectively, with DAPI (C) and blue overlay. The primary antibody negative controls are panels (A) and (E). The scale bars represent 100µm.

3.3.3.2 The relative contributions of GR and MR to CORT meditated phase re-setting

In order to define the relative roles of GR and MR I next used well established antagonists for these two receptors. In the case of RU486 (RU, GR antagonist) this drug is known to have partial agonistic effects on the GR receptor (Stevens et al. 2003) which increases p-ser^{211} (Wang et al. 2002) but is not considered to act on the MR. The MR antagonist spironolactone (spir) is not known to have partial agonistic effects but at 8µM (Ec50) and above spir engages the GR (as described in section 2.2.5.1). For this reason I have used 1µM spir in the experiments described below. For RU I also used 1µM as this dose has previously been reported to inhibit both primary tissue and cell lines.

RU significantly inhibited CORT-induced phase shifts in both the primary pulmonary tissue reporter and Rat-1 cells. In both Rat-1 and pulmonary tissue RU induced phase shifts which may be attributed to the partial agonistic effects of this compound (Fig 3.9c). However, in both systems the phase shifting effects of CORT were completely blocked by RU (Fig 3.9). Furthermore, RU blocked the CORT induced increase in luciferase expression in the pulmonary tissue and greatly attenuated the amplitude of the subsequent circadian oscillations in both tissue and Rat-1 cells (Fig 3.9d).

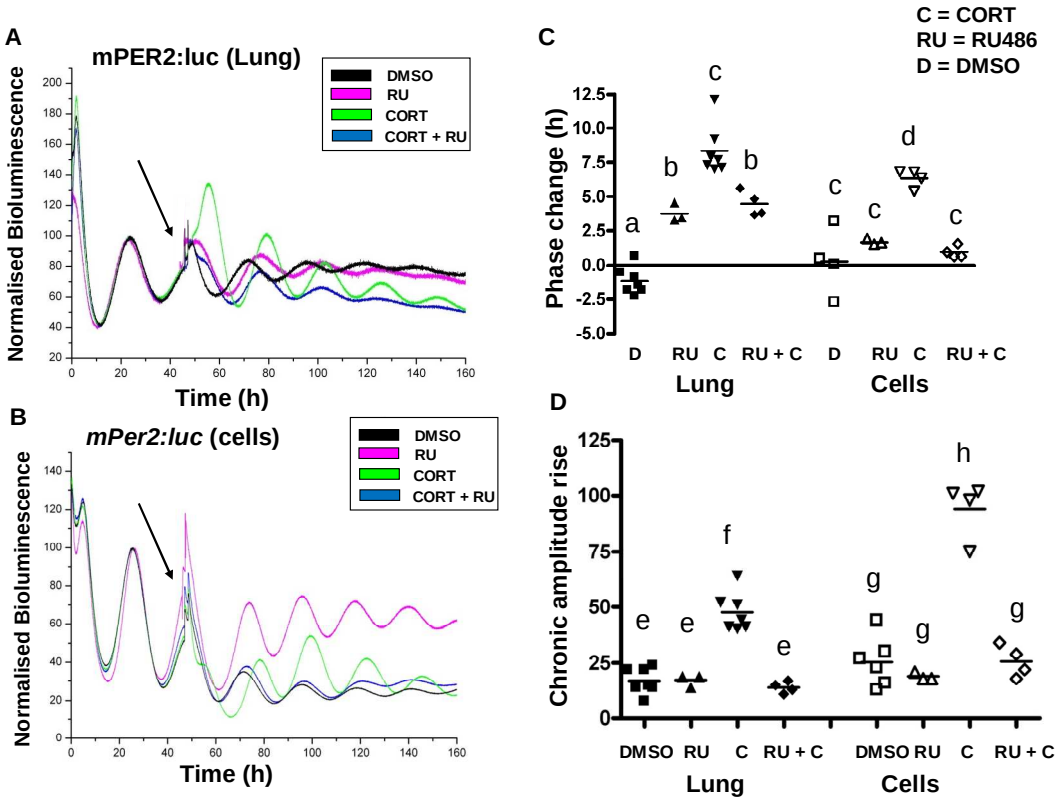

Figure 3.9 The relative contribution of GR to the CORT effect on the clock: Representative oscillations for the mPER2::luc lung (A) and *mPer2*::luc cellular (B) circadian reporters. The oscillations for CORT (C) treatment are shown in green; DMSO in black; RU with CORT (RU + C) in blue and RU only in magenta. The arrow shows the time of treatment (CT12). The resulting phase change is shown in (C) and the chronic amplitude rise (D). Each dot is a single experiment and the statistics are a One-way ANOVA (in both C and D: P< 0.0002) with a Bonferroni post-hoc analysis, (a) versus (b); (c) versus (d); (e) versus (f) and (g) versus (h) P<0.001.

The experimental procedures used to obtain data shown in Fig 3.9 were repeated using the MR antagonist. In both the pulmonary tissue and cell line reporters, antagonism of the MR had little effect on reducing the CORT-induced phase effects (Fig 3.10). However, the MR does have an effect as MR antagonism, with spir, blocked the CORT-induced luciferase increase, in the lung, seen immediately after CORT treatment (Fig 3.10a). MR antagonism also significantly attenuated the chronic amplitude of the subsequent circadian oscillation, in the lung but not the Rat-1 cell reporter (Fig 3.10d) in a similar manner to GR antagonism (Fig 3.9d). Thus phase-shifting effects of CORT appear to be mediated largely through the GR but the amplitude is both GR and MR mediated. In addition there is a significant difference between the phase effects of RU and spironolactone administration alone, in the PER2::luc lung section (student t-test P<0.002) but not in the *mPer2*::luc cell line reporter (student t-test P=0.47). Therefore, the effects of CORT are three fold: (1)

phase shifting; (2) strong initial rise in luciferase expression; (3) substantial high amplitude oscillations compared to DMSO. Furthermore, both GR and MR antagonism blocked the initial rise in luciferase expression and dampened the CORT-induced high amplitude oscillations, in the pulmonary reporter, but only the GR antagonism inhibited the CORT-induced phase change.

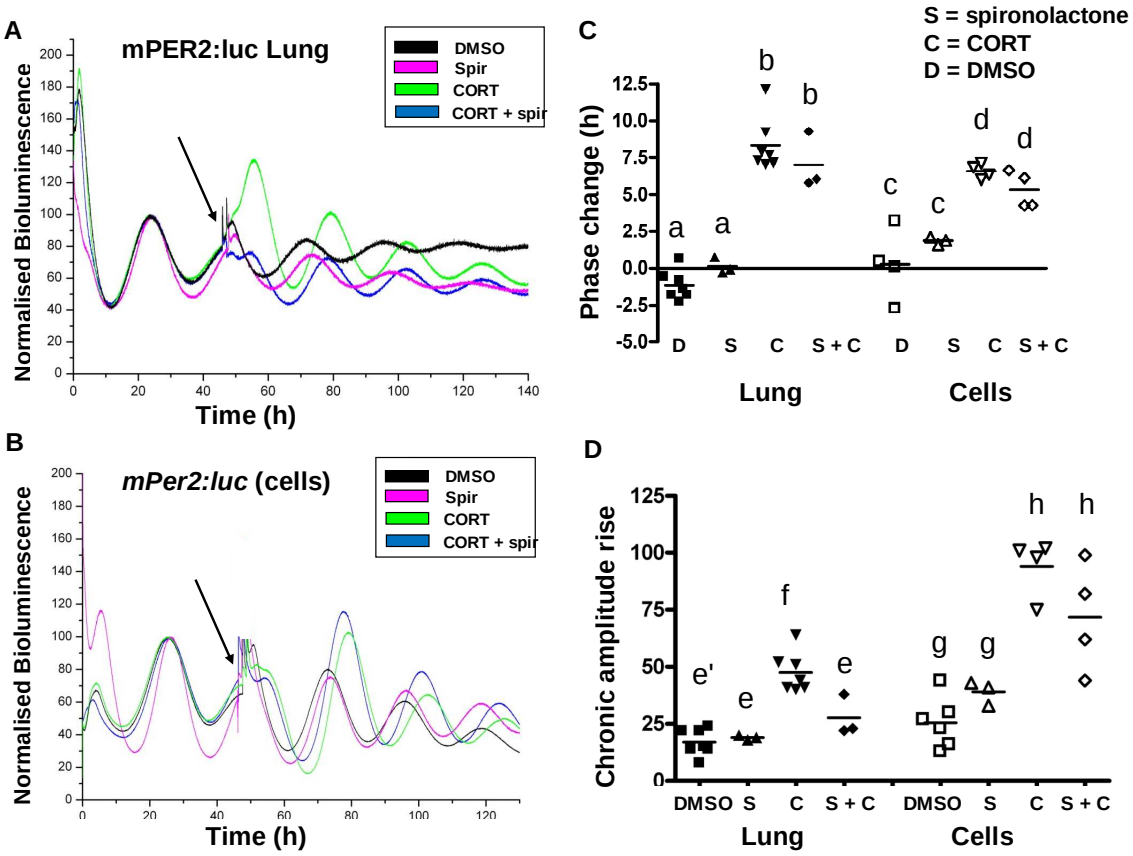

Figure 3.10: The relative contribution of MR to the CORT effect on the clock: Representative oscillations for the mPER2::luc lung (A) and *mPer2*::luc cellular (B) circadian reporters. The oscillations for CORT (C) treatment are shown in green; DMSO in black; spir with CORT (S+C) in blue and spir (S) only in magenta. The arrow shows the time of treatment (CT12). The resulting phase change is shown in (C) with the chronic amplitude rise (D). Each dot is a single experiment and the statistics are a One-way ANOVA (in C and D: $P<0.0002$) with a Bonferroni post-hoc analysis, (a) versus (b) and (c) versus (d) $P<0.001$; (e) versus (f) $P<0.01$ and (e') versus (f) $P<0.001$; (g) versus (h) $P<0.01$.

In addition to the phase and amplitude effects of CORT on the reporter constructs, period effects were also seen, as in Fig 3.5. The period shortening, as a direct result of CORT, is restricted to the lung as the Rat-1 cells do not show the same response (Fig 3.11). In the pulmonary tissue, CORT significantly shortened the period by approximately 2h relative to the RU treatment alone and the period shortening effects of CORT were significantly reduced when RU and CORT were co-administered (Fig 3.11a). However, there was no

significant interaction with DMSO control. However, no statistical differences were observed for MR antagonism with spir, in either the pulmonary or Rat-1 reporters. Together these data suggest that CORT can shorten the period of the pulmonary reporter and this effect is GR rather than MR mediated, as MR antagonism had no effect.

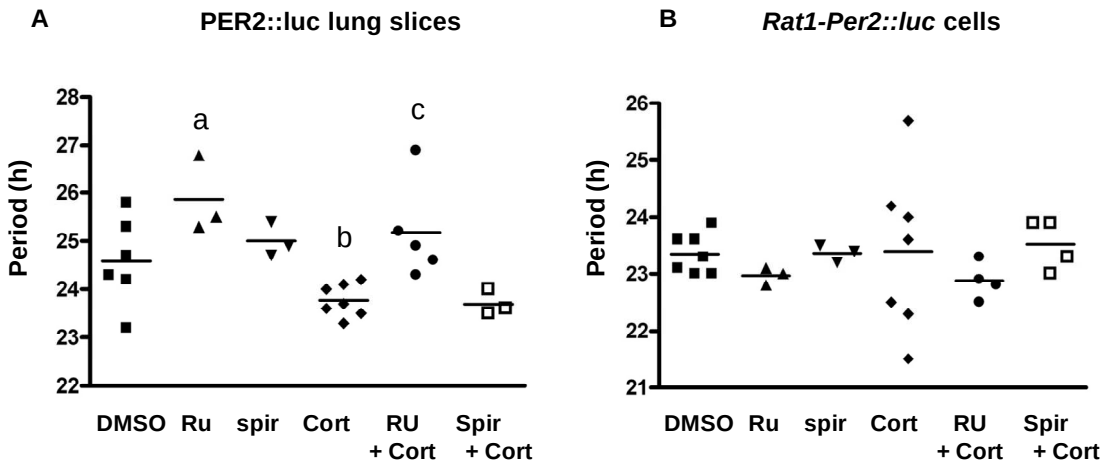

Figure 3.11: CORT induced period effects and the roles of GR and MR: The period analysis for the mPER2::luc lung reporter (A) treated with CORT or the respective GR or MR antagonist (RU or spir respectively) or the Rat-1 *mPer2*::luc cell reporter (B). Statistical analysis was by One-Way ANOVA (in A: $P<0.002$, in B: $P = 0.82$) with a Bonferroni post-hoc analysis, (a) versus (b) $P<0.01$, (b) versus (c) $P<0.05$ other comparisons are not significant. Dots represent individual experiments.

3.3.4 The effect of CORT on the *mPer1* GRE

The *Per1* promoter is the only clock gene to contain a functional GRE (Yamamato et al., 2005), therefore, a WT (section 2.3.3) and mutated form of the *mPer1* GRE (section 2.3.7 and Fig 3.12a), driving the luciferase reporter, was established in a Rat-1 cell line and used to characterise the effects of CORT on the phase and period of the oscillation. Comparison of the full length (WT) and mutated GRE is shown in Fig 3.12b, which also revealed that the CRE (induced in response to forskolin) remained intact in the mutated GRE construct. Using an acute response assay measuring bioluminescence 4h after Gc treatment it was seen that the mutation of the GRE greatly attenuated the amplitude of the response to both CORT and 2 novel non-steroidal GR ligands (67a and 69a) (Fig 3.12), which are described in more detail later (section 3.3.5 and Fig 3.17). In this assay, as in these described below, the mutation of the GRE did not impair the response to forskolin. The acute responses to CORT, or the GR ligands, are profoundly affected by the loss of the GRE.

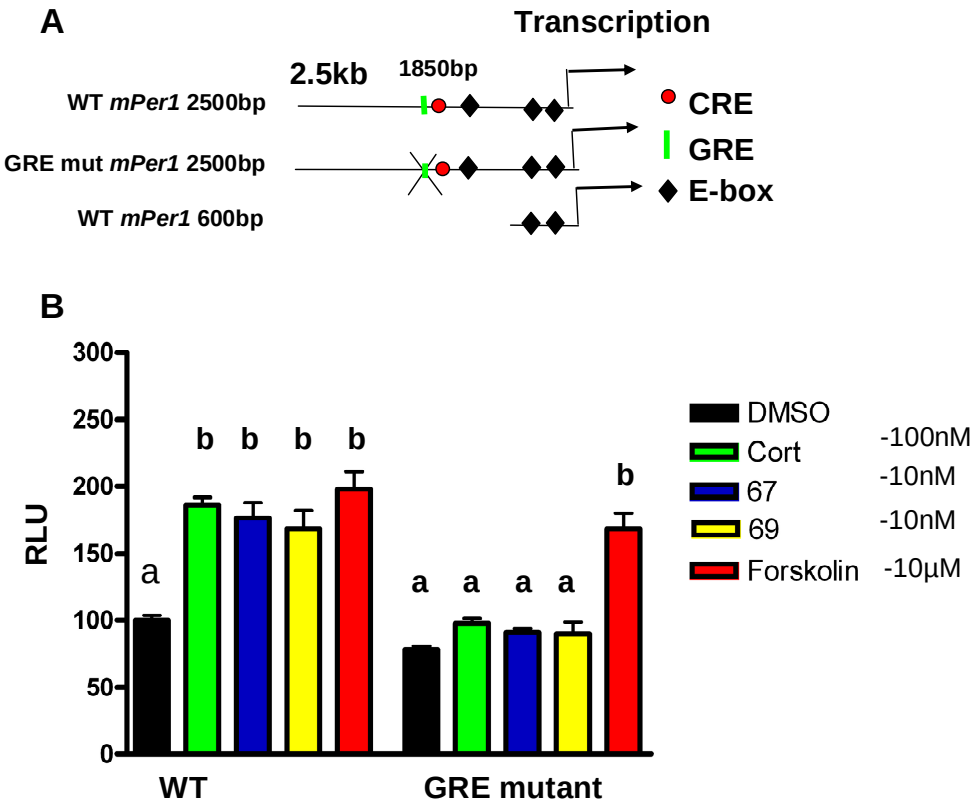

Figure 3.12: *mPer1* WT versus mutant GRE luciferase assay: (A) shows a schematic depicting the *mPer1* promoter 2500bp upstream of the transcriptional start site, showing the relative positions of the CRE (-1807bp-red), GRE (-1933bp-green) and E-boxes (-226bp, -589bp, -1335bp-black). Also represented is the *Per1* mutant GRE construct (also 2500bp upstream of the transcriptional start site) and the *Per1* 600 construct. The black cross represents the mutated GRE construct (section 2.3.7) which leaves the CRE intact. WT or mutant GRE *mPer1::luc* 2500 constructs were treated with either CORT, GR agonists (67a or 69a) or forskolin relative to vehicle control (DMSO) 4h before lysis and analysis (B). Units are relative luciferase units (RLU) normalised to WT vehicle. Statistical analysis is by Two-way ANOVA (for treatment and construct $P<0.0007$) with a Bonferroni post-hoc analysis. (a) versus (b) $P<0.001$ versus relative WT counterpart or DMSO control. Error bars are SEM with n=6.

The comparison of the mutated and WT forms reveals that mutation of the GRE causes a marked loss of circadian rhythmicity in the reporter cells (Fig 3.13 and 3.14 (a) compared to (b), black line). Indeed, a large number of replicates were needed to be run for cells containing the mutant GRE in order to obtain a measurable circadian period, approximately 50% of the time it was not possible to detect an oscillation in cells containing the mutant GRE. For replicates in which it was possible to detect period, phase and sustained amplitude rise there was no statistical significant effect of the loss of GRE on period, either in the mutated or truncated constructs (Fig 3.13b, 3.14b and summarised 3.16d). Following CORT treatment similar amplitude oscillations were observed in both

WT and mutant GRE reporters (Fig 3.15c). The loss of the GRE did not impair responses to forskolin treatment, similar to that seen in Fig 3.12. Truncation of the *mPer1* promoter to 600bp (which eliminated the GRE) caused significant attenuation of the circadian oscillation, prior to treatment (Fig 3.14c), despite retaining 2 of the 3 E-box elements, yet treatment with CORT, forskolin or DMSO stimulated an oscillation. In response to CORT treatment the truncated/mutated promoter element showed greatly attenuated phase shifts (Fig 3.15a), yet the phase was unaffected by forskolin treatment (Fig 3.15b).

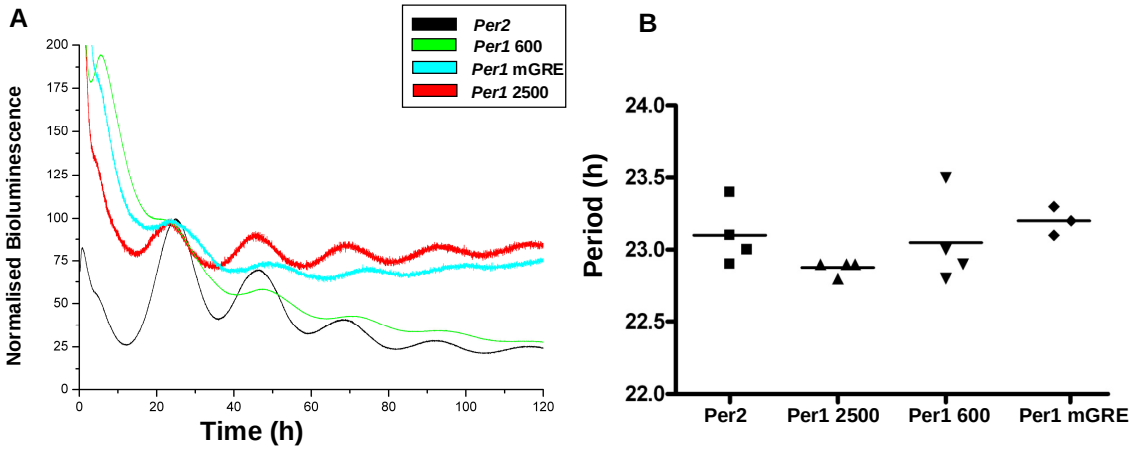

Figure 3.13: The contribution of the GRE to the period of the endogenous oscillation in the Rat1 cell line: The representative endogenous (untreated) oscillations are shown in (A) with the resulting periods analysed (B). Dots represent a single experiment. Analysis was carried using a one-way ANOVA (P= 0.25) but showed no significance between groups.

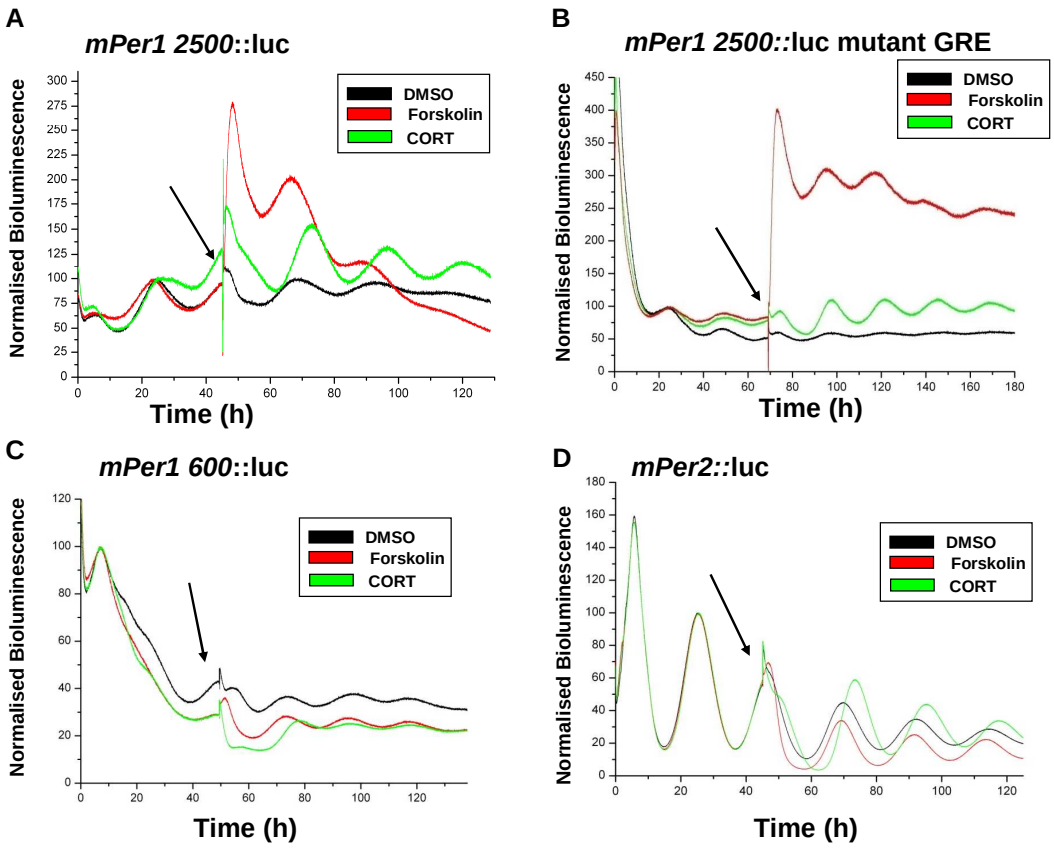

Figure 3.14: CORT and the *mPer1*::luc GRE: WT *Per1 2500* (A), mutant GRE (B), *Per1::luc 600* (C) and *Per2::luc* (D) representative oscillations are shown with CORT (green), forskolin (red) and vehicle (black). The arrow shows the time of treatment (CT12).

In contrast to the acute responses seen in Fig 3.12, chronic treatments with CORT sustain similar amplitude oscillations in both WT and GRE mutant cells (Fig 3.15c). This effect may be attributable to an indirect effect of CORT or other elements of the clockwork which then act on the 2500kp (WT or mutant) construct. Although earlier studies suggested that an additional GRE at -1226bp upstream of the transcriptional start site in the promoter is not important for the effect of DEX. It remains possible that this may contribute to the similar chronic effect that CORT has on the amplitude of the both WT and mutated GRE oscillation. Although the data might imply that the GRE element alone in the absence of CORT (Fig 3.13a and 3.14b) contributes to the amplitude effect on the circadian clock, it is possible that the random insertion of this reporter in to the genome is a contributing factor and it may be the case that the GRE itself is not contributing to these effects. There is no difference in period length with *Per2*::luc, *Per1 2500*::luc or *Per1 600*::luc after vehicle, CORT or forskolin treatment (Fig 3.16), yet there is a significant

shortening of period after forskolin treatment with the *Per1*::luc mGRE construct (Fig 3.16d).

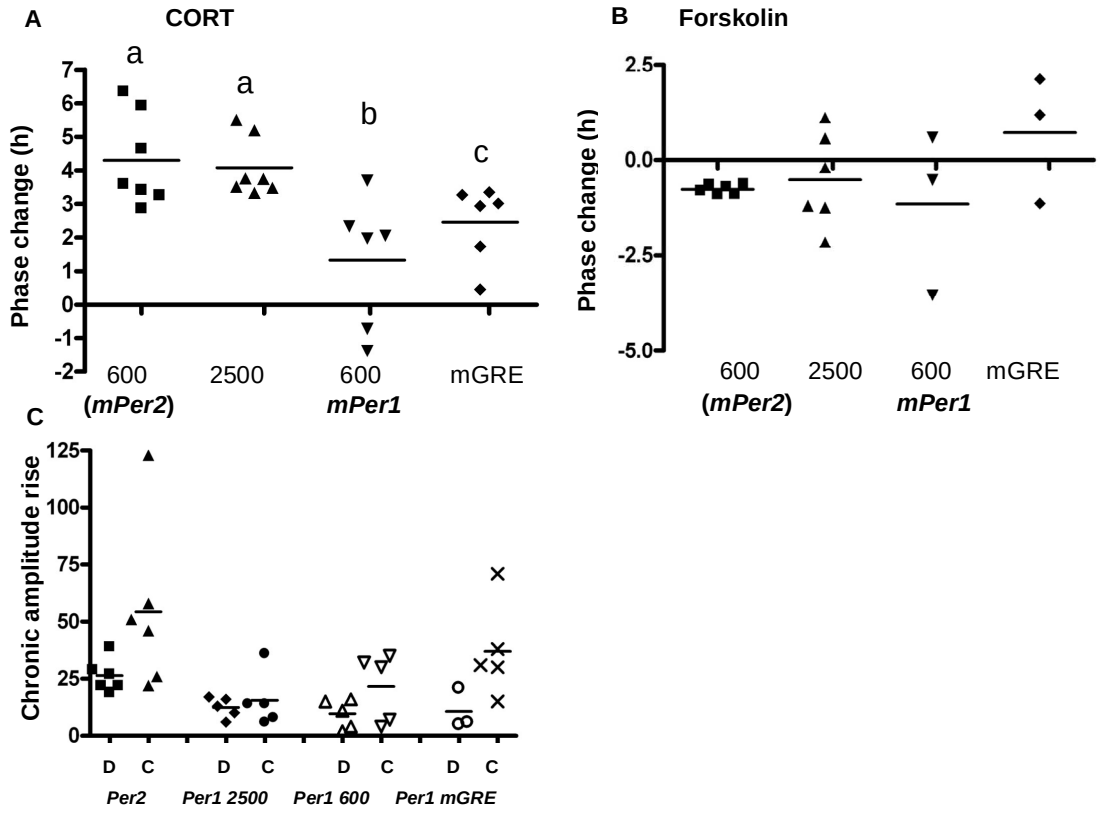

Figure 3.15: Phase effects of CORT and forskolin on the endogenous GRE: The CORT induced phase change (A) and the forskolin induced change (B) on the cellular promoter reporters with and without an endogenous GRE. The chronic amplitude rise is shown (C), DMSO (D) and CORT (C). Dots represent a single experiment. Analysis was carried using a one-way ANOVA (A: $P<0.0005$; B: $P=0.74$; C $P=0.0034$) with a Bonferroni post-hoc analysis, (a) versus (b) $P<0.01$, (c) versus (a) $P<0.05$.

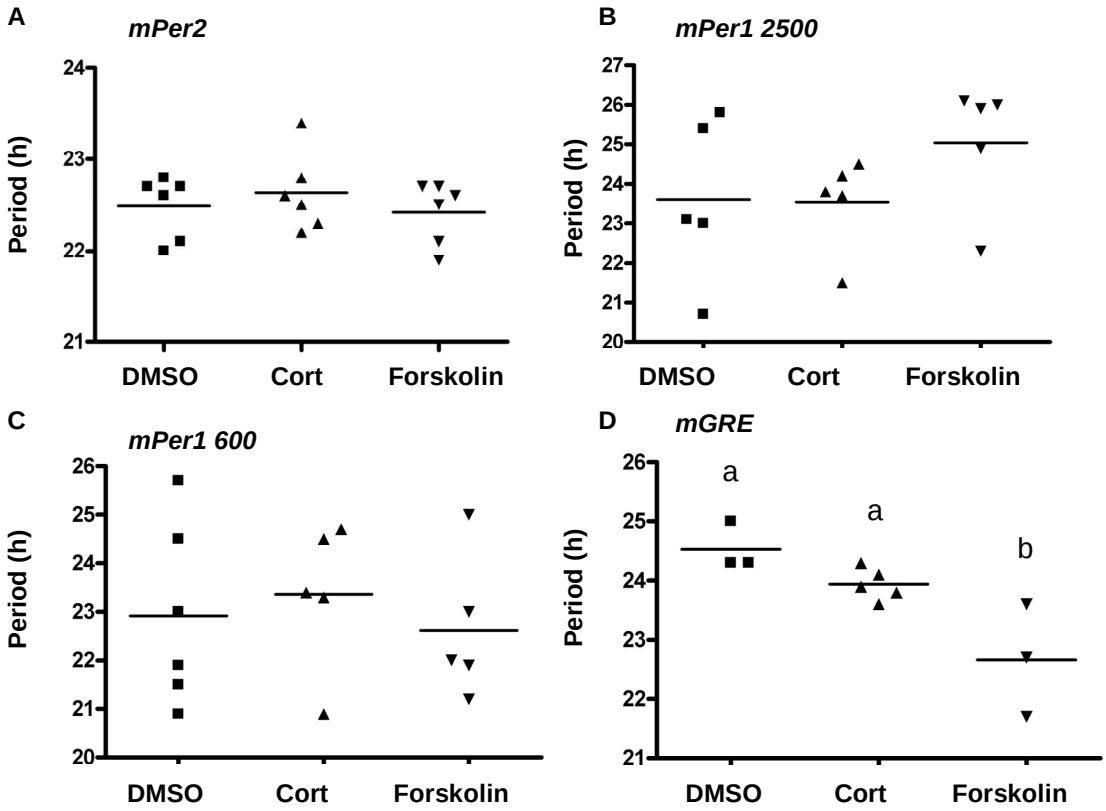

Figure 3.16: Periods of the circadian reporters after treatment: The periods for *Per2::luc* (A), *Per1::luc 2500* (B), *Per1::luc 600* (C) and the *Per1::luc* 2500 mutant GRE construct (D) were analysed after CORT or forskolin treatment relative to the vehicle control. Dots represent single experiments. Statistical analysis was by One-way ANOVA (in A: P = 0.6; in B: P = 0.31; in C: P=0.78; in D: P= 0.0086) with a Bonferroni post-hoc analysis (a) versus (b) P<0.05.

3.3.5 The non-steroidal GR specific agonists

3.3.5.1 GR and MR transactivation assays

Novel non-steroidal GR specific agonists were used to further the studies (set out in Fig 3.9 and 3.10) regarding the role of GR in mediating the phase and amplitude effects of CORT. They were designed to have no MR activity and high GR potency (low EC_{50}); however, they were untested at the time of receipt. The high potency and lack or MR activity was demonstrated using transactivation assays with both GR and MR, (Fig 3.17). Both, 67a and 69a, were shown to be highly potent GR agonists with EC_{50} values in the sub nM range, 0.7nM and 0.5nM respectively, compared to the DEX control, (EC_{50} of 19.96nM). This suggested that these non-steroidal agonists were around 20-fold more potent that DEX. The two GR agonists had no MR activity as the assay showed basal levels of

activation at all concentrations compared to the CORT control, (EC_{50} = 0.31nM), (Fig 3.17b).

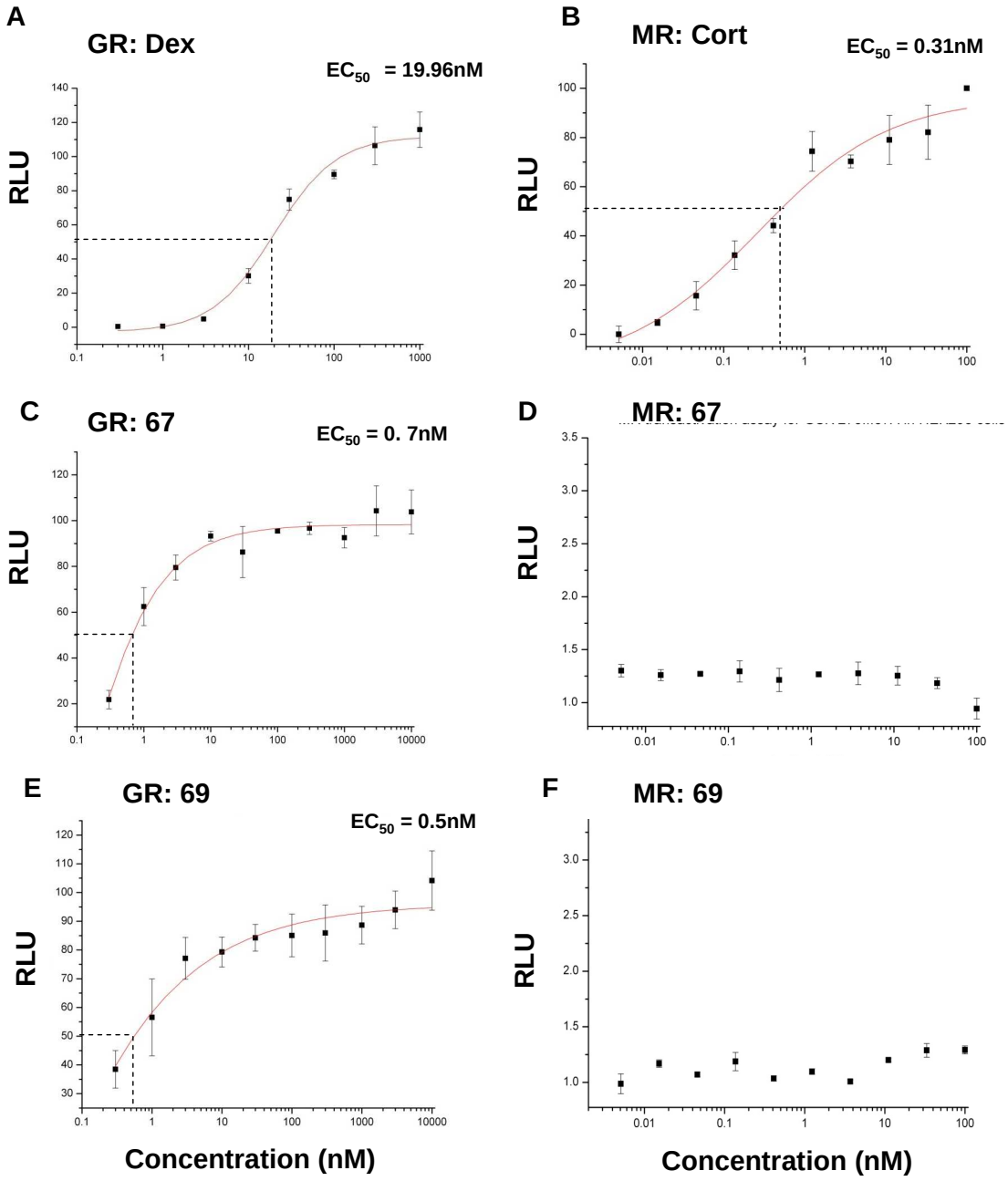

Figure 3.17 GR and MR transactivation assays with non-steroidal GR agonists: GR transactivation assays for the non-steroidal agonists (C and E) relative to the DEX control (A). MR transactivation assays for the non-steroidal agonists (D and F) relative to the CORT control (B). The EC_{50} values are given in the upper right. RLU are normalised between 0 (no activity) and 100 (full activity) and the EC_{50} values were determined by GraphPad Prism software, n=3. The GR assays were carried out by Dr. Midori Kayahara.

3.3.5.2 Non-steroidal GR agonists and their effect on the phase of the lung oscillation

The GR agonists were used with the pulmonary tissue and Rat-1 circadian reporters (like CORT, Fig 3.9), with the assurance that these compounds were GR specific. The agonists, used at a GR saturating concentration (x10 EC_{50} concentration), were able to advance the phase of the PER2::luc oscillation in the pulmonary reporter. However, the acute amplitude effect was different to that seen with CORT alone, as neither agonist showed an immediate amplitude rise (Fig 3.18). Instead the agonist treated slices continued into a trough, similar to the vehicle control, but rose to the acrophase earlier than the vehicle, therefore, advancing the phase (Fig 3.18c). The time to the fist peak after treatment occurred approximately 10h and 7h after CORT peaked for 67a and 69a respectively, and suggested a very different immediate mechanism of action on the PER2::luc reporter. The chronic amplitude of the oscillation post treatment with either agonist or CORT was not significantly different to the vehicle control (Fig 3.18d) although the average amplitude response for CORT was similar to that seen in Fig 3.9 compared to Fig 3.19.

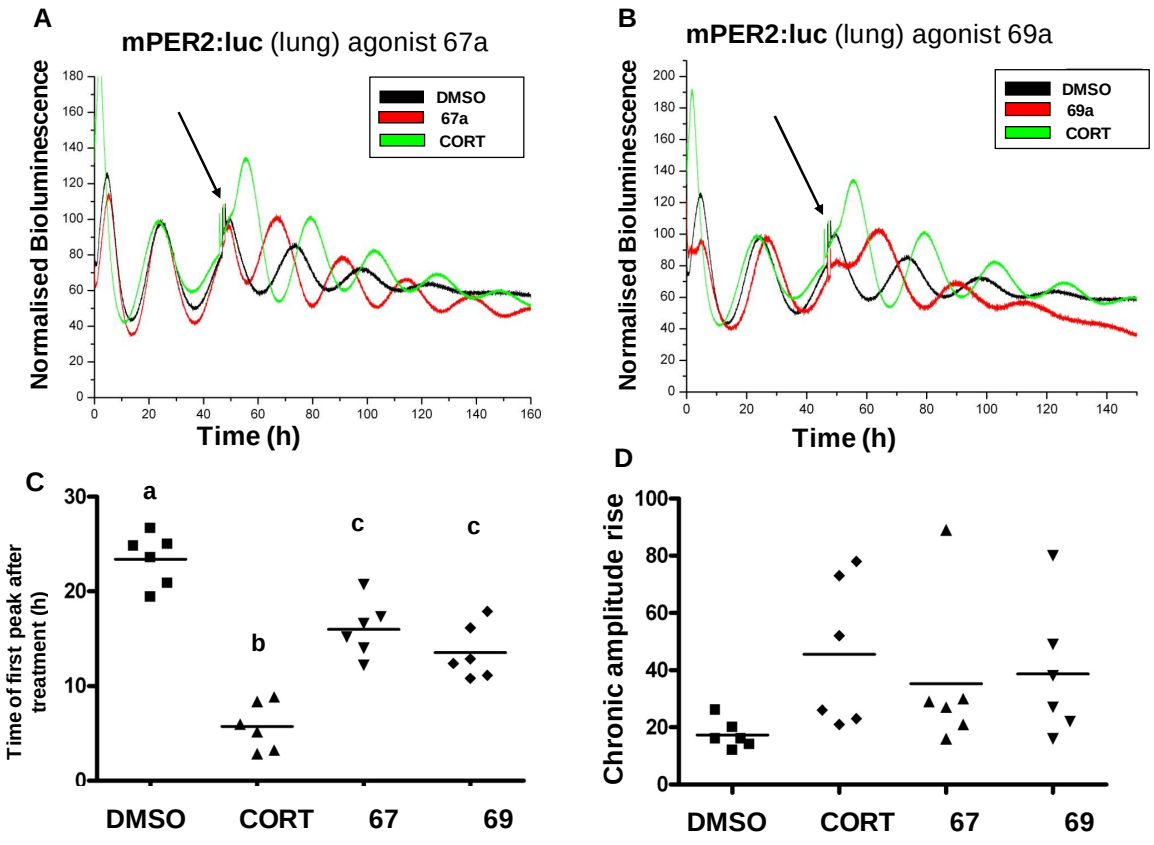

Figure 3.18: Comparative differences between CORT and the non-steroidal GR agonists: Representative mPER2::luc lung slices were treated, at the arrow (CT12), with DMSO (black), CORT (green) and either agonist 67a (A; red) or 69a (B; red) and the time to first peak after treatment was analysed (C) as well as the chronic amplitude rise (D). Dots represent individual experiments and analysis was by One-Way ANOVA (in C: $P<0.0001$, in D: $P=0.28$) with a Bonferroni post-hoc analysis, (a) versus (b) and (c) $P<0.001$ and (b) versus (c) $P<0.001$.

The two agonists responded to GR antagonist treatment, similar to CORT (Fig 3.9), even though there was a different temporal response in the immediate few hours following treatment. The effect of the GR antagonist (RU) in conjunction with the GR specific agonists was similar to that seen with CORT (Fig 3.9 and 3.19). The resulting oscillation peaked later than the vehicle control at a time similar to that of the pulmonary tissue treated with RU only, approximately 4h after the control (Fig 3.9), suggesting that RU completely antagonises the effects of the GR agonists (Fig 3.19 a and b). The resulting periods following treatment with either CORT (taken from the oscillations in Fig 3.18), agonist or by pre-treating with RU showed no difference between groups (Fig 3.19c). (The period for lung slices treated with RU is approximately 26h, shown in Fig 3.11). The chronic amplitude of the oscillation post agonist treatment was not significantly increased above vehicle control with either 67a or 69a nor did RU reduce the subsequent amplitude with respect to 67a but it was significantly reduced with 69a (Fig 3.19d). Antagonism of the GR by RU in the presence of CORT results in an approximately 4.5h phase change (Fig 3.10) compared to a 4h and 2.9h phase change in the presence of 67a and 69a respectively (Fig 3.19), yet neither of these comparisons are significantly different (student t-test P=0.47 for 67a and P=0.08 for 69a).

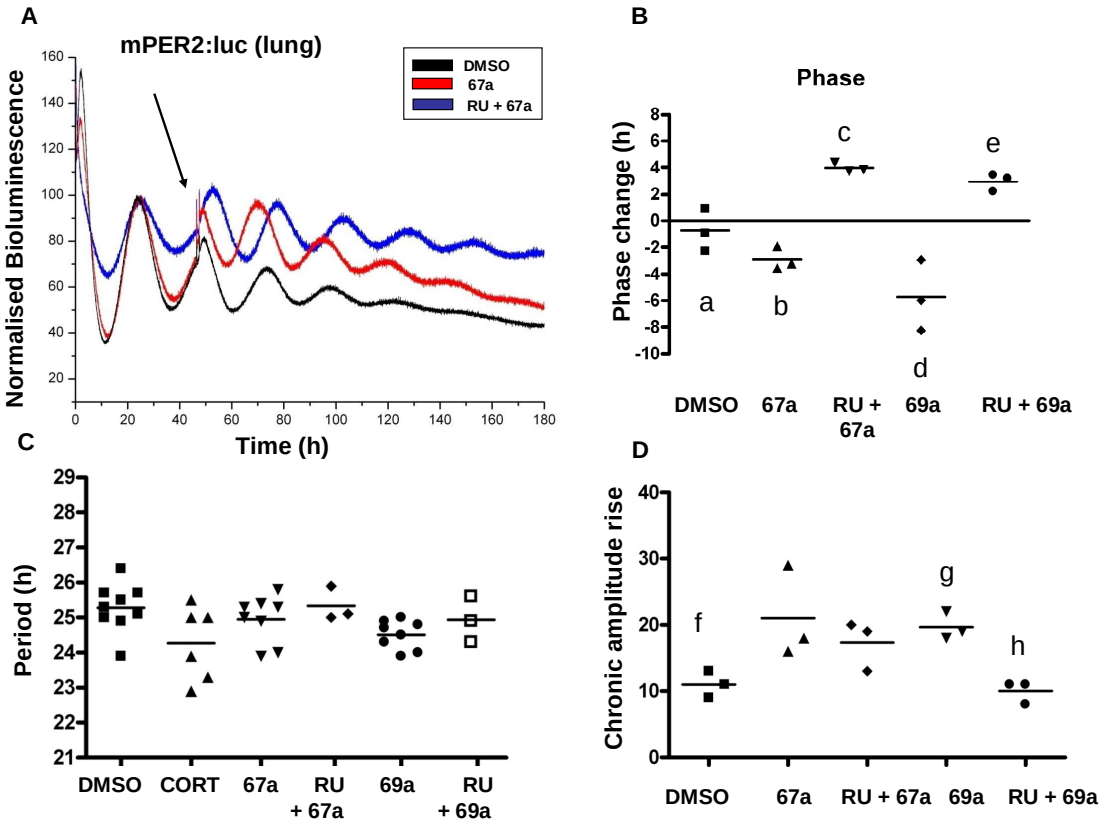

Figure 3.19: Antagonising the effects of the non-steroidal agonists: A representative mPER2::luc lung oscillation (A) shows 67a (red), RU with 67 (blue) and DMSO (black). The phase analysis (B) shows results for both 67a and 69a with and without RU pre-treatment. Overall period analysis for CORT, 67a, 69a and RU treatments is shown in (C). The chronic amplitude rise is shown in (D). Dots represent individual experiments and analysis is a One–way ANOVA (in B: $P<0.0001$; in C: $P=0.08$; in D $P=0.017$) with a Bonferroni post-hoc analysis. Regarding B: (a) versus (b) not significant, (a) versus (c) and (d) $P<0.05$, (b) versus (c) $P<0.01$ and (d) versus (e) $P<0.001$. Regarding D: (f) versus (g) and (g) versus (h) $P<0.05$, (f) versus (h) is not significant. The oscillation for 69a was the same as (A) but is not shown.

3.3.5.3 Non-steroidal GR agonists and their effect on the phase of the Rat-1 oscillation

To complement the effects of the agonists on the pulmonary reporter, both were administered to the *Rat-1- Per2:luc* reporter. As in the PER2::luc pulmonary reporter, the agonists resulted in a phase change but this time resulted in a peak after the vehicle control, constituting delay (Fig 3.20a and c). However, when the cells were pre-treated with RU the phase effects were significantly reduced by approximately 2h, with both agonists, unlike in the presence of CORT where the phase change was reduced to almost basal levels (Fig 3.9). Antagonism of the GR by RU in the presence of CORT results in an approximately 1h phase change (Fig 3.9) compared to a 2.6h and 3.8h phase change in the

presence of 67a and 69a respectively (Fig 3.20), which are significantly different (student t-test P=0.026 for 67a and P=0.001 for 69a).

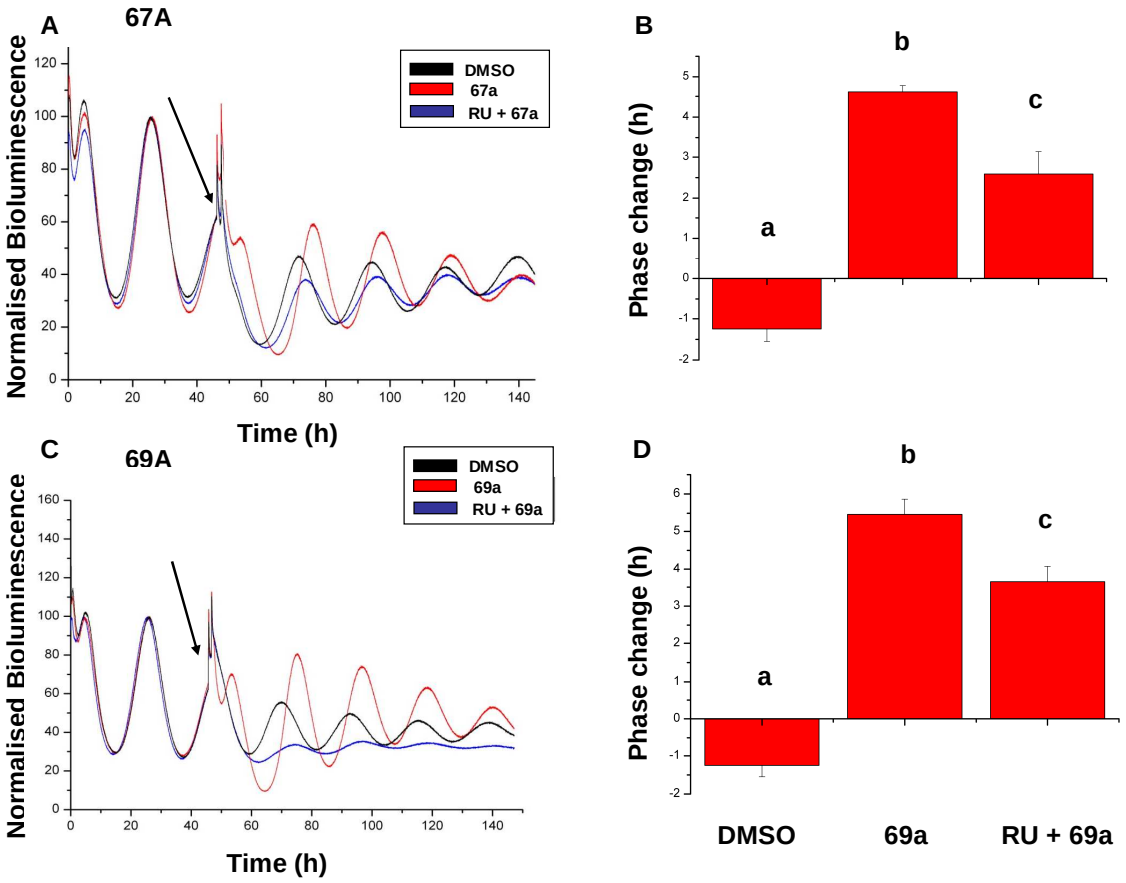

Figure 3.20 Agonist effects on the *Rat-1:Per2::luc* promoter reporter: Agonists, 67a (A) and 69a (C), were added to the Rat-1-*Per2* reporter alone or after pre-treatment with RU. Treatment time is shown by the arrow (CT12). The phase analysis is shown for 67a (B) and 69a (D). DMSO (black), 67a or 69a (red) and RU with 67a or 69a (blue). Statistical analysis was by One-Way ANOVA (in B and D: $P<0.0001$) with a Bonferroni post-hoc analysis, (a) versus (b) and (c) $P<0.001$; (b) versus (c) $P<0.05$. Error bars are SEM from n=3 repeats.

In addition to the effects on phase, the agonists had large effects on the period of the post treatment oscillations, unlike with the agonist treated pulmonary reporter where the post treatment periods were not statistically different between treatment groups (Fig 3.19c). Both 67a and 69a significantly reduced the period length, by approximately 1h relative to the vehicle control, which was inhibited by pre-treatment with RU in the presence of both 67a and 69a (Fig 3.21). The chronic amplitude rise of the Rat-1-*Per2::luc* reporter did not significantly increase in the presence of the either agonist relative to the vehicle control but in the presence of RU the amplitude effects were significantly attenuated (Fig 3.21b).

Together suggesting that these potent agonists show subtly different effects resulting from type of reporter used to study them, be it a protein or promoter reporter.

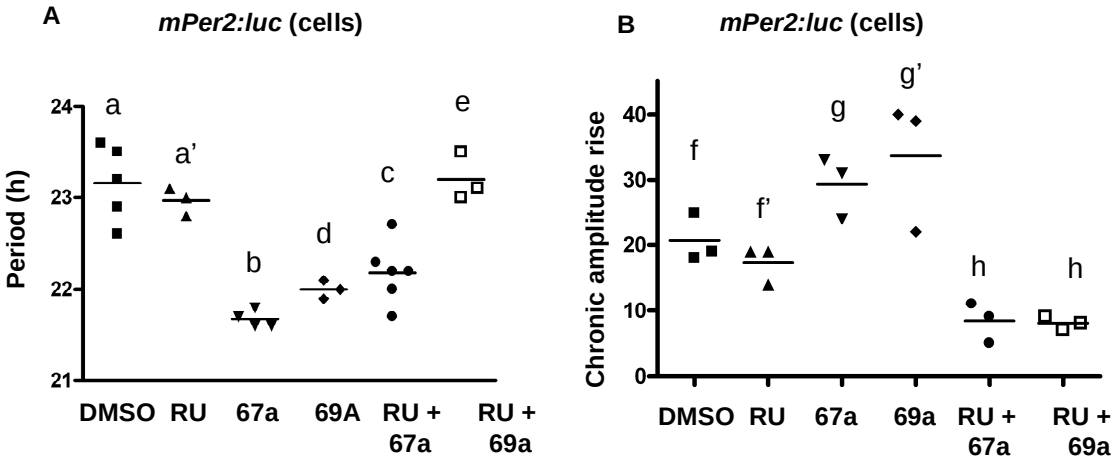

Figure 3.21 Agonist induced period change in the m*Per2::luc* promoter model: Period was analysed after agonist treatment with or without the presence of RU. Dots represent a single experiment. Analysis is by One-way ANOVA (P<0.0002) with a Bonferroni post-hoc analysis. Regarding (A); (a) versus (b), (c) and (d) by P<0.001; (a') versus (b) P<0.001; (a') versus (d) P< 0.01; (a') versus (c) P<0.05; (a and a') versus (e) not significant; (b) versus (c) not significant and (d) versus (e) P<0.001. Regarding (B); (g) versus (h) P< 0.001; (f') versus (g') P<0.05; (f) and (g) are not significant and (f) and (h) are not significant.

3.3.6 Analysis of the pulsatile nature of Gc on the circadian clock

3.3.6.1 Pulse duration with a constant Gc concentration

The pulsatile nature of Gc release was explored with the development of a flow-through culture system linked to the PMT (section 2.4), where a pulse of Gc could be administered, for a set length of time, and then removed without disturbing the PMT recordings. In order to develop a working model, the pulse duration was addressed while the concentration of the Gc remained constant. In general, since n=1 for all the following pulse experiments, no conclusions can be made about the results, but they form a useful description of developing a technique.

Pulse durations of 15, 30 and 60 min were used, on the PER2::luc pulmonary model, while keeping the concentration of DEX constant, at 200nM (Fig 3.22). DEX was used as it is a potent Gc analogue and was thought likely to produce more robust results in the early stages of this model, compared to CORT. It was evident that pulse durations of either 15 or 30 min, even though produced an amplitude rise and a phase change, were not as robust

as the 60 min pulse (Fig 3.22c). The time to the first peak after each pulse was within the same dynamic range as seen with the static PMT experiments (6-8h) (Fig 3.18c). The post treatment periods were slightly longer than those observed previously (Fig 3.11) at 24.3h, 24h and 25.4h for the 15 min, 30 min and 60 min pulse respectively but still well within the endogenous range seen with DMSO (Fig 3.2c and 3.11). The larger amplitude of the 60 min pulse meant that this duration was carried forward. Furthermore, the flow rate was 6.8ml/h with roughly a 2ml residual volume in the well, therefore, a 60 min pulse would see three full media changes with 200nM DEX, whereas the 30 min pulse would only see approximately 1.5 changes and the 15 min pulse less than1 change. Calculations of fluid dynamics were not undertaken, but a 60 min pulse would allow the lung slice to see a concentration of 200nM DEX for a set period rather than it being diluted in the existing media and then removed.

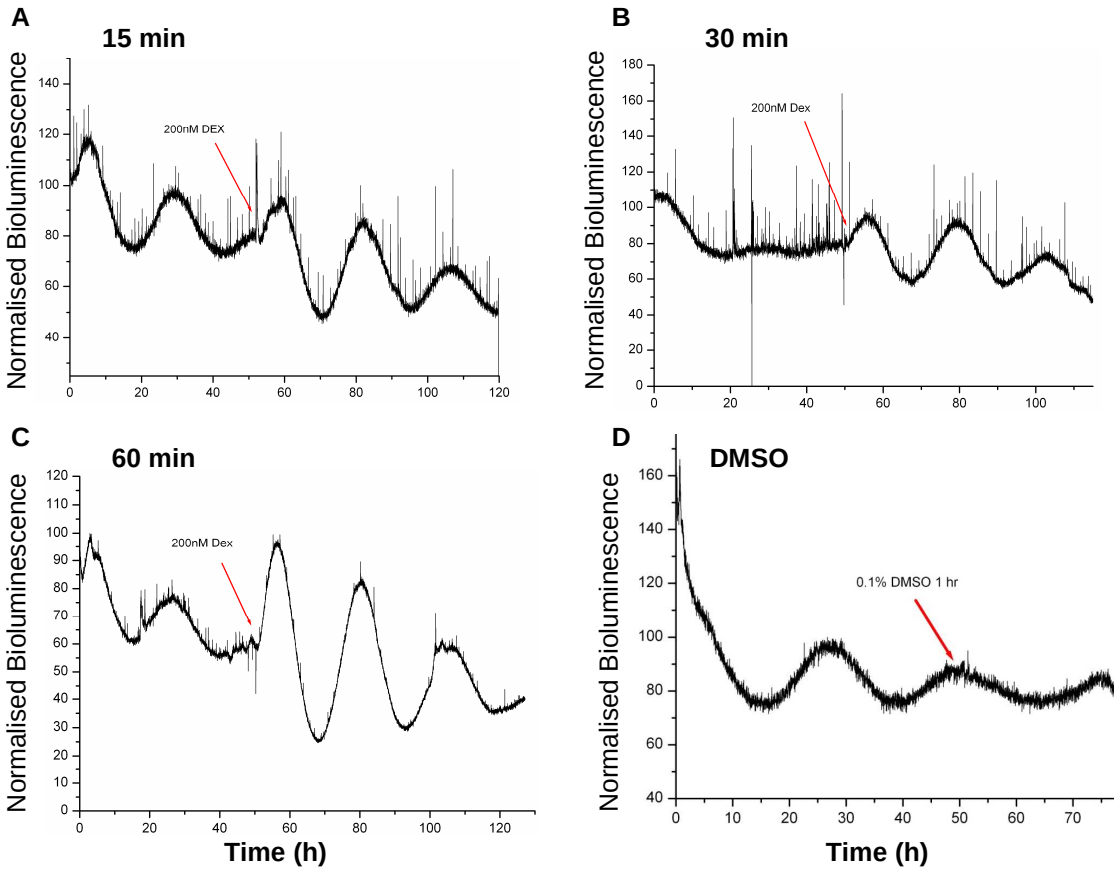

Figure 3.22: Pulse duration of the flow-through circadian mPER2::luc reporter model: A timed pulse of 200nM DEX was given to the mPER2::luc lung model, under flow through conditions. Pulse durations of 15 min (A), 30 min (B) 60 min (C) and a DMSO pulse (D) are shown by the arrow (CT12). A flow rate of 6.8ml/h was used throughout. Each oscillation is a single experiment, (n=1).

3.3.6.2 Gc concentration with a constant pulse

Following the determination of the optimal pulse durations, different DEX concentrations were used while keeping the pulse duration constant. Pulse duration of 60 min was used and 10, 500 or 1000nM DEX was added (60 min pulse; 200nM DEX was performed in Fig 3.22c). Although each concentration produced a phase effect and an amplitude response, the continuation of the recordings was problematic; therefore, the 500 and 1000nM experiments were terminated (Fig 3.23 b and c). The 10nM DEX pulse does, however, produce a robust oscillation after the pulse and washout, with a period of 23.5h, together suggesting that the pulse duration is more influential than the concentration of Gc, at least using DEX.

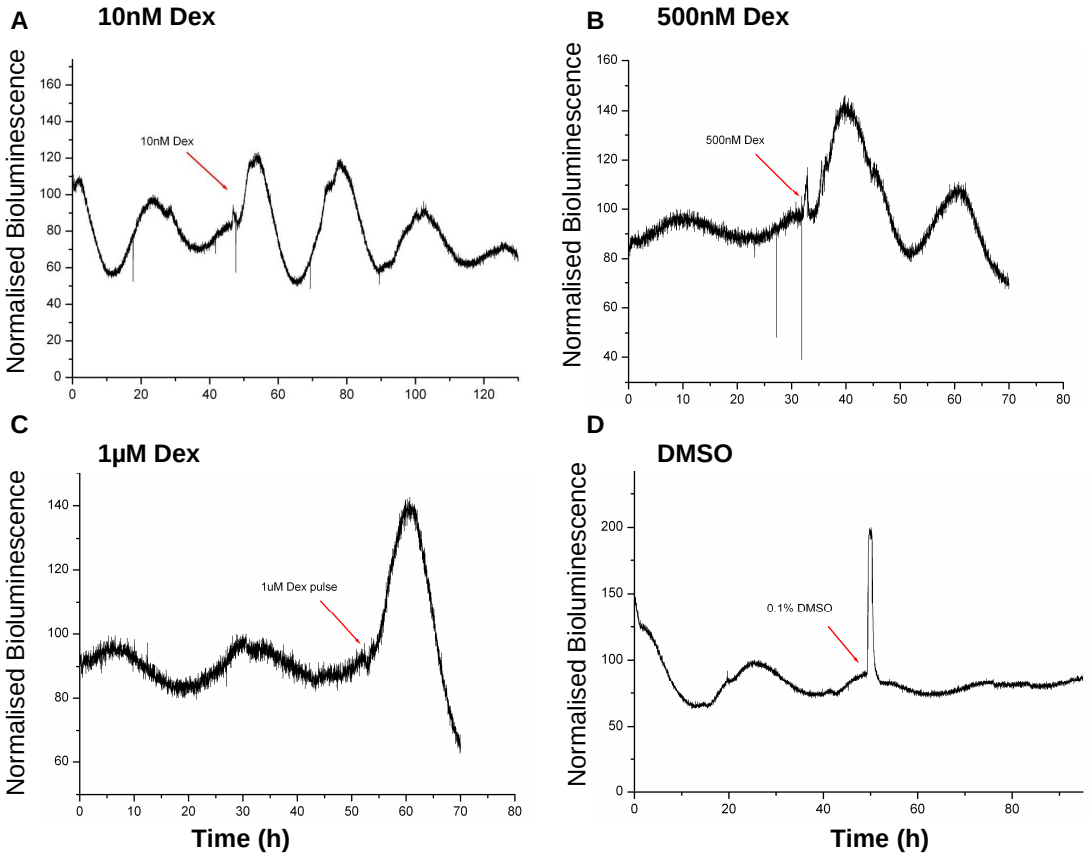

Figure 3.23: Gc concentration effects on the flow-through mPER2::luc model: Different concentrations of DEX were administered for a 60 min pulse as shown by the arrow (CT12), 10nM (A), 500nM (B) and 1μM (D), a DMSO vehicle was also pulsed (D). A flow rate of 6.8ml/h was used throughout. Each oscillation is a single experiment, (n=1).

The initial work on Gc pulsatility used DEX as the model Gc (Fig 3.22 and 3.23) but the endogenous Gc CORT was required as to best mimic the pulsatility of Gcs on the

peripheral clock. Pulse duration of 60 min was used with 50 or 200nM CORT, again using the PER2::luc pulmonary reporter. Both concentrations had an effect on the phase of the oscillation, with the time to first peak being between 6-8h, but the 200nM pulse produced a far greater amplitude response (Fig 3.24a). The resulting post treatment periods were 23.9h and 24.5h for the 200nM and 50nM CORT pulse respectively. This suggested a difference in the dynamic range of action between DEX and CORT. Therefore, it was concluded that the model worked with both DEX and CORT but for subsequent experiments 200nM CORT; 60 min pulse was required.

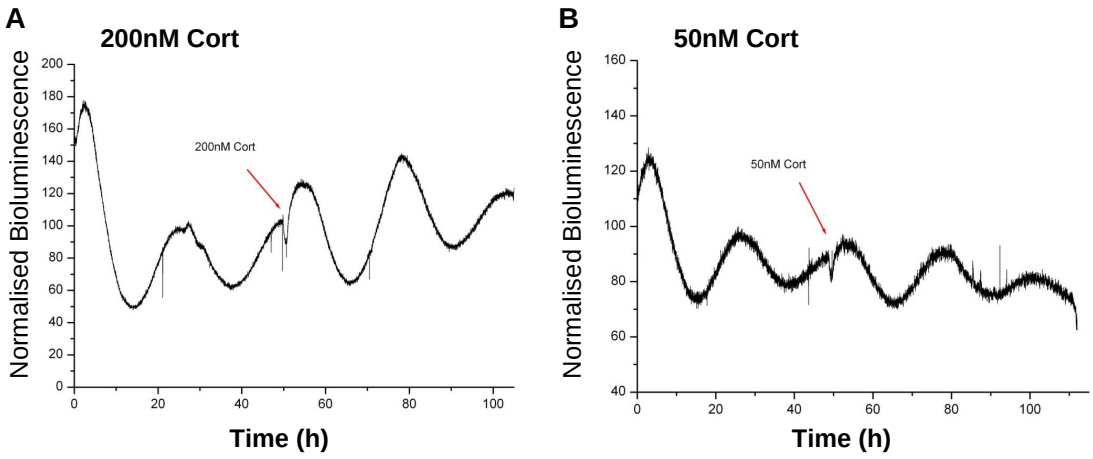

Figure 3.24: CORT induced effects under flow-through conditions using the mPER2::luc model: CORT concentrations were administered through a 60 min pulse to the PER2::luc lung slice, 200nM (A) or 50nM (B) as shown by the red arrow (CT12). A flow rate of 6.8ml/h was used throughout. Each oscillation is a single experiment, (n=1).

3.3.6.3 Multiple Gc pulses on the same sample

The overall aim for this study was to address the effects of the pulsatile nature of Gcs on the pulmonary clock and by setting up the flow–through system this could be achieved. Using a 60 min pulse duration and 200nM CORT, two pulses were added to the same lung slice approximately 7h apart. The aim was to pulse at the peak of the PER2::luc rhythm and then pulse a second time at the acrophase of the CORT-induced peak, in order to determine a) if the second CORT pulse would result in a further induced phase change and amplitude rise or b) if there was an inbuilt refractory period and the clock would not respond to the second pulse and continue with the oscillation and into the trough. It was apparent that the second hypothesis was the biological result as the second pulse (B in Fig 3.25), again 200nM CORT for 60 min, did not induce a second phase change or amplitude response and the oscillation continued into the decline and to the trough (Fig 3.25).

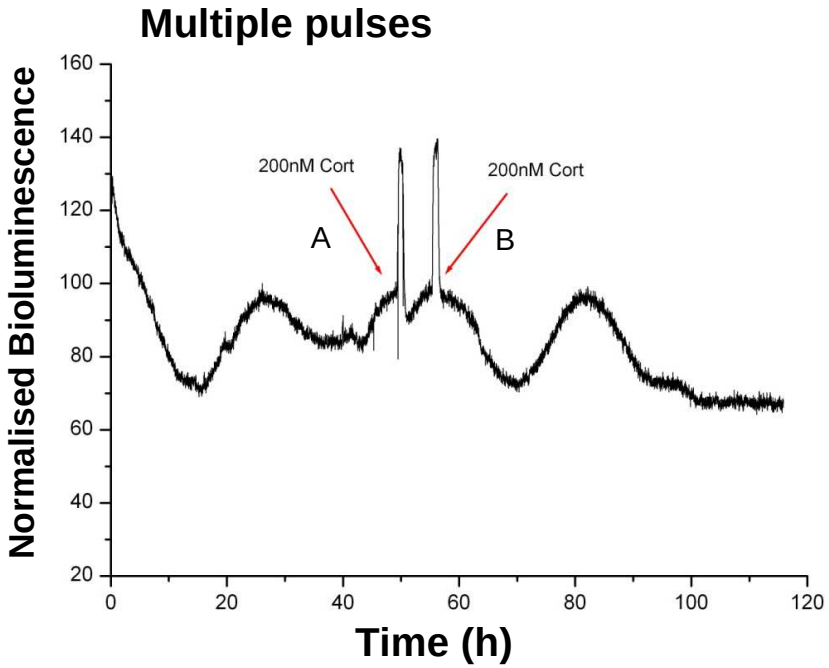

Figure 3.25: Multiple CORT pulses on the same mPER2::luc lung sample: Two 60 min 200nM CORT pulses were administered to the same PER2::luc lung slice, labelled (A) and (B), 7h apart at the arrows (CT12 and CT19). This oscillation is a single experiment where n=1.

Further multiple pulse challenges were carried out with the second pulse given at different phases of the first Gc induced rhythm; at the trough and mid-way through the decline but due to complexities in the flow-through model as a reporter system the results were of poor quality (appendix D). Also in an attempt to improve the quality of the flow-through oscillations multiple slices were used at the same time (appendix D), but again there were problems with reproducibility. Likewise flow-through culture with a Rat-1 cellular reporter itself proved problematic, as cells readily became detached and the rhythms quickly became dampened (appendix D). A more detailed analysis of the pitfalls and drawbacks of the flow-though systems are discussed in section 3.4.4.

3.4 Discussion

3.4.1 The effects of Gc on the peripheral clock

Gcs, or more precisely CORT, have been shown here to be potent regulator of the circadian clock, having effects on the phase, period and amplitude of the oscillation, in the two circadian reporters used here. Initial work, which clearly demonstrated the role of Gcs in synchronising the external environment and SCN with the periphery (Balsalobre et al.

2000a), used the potent Gc analogue DEX, initiating an increase in the transcript abundance of *Per1*, but not *Per2*. Later work provided evidence that *Per2* was itself directly regulated by DEX (So et al. 2009) but the model system used in this study was a primary mesenchymal stem cell, which suggested that model system used had a large effect on the outcome of the result. It is also of interest to note that even though Gcs show both circadian and ultradian rhythms *in vivo* (Windle et al. 1998b), the Gc only needs to be present, and does not need itself to be rhythmic, to initiate a robust cell cycle rhythm (Dickmeis et al. 2007), which is how these studies were conducted; that is with a one time treatment that remained in the culture. It has recently been hypothesised that temperature oscillations could be the way through which the periphery is synchronised with the SCN and external environment. The authors report that there is differential sensitivity to temperature in the SCN i.e. some SCN neurons is sensitive to temperature change where others are not, which allows the SCN to drive circadian rhythms in body temperature. This temperature rhythm can then act as a signal for the entrainment of other oscillators throughout the body, in tissues such as the liver, lung, and heart. In the study by Buhr et al., 2010 the authors suggest that although there are many ways to synchronise the periphery e.g. Gcs, food and light they each have an effect of temperature. For example Gcs inhibit the transcription of the inactive GR chaperone proteins Hsp70 and Hsp90, restricted feeding is associated with a decrease in body temperature and pathways involving cAMP and Ca^{2+} also activate heat shock pathways. In this study, reported above, two circadian reporters were used, the Rat-1 cell line, for promoter studies and PER2::luc pulmonary protein reporter. It is clear from the two reporters that CORT has an effect on both the transcript and protein rhythms, resulting in a dynamic response at all phases of the circadian cycle (Fig 3.5), with regard to the phase re-setting effects. The PRC response falls into the biphasic type 0 PRC class with both an advance and delay seen, unlike that of the SCN which, in response to light, forms an unresponsive 'dead zone' (Takahashi et al. 1984). The absence of the dead zone in the peripheral model means that it can remain responsive to Gcs at any time of the circadian cycle, and can therefore remain sensitive to synchronisation from the central oscillator. The PER2::luc CORT-induced PRC complements earlier work that showed a similar amplitude and dynamic response of a *Per2::luc* promoter reporter in response to DEX (Gibbs et al. 2009; Izumo et al. 2006). An explanation for the dynamic response to CORT that results in both an advance and delay phase is the local chromatin environment and the state of histone acetylation. The key point of the curve is the transition between delay and advance (CT10-12), this rapid switch

also occurs in the Izumo (2006) curve to DEX between 32 and 36h, after serum shock. This time corresponds exactly with maximal histone H3 acetylation, therefore an open chromatin structure, on the *mPer2* promoter (Naruse et al. 2004), suggesting that the accessibility of the local transcription environment is the cause of the advance or delay output. In addition to this, the 'dead zone' observed in the SCN in response to light in the subjective light phase (CT0-12) (Takahashi et al. 1984) is likely to be caused by a lack of acetylation and therefore a closed structure. Although histone H3 acetylation was not directly shown H3 phosphorylation, considered to significantly enhance acetylation, was shown to occur after a light pulse in the dark phase, in the SCN of mice kept under constant dark, but not when pulsed in the light phase (Crosio et al. 2000) altogether suggesting that the chromatin environment and histone acetylation is key to the circadian response to both light and hormonal cues. However, it cannot be assumed that histone modification is the only way through which the PRC is formed but likely through numerous other interconnecting pathways such as activation of the NMDA receptor, in the SCN, which can closely mimic the light induced PRC when administered over the circadian day in the hamster (Mintz et al. 1999). Yet glutamate administration appears to mimic the effects of dark pulsing suggesting that this particular pathway is not involved, and that the PRC is pathway specific (Meijer et al. 1988). There could also be overlap with other pathways such as those activated by neuropeptide Y, which has been shown to induce phase shifts in the firing rate of SCN neurons and behavioural responses in the intact animal (Shibata and Moore 1993). Other pathways could also be involved such as those leading from voltage gated calcium channels, particularly the L-type and T-type channels which are known to be under circadian control (Ko et al. 2009). In particular, expression of the T-type channel, in the SCN, is greatest at the transition point from day to night suggesting involvement in phase re-setting (Nahm et al. 2005).

The PER2::luc pulmonary model revealed that CORT had an immediate temporal role on the amplitude of the protein (as seen by the surrogate luciferase signal (Fig 3.3), which suggests the possibility that CORT has a non-genomic role on circadian timing. However, the blockade of this response with the addition of either a GR or MR antagonist suggests that the response remains receptor mediated; but the immediacy of the response suggests the possibility of cytoplasmic, rather than nuclear, function, that could be due to rapid phosphorylation events. Interestingly, the acute and chronic amplitude rises (Fig 3.3) occur at 0.1µM and above, in the pulmonary reporter, whereas the phase change is still

observed at 10nM and partly at 1nM. Therefore this indicates that the two are exclusive events and that the amplitude effects are mediated via a divergent pathway to the phase re-setting events. Furthermore, CORT induces a similar chronic amplitude increase, seen over the duration of multiple cycles in the Rat-1-*Per2::luc* reporter, which is attenuated by GR rather than MR antagonism (Fig 3.9 and 3.10).

CORT has been shown to affect both the phase and amplitude response of the PER2::luc rhythm but it has a period effect too, which appears (at least in the lung) to result in a significant reduction in period length at CT 0, 6, 8 and 14, (Fig 3.5) although a reduction in period was seen at all times. Other CT values were not significantly different to the vehicle control, including CT12 (the time used for further antagonist based study). Interestingly, antagonism of the GR, but not the MR, resulted in a significant period lengthening when compared to CORT treatment at CT12, with almost a 2h difference. Consequently GR antagonism can inhibit the period shortening action of CORT, (Fig 3.11) and together provides evidence that the GR is important in the phase effects of CORT and the endogenous period of the clock. The period shortening effects of CORT, at times previously mentioned (CT0, 6, 8 and 14), are of similar magnitude to that seen when GSK3β is inhibited through siRNA (Hirota et al. 2008). It is also know that the Gc analogue DEX can induce GSK3β phosphorylation and inactivation through PKB, via PI3K pathway and inhibition of PI3K or GR can block PKB activation (Matthews et al. 2008). These data provide very promising evidence that the effects of CORT on the period are both GR and GSK3β mediated.

A functional GRE in the *Per1* promoter was demonstrated through DEX induction of a cloned construct (Yamamoto et al. 2005), and a reduction in expression through its mutation, which was repeated in this study (Fig 3.12). However, a circadian analysis and the importance of this element to the underlying circadian oscillation was not characterised and, therefore, explored in this study. The GRE affected the magnitude of the CORT-induced phase change, as the phase change was reduced when mutated or through construct truncation, but it had no effect on the period length (Fig 3.13 and 3.16) or the amplitude of the oscillation following CORT administration (Fig 3.15). This is in direct contrast to other reports where the chronic treatment of cell lines and mice with prednisolone, another Gc analogue, resulted in constitutively high levels of *Per1* while repressing the oscillations of the other clock genes, such as *Per2* (Koyanagi et al. 2006a).

Perhaps an effect as a direct result of the half life ($t_{1/2}$) of the Gc analogues used, as the $t_{1/2}$ of plasma CORT is 25 min (Sainio et al. 1988); for DEX it is 200 min and for prednisolone it is between 115 and 200 min (Cevc and Blume 2004), and in part explaining why CORT was used in this thesis rather than other, more potent, analogues. Interestingly, the presence of the GRE did not induce a large amplitude rise after Gc addition, unlike that of the CRE after forskolin treatment (Fig 3.13), which was the same with the mutant construct. This is in direct contrast to the luciferase time point assay where Gc (CORT and GR agonists) and forskolin induced a similar induction of *mPer1::luc* which was lost in the mutant form (Fig 3.12). An explanation for this apparent discrepancy and maintenance of a robust rhythm in the mutated *Per1::luc* construct could be the compensatory effects of a further GRE, more proximal to the GRE cloned in this study and, though shown by Yamamoto et al. (2005) not to be functional in a luciferase assay, can still bind to the liganded GR in both a cellular assay and in the liver in response to stress or the presence of other circadian proteins on the promoter construct. The period lengths post treatment were constant for all constructs; *Per2*, *Per1 2500* and *Per1 600*, however, there was a shortening of period length with the mutant GRE after CORT and forskolin but these data did not reach significance.

3.4.2 GR MR and the peripheral clock

The data shown here suggests that the GR is the more dominant receptor involved in the CORT mediated phase re-setting of the peripheral clock, however, the MR does have a role in the amplitude effects of CORT on PER2::luc (Fig 3.9 and 3.10). Interestingly RU486 alone can alter the phase of the oscillation by approximately 3.7h, in the PER2::luc lung slice which is significantly different to DMSO, suggesting that it is indeed acting as a partial agonist previously described by Stevens et al., 2003 and Wang et al., 2002 (Fig 3.9c). Although a 1h phase change is observed in the *Per2*::luc cell line reporter this is not significantly different to DMSO. The MR has an approximately x10-fold higher kd for CORT than for the GR, (0.5-1nM for MR and 2.5-5nM for GR (De Kloet et al. 1998) which suggests that the MR will be saturated by CORT at physiological concentrations and only effective when CORT is at very low amounts. The GR itself seems not to be under circadian control, as seen at the protein level in the mouse lung (Gibbs et al. 2009) and at both transcript and protein level in the Zebrafish (Dickmeis et al. 2007), however, similar studies for the MR have not been undertaken. Interestingly, both the GR and MR transcript have been shown to oscillate in an ultradian fashion, with an 8h and 12h rhythm

respectively in muscle (Yang et al. 2006). This is similar to the reported circadian harmonic oscillations of a variety of transcripts which cycled with 8h and 12h periods, however, neither MR nor GR were reported here, although some heat shock proteins did show a 12h oscillation (Hughes et al. 2009).

It has been shown that the GR and MR can heterodimerise to form CORT mediated transrepression via a negative GRE (nGRE) (Ou et al. 2001) and with work by Torra et al. (2000) the *Rev-erbα* promoter is thought to contain a nGRE showing a direct association with a known circadian regulated gene. However, further work on their transactivation properties has not yet been revealed. In a more recent study it was found that aldosterone could induced *Per1* mRNA expression in a mouse cell line and in the kidney, which could, at least in part, be blocked by siRNA knock down of MR but fully inhibited by knock down of the GR (Gumz et al. 2009). A further study also demonstrated aldosterone induced transcription of *Per2*, as well as *Per1* in a cardiomyoblast cell line, which was inhibited by the MR antagonist spironolactone (Tanaka et al. 2007). Although the work in Fig 3.10 suggested that the MR does not have a functional role in the CORT mediated phase re-setting of the clock, this may just be a result of the reporters used, as the lung especially is not an aldosterone (MR) target tissue and may, therefore, have a different cellular biology to other MR target organs, such as the kidney. It would be of particular interest to repeat the experiments used in Fig 3.9 and 3.10 in kidney, ovary or heart sections and to make use of gene knock down techniques to complement antagonist treatment, perhaps in this case a more prominent role for the MR would be discovered. Furthermore, recent evidence has suggested that the MR is indispensable for CORT mediated non-genomic signalling in the hippocampal neurons, where it is responsible for glutamate release (Karst et al. 2005). In this work it was shown that GR had surprisingly little effect on the rapid signalling events. This idea links with work in this thesis as the immediate amplitude effects seen on PER2::luc after CORT administration, although can be inhibited by a GR antagonist, can be inhibited by a MR antagonist. This immediate effect is considered to be too rapid for genomic signalling and the potential non-genomic signalling effects of CORT are further explored in Chapter 4.

3.4.3 Non-steroidal GR agonists

The selectivity of these two agonists is an invaluable tool in order to try and differentiate between the activity of the GR and MR. The two agonists used in this thesis are both very

potent (sub nM kd) and GR specific, that is they have no MR activation potential (Fig 3.17), and so, whereas with CORT, the observed effects were always a mix of GR and MR activity with the agonists the effects are GR mediated only. The observed differences are substantial, particularly in the immediate few hours after treatment. The two agonists respond as expected to pre-treatment with RU (Fig 3.19) but when added to the lung slice neither agonist resulted in the immediate amplitude effects seen with CORT and it took 10-15h before the PER2::luc protein first peaked after agonist treatment (Fig 3.18), yet the peak still occurred before the vehicle control, resulting in a phase advance. These agonists provide further evidence that there is a non-genomic element to the effects of CORT on the clock, or at least PER2, and that there is potential for receptor selectivity. However, the agonists are non-steroidal (Biggadike et al. 2009), that is they do not have the cholesterol backbone of CORT and, as a result, cause the GR binding pocket to take on a different confirmation on agonist binding, potentially leading to downstream binding difficulties with other proteins and resulting in the different characteristics seen in the immediate few hour post treatment. However, this does link well with the MR, rather than GR, mediated non-genomic effects on glutamate release from the hippocampus (section 3.4.2). Furthermore, in the lung there was no period shortening in the presence of the agonist relative to the period of agonist and the GR antagonist (RU) (Fig 3.19) unlike that seen with CORT (Fig 3.11). However, in the Rat-1-*Per2::luc* cellular reporter both agonists, 67a and 69a, significantly shortened the period relative to DMSO, which was attenuated by RU (Fig 3.21). There was no effect of 67a on the sustained (chronic) amplitude after treatment in the lung and nor was the resulting amplitude attenuated by RU administration; however 69a did show a sustained response which was attenuated by RU (Fig 3.18d and 3.19d). This suggests that the two agonists have subtly different effects on the amplitude of the PER2::luc rhythm. However, administration of RU did attenuate the chronic amplitude effects of both agonists in the Rat-1 cellular reporter (Fig 3.21b).

3.4.4 Flow-through culture

The flow-through culture system was a novel set-up designed to mimic the endogenous pulsatile nature of Gc release in the rodent. A constant flow of media through the system meant that CORT could be added and removed from the system without disturbing the tissue slice, as removing and replacing media manually would itself act to re-set the oscillator and CORT mediated effects would be lost. The work in this thesis was carried out on a single lung slice, as cell lines were not robust enough to cope with the flow-rates

used. As this system was essentially developed by the author, although building on previous work by Dr. Andrew McMaster, there were many technical difficulties to deal with; the main issue being an inefficient out-flow. The issue here was ensuring that the out-flow was at the same rate as the in-flow, the two rates were theoretically the same as they were controlled by the same pump, but any slight discrepancy meant that the media would build up and flood the system. This reason meant that the results shown here (section 3.3.6) were just an n=1 experiment and the system itself proved very unreliable. Furthermore, electronic spiking was an issue, seen to some extent in Fig 3.22, perhaps as a result of tissue movement due to different flow-rates; this also resulted in the loss of data and remained unresolved. Improvements to the system were made and issues were in part resolved, but the whole process was very much work in progress. The 35mm dish (Fig 2.6) was replaced with a Perspex block with fixed bore holes for the tubing, yet an anomaly regarding the flow rates still existed, even though a large improvement on the 35mm dish. Perhaps a greater investment of time into the pump and flow-rates would yield a more reliable model. A similar technique was established by Stavreva et al. (2009) where they used pulsatile induction by CORT to stimulate gene transcriptional events and GR signalling, rather than investigating clock gene regulation (Stavreva et al. 2009), suggesting that the technique can work. However, the results from section 3.3.6, do suggest that the pulsatile nature of CORT can induce a phase and amplitude response in PER2, like with the static culture (where CORT was added and left throughout the experiment), using a 60 min pulse of 200nM CORT. The effect of DEX, although a more robust model with an effective concentration as low as 10nM, being better than CORT, was not taken forward as DEX is a potent Gc analogue with a very long $t_{1/2}$ compared to CORT.

The ultimate aim with this work was to expose the same tissue slice to multiple pulses of CORT, and to address the issue of how the clock responds to the pulsatility of endogenous CORT release. The ultradian pulse of CORT occurs once every 50 min (Windle et al. 1998b), yet to establish methods the multiple pulse experiments were first attempted at the peak of PER2::luc and at the subsequent induced peak. The result from this provided evidence for either further induction of phase and amplitude or an inbuilt refractory period, with the latter being the case (Fig 3.25). The peripheral clock is able to ‘ignore’ the secondary signals from circulating CORT once it has been re-set and the phase of the oscillation changed. This model makes sense because if the clock responded to each pulse,

one every 50 min, there would be no circadian rhythm and peripheral clocks (liver, lung) would by arrhythmic. However, due to the technical difficulties associated with this model system further investigations into the dynamic range of this refractory period were not revealed. Appendix D does, however, show secondary pulses given at the trough and mid-way through the decline, but clear analysis was difficult. It would be nice to hypothesise that there is a window of 12h where there is no response to secondary signals, whatever the phase of the initiating one.

3.5 Conclusions

Work here has shown the potent effects of Gcs on the peripheral clock which can induce a significant response at all times of circadian day and can alter both phase and period of the circadian oscillation. These effects have been shown here to be GR rather than MR mediated. CORT also has an immediate effect on the PER2::luc protein; seen as a rapid amplitude rise as well as a chronic (sustained) amplitude effect, which can be blocked through the inhibition of both the GR and MR, therefore implicating the MR, in the amplitude effects of the Gc on PER2.

For the first time a *mPer1::luc* reporter construct containing a functional GRE has been cloned into a circadian cell line, as well as a mutant GRE construct, to address the affect that the GRE had on the underlying oscillation. The GRE had no effect on the period or amplitude of the oscillation but has a significant affect on the phase change in the presence of CORT.

The use of highly potent novel, non-steroidal GR specific agonists has revealed, for the first time, large temporal differences in the amplitude response of PER2::luc with respect to CORT, in that there was no acute amplitude effect with the agonists, yet a chronic amplitude increase was observed with regard to 69a rather than 67a. No chronic amplitude effect was observed in the Rat-1 cellular model relative to DMSO but RU did significantly attenuate the effect. The GR agonists still induced a phase change, which was GR mediated, but with no period shortening in the lung, unlike that seen with CORT. There was an agonist induced period shortening effect in the cell lines but not in the presence of CORT.

A novel flow-through system was developed which linked an existing flow-through culture system with a transgenic luciferase tissue reporter to a bioluminescent recording system, in order to study the real-time pulsatile effects of Gc on the peripheral clock. This model was achieved and a single lung slice was subjected to two 1h pulses CORT pulses, timed at subsequent peaks of the PER2::luc rhythm. Most importantly, though, the micro environment of the slice was not disturbed by the operator during the process, that is the exchange from media - to media with CORT and back to media was constant. This is a potential breakthrough in the development of a real time mimic of an endogenous process.

3.6 Future directions

- A more thorough investigation into the role of the MR in phase, period and amplitude effects on the clock, with particular regard to PER2, but in a MR target tissue such as the kidney or liver (or isolated cell cultures), as the limited MR effects seen here could be due to the chosen tissue, the lung, rather than the MR itself. Furthermore, use of siRNA or shRNA to both GR and MR would be advantageous as pharmacological inhibition, although a very useful tool may not be portraying the full picture.

- The circadian field, in general, has very few commercial antibodies and even less that have been proven to work repeatedly well for both western (immuno) blotting and immunohistochemistry studies, which is why there has been a reliance on luciferase based reporters and transcript expression in this thesis. Therefore, the production of an array of antibodies would be very beneficial, to study sub-cellular localisation, protein stability (Chapter 3 and 4) and protein binding studies; answering the question, ‘does protein ‘A’ bind to PER2? and at what time of the circadian cycle does this occur? A lot of work on post-translational modification events and binding partners could be achieved with antibody developments, including work within this thesis.

- The work in this thesis, and circadian work in general, would benefit from having other clock protein luciferase reporter, or other fluorescent reporters, to complement the PER2:: luc mouse, so as to determine if the results obtained with the PER2 reporter are maintained or different to that of CRY1 or BMAL1 and to

determine clock component specificities and similarities in the ever, rapidly evolving circadian field.

- The work on the flow-through system and the refractory state of the clock, in response to CORT, would benefit greatly from more time spent developing a robust mechanical system. Unfortunately, a quick fix will not work here and serious time must be spent to achieve a greater than 80% experimental success rate, identifying and resolving the problems created through trial and error. If this is done properly great advances could be made, not only in observing rapid pulsing of CORT on the clock, or any other compound, but the need for experimenter manipulation would be removed and a much more *in-vivo* like model would be created.

- Further work with regard the potential non-genomic effects of CORT were explored in Chapter 4 so future directions will be discussed in that summary.

Chapter 4: Non-genomic glucocorticoid regulation of circadian timing

4.1 Introduction

Gcs act, in the mammalian system, through the ubiquitously expressed GR, but are also known to bind to the MR (Funder 1992; Krozowski and Funder 1983). Work in this thesis has shown that the MR has little influence on the phase and period response to Gc but is involved in the amplitude response (both acute and chronic) (Chapter 3). Gcs bind to the unliganded GR within the cytosol, the GR will rapidly translocate to the nucleus (Nishi et al. 2001), where a GR monomer will bind to the GRE containing genes, such as *Per1* and *Per2* with regard to circadian timing (So et al. 2009; Yamamoto et al. 2005). A second monomer will then bind to form a homodimer and this will induce transcription. Although a classical pathway involving the genomic action of GR is now well defined it is also known that responses can occur within minutes and these later responses are insensitive to transcriptional inhibition and cannot therefore be a consequence of *de-novo* transcription. This action is referred to as non-genomic (Croxtall et al. 2000; Croxtall JD 2002). The non-genomic action of Gcs is mediated via signalling molecules such as PI3K (Hafezi-Moghadam et al. 2002) and MAPKs (Solito et al. 2003). Studies have shown that the non-genomic actions of Gcs are, at least in part, associated with membrane bound GR (Gametchu et al. 1993) and this class of receptors has been shown to associate with CAV-1, a protein found in cholesterol rich regions of the lipid bilayer called lipid rafts, or more precisely a subset of these termed caveolae, which are involved in signalling pathways (Matthews et al. 2008). Matthews et al. (2008) showed that the Gc analogue DEX could induce rapid kinase phosphorylation events in minutes and that these were mediated through CAV-1. DEX induced the phosphorylation of CAV-1, Akt and GSK3β within 10 min of treatment, which was lost in *Cav-1* knock-out cells (i.e. no CAV-1 expression and constitutive phosphorylation of both Akt and GSK3β). More importantly, this had no affect on the transactivation potential of GR, so a definite non-genomic signalling effect. GSK3β has also been shown to phosphorylate the central circadian proteins, such as PER2 (Iitaka et al. 2005) and BMAL1 (Sahar et al. 2010), suggesting direct links between the non-genomic actions of Gc, with membrane bound GR, and circadian function. Experiments described in Chapter 3 revealed a very rapid response to CORT for the PER2::luc construct in a time course incompatible with classical genomic signalling. Experiments described in this chapter explore this rapid response system in more detail.

4.2 Aims

- To explore potential non-genomic action of CORT on the clock (outlined in Chapter 3), in particular the mPER2::luc protein reporter.

- To characterise the role of the membrane protein CAV-1 in the action of CORT on the *Per2* transcript and its protein product through the mPER2::luc pulmonary model.

- To build on previous literature and examine links between CAV-1, Akt and GSK3β signalling following CORT administration,

4.3 Results

4.3.1 Cort mediated PER2 protein stability

4.3.1.1 Translational inhibition

To further explore the immediate and potential non-genomic, amplitude effects of CORT on PER2::luc, cycloheximide (CHX) was used to inhibit *de novo* protein synthesis, in the presence or absence of CORT. This technique has been previously used, with PER2::luc to study degradation rates in conjunction with immuno blots (Meng et al. 2008a). Because antibodies to PER2 were not available for immuno blotting the luciferase signal was used. The goal was to test the hypothesis that immediate changes in PER2::luc following CORT treatment were a consequence of altered degradation rates of PER2. Since studies with the non-steroidal agonists induce strong phase shifts but without the concordant rise in PER2::luc expression (Fig 3.18 and 3.19) a further prediction would be that CORT and the non-steroidal agonist may have different impacts on PER2::luc degradation rates following CHX treatment. The results from these experiments clearly reveal that there is no significant difference between the decay rates of the PER2::luc signal in lung slices treated with CORT or the non-steroidal agonist (67a) (Fig 4.1 and 4.2 respectively). In all cases the $t_{1/2}$ was approximately 1.75h. Furthermore, following CORT treatment there was no immediate sustained rise in PER2::luc signal in the CHX treated slices.

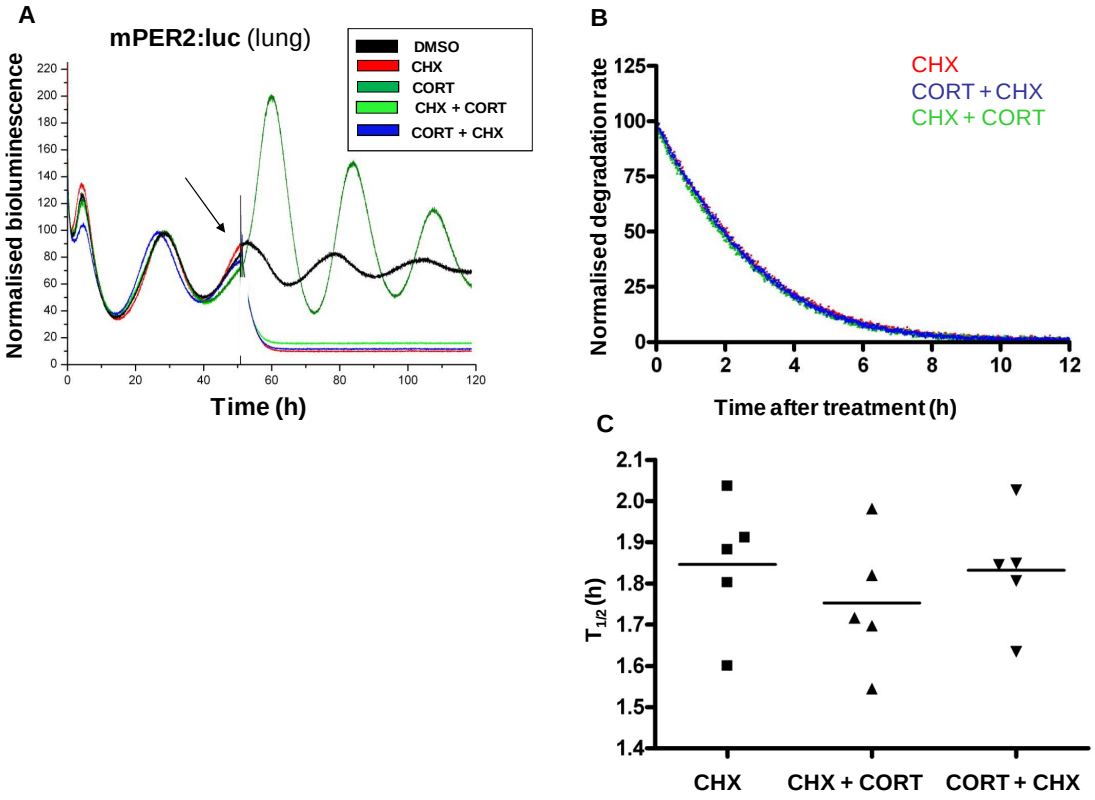

Figure 4.1: Effect of translational inhibition on CORT induced mPER2::luc stability: A representative oscillation of mPER2::luc lung slices after 20µg/ml cycloheximide (CHX) induced translational inhibition with CORT treated either 30 min before or 30 min after CHX (A), with CORT only (dark green) and DMSO (black). Treatment indicated by the arrow (CT12). The degradation rates are shown in (B) with CHX (red), CHX with CORT pre-treatment (blue) and CORT with CHX pre-treatment (light green). The resulting degradation rates are shown in (C) and were calculated using a one phase exponential decay algorithm (GraphPad Prism). Statistical analysis was by One-way ANOVA P= 0.8.

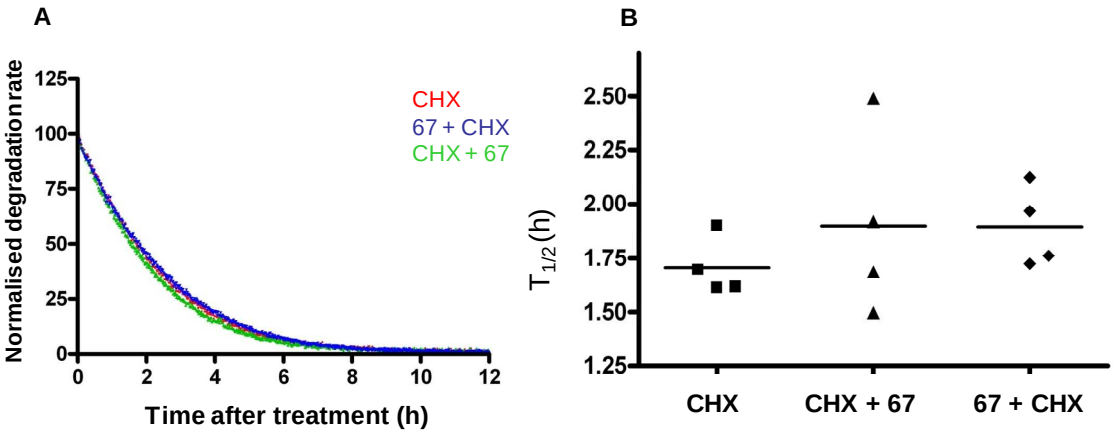

Figure 4.2: Effect of translational inhibition on agonist induced mPER2::luc stability: The degradation rates are shown in (A) with 20µg/ml CHX (red), CHX with agonist (67a) pre-treatment (blue) and 67a with CHX pre-treatment (light green). The slices were treated as previous at CT12 (oscillation not shown). The resulting degradation rates are shown in

(B) and were calculated using a one phase exponential decay algorithm (GraphPad Prism). Statistical analysis was by One-way ANOVA P= 0.5

4.3.1.2 Transcriptional inhibition

The above studies suggested that PER2::luc protein stability rates are not altered by Gc signalling. In order to test the role of the transcription mechanism underpinning the responses, similar studies to those described above were undertaken. In these experiments CORT was added to PER2::luc expressing lung slices 30 min before or after actinomycin D (Act D) treatment. It can be seen (Fig 4.3a) that transcriptional inhibition blocked the immediate amplitude rise in PER2::luc expression following CORT administration. Comparing normalised PER2::luc degradation rates it can be seen that there are no significant differences consequent on the order Act D was administered (Fig 4.3b). Blockade of translation by CHX, without any effect by CORT (Fig 4.1) suggests that the immediate amplitude rise is not due to post-translational modification. Furthermore, blockade of transcription by ActD, without any effect of CORT, suggests that the acute amplitude rise is transcriptionallly mediated and requires *de novo* transcription of *mPer2*. Translational inhibition of mPER2::luc by CHX has previously been used to show more rapid degradation of the mPER2::luc tau protein compared to the WT protein (Meng et al., 2008a), which suggests that both PER2 and the LUCIFERASE proteins are being degraded at the same rate, rather than PER2 being degraded much faster than the LUCIFERASE protein and leading to a false negative result.

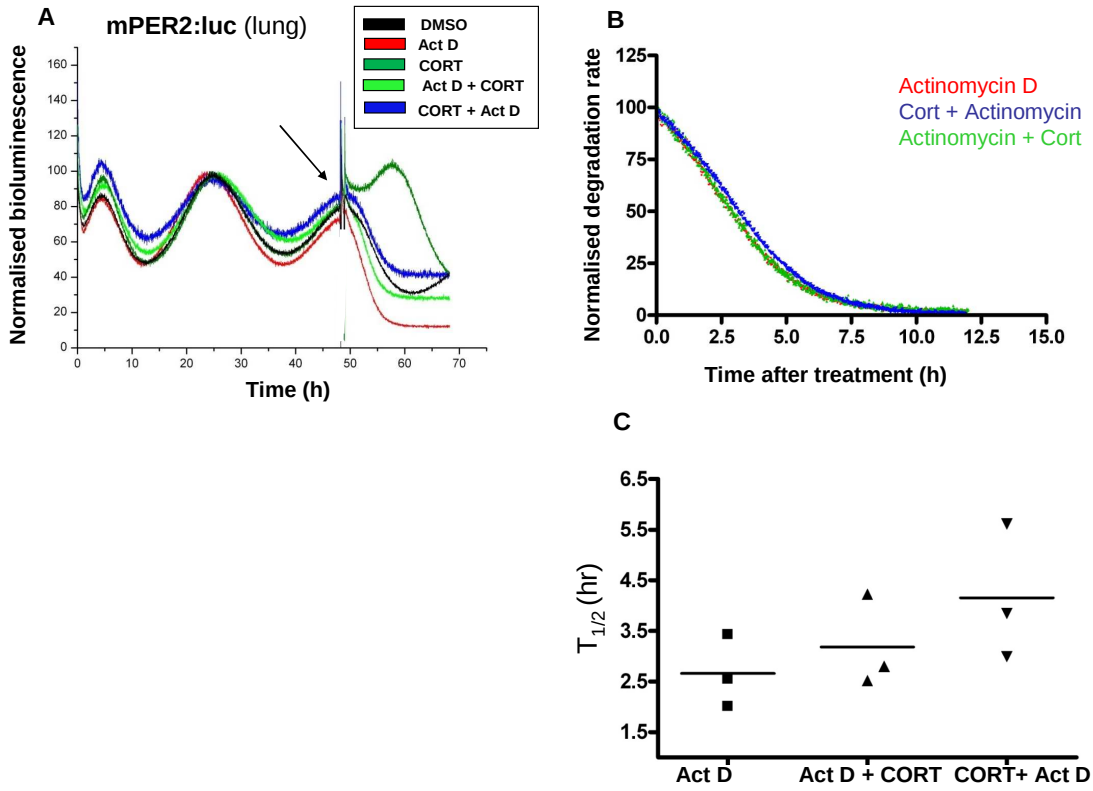

Figure 4.3: Effect of transcriptional inhibition on CORT induced mPER2::luc stability: A representative oscillation of lung slices after 10µg/ml act D induced transcriptional inhibition with CORT treated before or after act D (A), with CORT only (dark green) and DMSO (black). Treatment time is indicated by the arrow (CT12). The degradation rates are shown in (B) with act D (red), act D with CORT pre-treatment for 30 min (blue) and CORT with act D pre-treatment for 30 min (light green). The resulting degradation rates are shown in (C) and were calculated using a one phase exponential algorithm (GraphPad Prism). Statistical analysis was by One-way ANOVA (P= 0.27).

4.3.2 CORT mediated *Per2* transcript induction

The previous section (4.3.1) showed no significant effects of CORT on the degradation rate of the PER2::luc signal (a surrogate measure for PER2 protein turnover) and so here studies were extended to measure the CORT effect on *Per2* mRNA, as the results (Fig 4.3) suggested *de novo* mRNA synthesis. Lung sections were treated at either the projected peak or trough (24 or 36h after synchronisation, section 2.2.5.2) of the PER2::luc protein rhythm (a representative oscillation was not used), to assess the response of CORT in the induction of *Per2*. Lung sections were collected 4h later and the RNA extracted; *mPer2* and *mGILZ* transcripts were quantified by qPCR. The lung slices showed a significant 4-5 fold increase in transcriptional expression to Gc as measured by the induction of the glucocorticoid responsive gene, *mGILZ* (Fig 4.4a). *mGILZ i*s used here as a positive marker of GR mediated genomic activation (D'Adamio et al. 1997). The induced response

was equivalent at both the peak and trough of the PER2::luc rhythm, and with no difference between CORT and the agonist (67a). In contrast, the induction of *mGILZ* to both CORT and the non-steroidal agonist (67a) was completely blocked by the GR antagonist RU, clearly indicating that the responses were largely mediated through the classical genomic signalling pathway (Fig 4.4a). In contrast, the relative induction of *mPer2* mRNA was of lower magnitude to that of *mGILZ* with an approximate 2-fold response to 100nM CORT (comparison of treatment administered at the trough of the cycle, Fig 4.4b). RU had relatively less effect than that observed for mGILZ and consequently did not significantly reduce the *mPer2* response to CORT (Fig 4.4b).

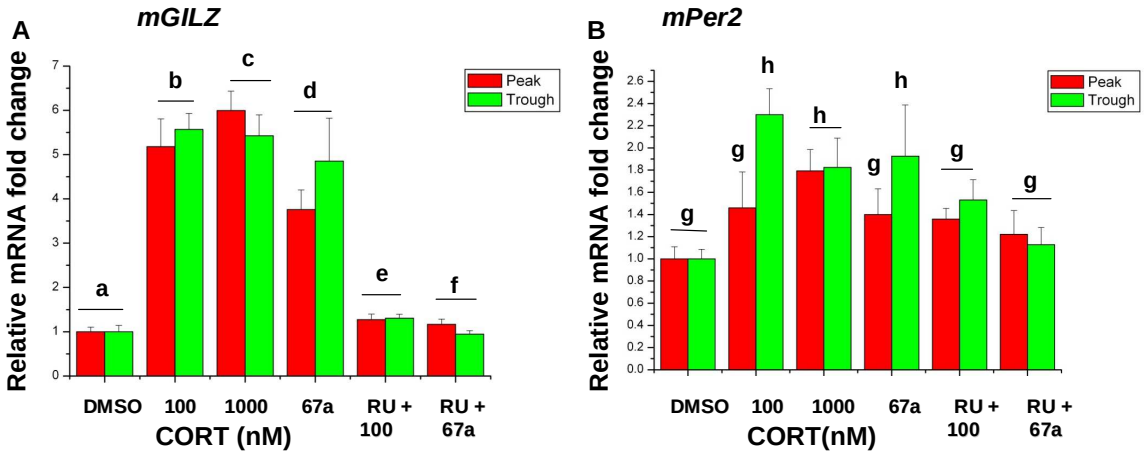

Figure 4.4: CORT and agonist induction of *mPer2* transcript in the lung: CORT or GR agonist (67a, 10nM) was used to treat lung sections, at either the peak (CT12, red) or trough (CT0, green) of the mPER2::luc protein rhythm alone or with 1h pre-treatment with 1µM RU486 (RU), for 4h prior to the RNA extraction. Transcript expression for the positive control *mGILZ* (A) and *mPer2* (B) are shown as fold change relative to the DMSO control. Both *mGILZ* and *mPer2* were assayed from the same RNA sample. Analysis was by Two-Way ANOVA (there is no interaction with regard to time in A: P=0.58 or B: P=0.074) with a Bonferroni post-hoc analysis for treatment. Error bars are SEM of n=3 for each treatment. (a) versus (b), (c) and (d) P<0.001; (b) versus (e) and (d) versus (f) P<0.001; (g) versus (h) P< 0.05.

These experiments were next repeated using Rat-1 cells carrying the *Per2::luc* mRNA reporter (section 2.3.9.2). In these experiments CORT and 67a were administered at the peak of the *Per2::luc* rhythm (24h after synchronisation). CORT was administered here at the peak, as the previous work treating at both the peak and trough of the mPER2::luc rhythm (Fig 4.4) had shown there to be no interaction with regard time (One-Way ANOVA P=0.58 and P=0.074 for *mGILZ* and *mPer2* respectively). Also, all previous experiments were carried out at the peak of the *mPer2* rhythm (either protein or transcript) and robust and reproducible results were observed, and if there was an effect of CORT at the transcript level then it would occur readily at the peak. Although CORT, administered

at the trough, induced a greater *mPer2* response at 100nM CORT this was not replicated at 1000nM nor with regard RU486 treatment or in the *mGILZ* response. Together these data suggest that CORT administration at the trough of the *Per2*::luc rhythm was no different to peak administration.

Similar to the experiments above, using lung tissue, *rGILZ* exhibited a robust response to CORT and 67a and these were totally blocked by pre-treatment with RU (Fig 4.5a). In contrast *rPer2* mRNA induction was of lower amplitude but a significant difference was observed between DMSO and 100nM CORT which was blocked by RU (Fig 4.5b). (A similar magnitude of response was seen with 67a the induction or blockade by RU did not reach significance. Together the results from Fig 4.3, 4.4 and 4.5 suggest that the immediate amplitude rise seen with CORT (and not with the agonist) (Fig 3.18) is as a result of induced *de novo Per2* transcription, yet there is no significant difference between CORT and agonist induction at the transcript level (Fig 4.4 and 4.5), and therefore this difference must be a result of some other, uncharacterised mechanism capable of an immediate response.

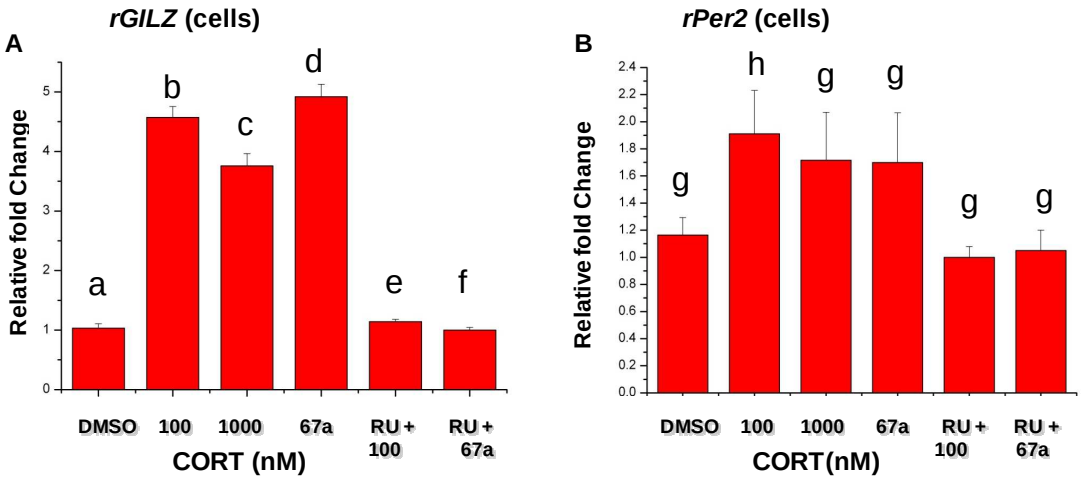

Figure 4.5: CORT and agonist induction of *rPer2* transcript in the Rat-1-*Per2::luc* cell line: CORT or GR agonist (67a, 10nM) was used to treat the Rat-1 cells, at the peak (CT12) of the *mPer2* transcript rhythm alone or with pre-treatment with 1µM RU486 (RU), for 4h prior the RNA extraction. Transcript expression for the positive control *rGILZ* (A) and *rPer2* (B) are shown as fold change relative to the DMSO control. Both *rGILZ* and *rPer2* were assayed from the same RNA sample. Analysis was by One-Way ANOVA (in A: $p<0001$, in B: $P=0.0056$) with a Bonferroni post-hoc analysis. Error bars are SEM of n=3 for each treatment. (a) versus (b), (c) and (d) $P<0.001$; (g) versus (h) $P<0.05$.

4.3.3 The disruption of lipid raft signalling on the circadian oscillation

The studies above suggest that CORT acts on the clock largely through a transcription mediated process. Yet since changes in the PER2::luc protein signal are acute and rapid (Fig 3.3, 3.4, 3.9, 3.10, 3.18 and 4.1) this still suggests a non-genomic mechanism. In all the figures above, it can be seen that increases in bioluminescence are detected within 30 min of treatment (PMT resolution is per min). In order to test whether a rapid non-genomic mediated signalling pathway may be involved the potential involvement of membrane-bound lipid rafts (Caveolae) was investigated. Using the lung slice model the cholesterol rich lipid raft region of the membrane was disrupted with methyl cyclodextrin (section 2.2.5.1). Pre-treatment of lung slices with methyl cyclodextrin followed by CORT completely blocked the expected acute rise in the PER2::luc signal (Fig 4.6a). Following methyl treatment the expected CORT induced phase shift was also blocked (Fig 4.6a and b). There was no significant difference between DMSO and methyl alone. There was no overall period effect of methyl cyclodextrin (with or without CORT, Fig 4.6c), but methyl cyclodextrin did reduce the chronic CORT-induced amplitude effect, similar to the effect with RU (Fig 3.9). In these experiments it was necessary to use a relatively high DMSO concentration to dissolve the methyl cyclodextrin (1% versus the normal 0.1%, a 10-fold increase).

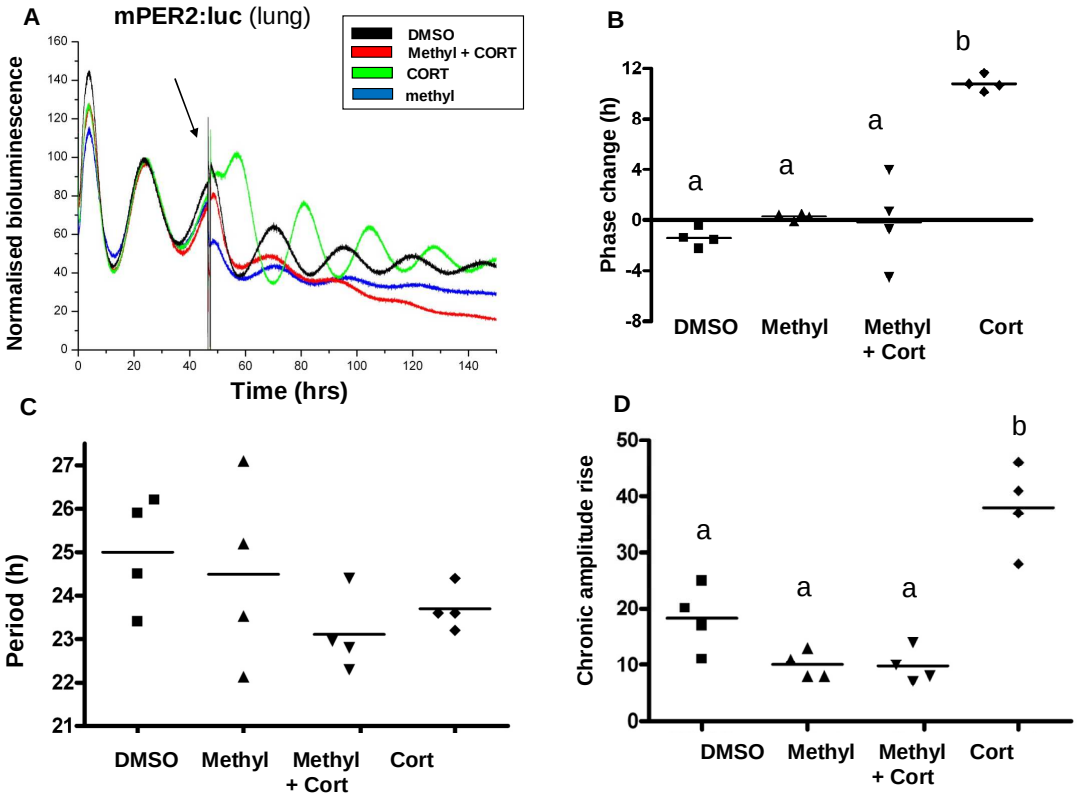

Figure 4.6: Disruption of lipid raft signalling on circadian oscillation of the mPER2::luc protein reporter: A representative oscillation (A) with CORT (green), pre-

treated with 5mM methyl for 30 min then with CORT (red), methyl only (blue) and DMSO (black). Treatments were administered at the arrow (CT12). The phase change relative to the vehicle control is shown (B) and the post treatment periods are shown in (C) with the chronic amplitude rise shown in (D). Dots represent single experiments and statistical analysis is by one-way ANOVA (in B: $P<0.0001$, in C: $P=0.26$, in D: $P<0.0001$) with a Bonferroni post-hoc analysis; (a) versus (b) $P<0.001$.

The above experiments were next repeated using the Rat-1-*Per2::luc* cells (Fig 4.7). Here methyl did not significantly attenuate the CORT-induced phase shift ($P>0.05$), although it did reduce the phase change by approximately 2h. In these data there was a significant interaction between methyl and CORT - the phase shifts such that methyl and methyl with CORT treatment differed significantly (Fig 4.7b), yet there is no significant difference between DMSO and methyl alone. Methyl also significantly reduced the amplitude of the chronic circadian oscillation in these cells (Fig 4.7d). In contrast to the lung slices CORT (with and without methyl) significantly shortened the period of the Rat-1-*Per2::luc* oscillation by approximately 1h (Fig 4.7c). These experiments described above implicate lipid rafts in the rapid responses of PER2::luc to CORT.

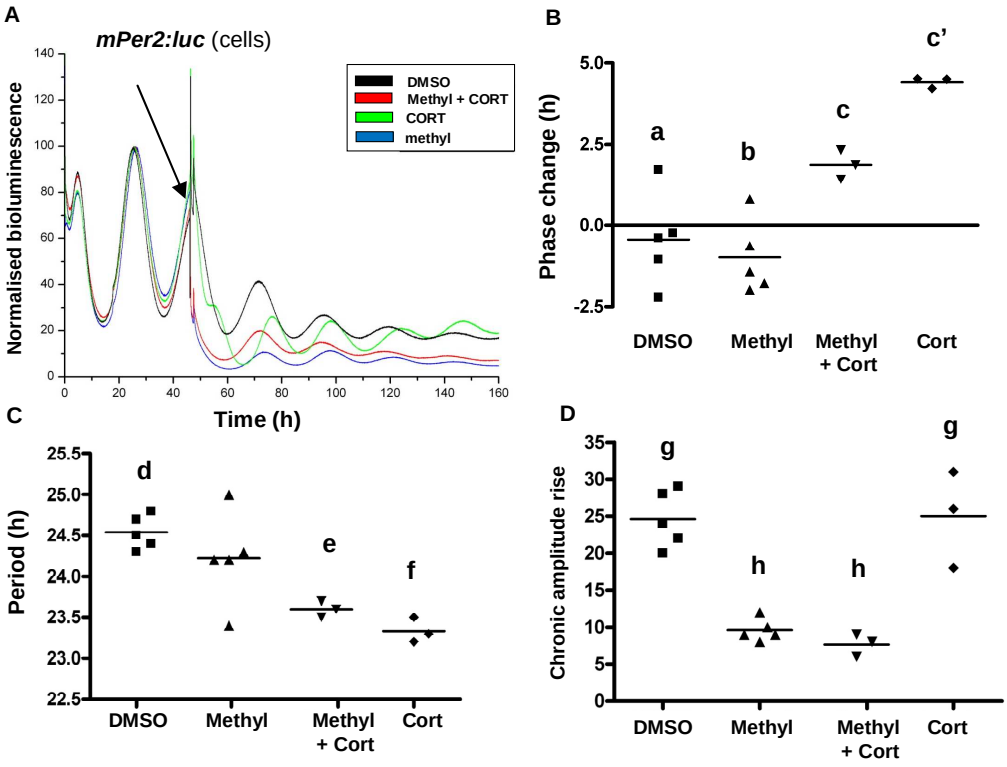

Figure 4.7: Disruption of lipid raft signalling on the circadian oscillation of the *Rat-1-mPer2::luc* promoter reporter: A representative oscillation (A) with CORT (green), pre-treated with 5mM methyl for 30 min then CORT (blue), methyl only (red) and DMSO (black). Treatment time is indicated by the arrow (CT12). The phase change was relative to the vehicle control (B) and the post treatment periods are shown in (C) with the chronic amplitude rise shown in (D). Dots represent single experiments and statistical analysis is

by One-way ANOVA (in B, C and D: P<0.0001) with a Bonferroni post-hoc analysis. In B: (a) versus (b) and (c) not significant; (a) versus (c') P<0.001; (b) versus (c) P<0.05, (b) versus (c') P<0.001 and (c) versus (c') not significant. In C: (d) versus (e) P<0.05 and (f) P< 0.01. In D: (g) versus (h) P<0.001.

4.3.4 *Cav-1* WT and -/- cells and circadian gene expression

4.3.4.1 Differential expression of circadian genes in the *Cav-1* WT and -/- cells

The fibroblast cell line was derived from the thymus gland of *Cav-1* WT and -/- mice (section 2.3.1). Since these cells lack any circadian reporter it was necessary to determine whether loss of *Cav-1* impacted directly on the core circadian clock work. Primary cells are difficult to transfect and the *Cav-1* -/- cells show no contact inhibition in cell culture growth, and for this reason the insertion of a conventional luciferase reporter was not attempted. Accordingly, *Cav-1* WT and -/- cells were synchronised for 1h by forskolin treatment, cells were washed and left in culture for 24h prior to sampling at the presumptive CT0 and CT12. qPCR determination of *mBMAL1* showed high amplitude differences between CT0 and CT12 in WT cells and a reduced but significant difference in *Cav-1* -/- cells (Fig 4.8a). *mPer2* expression revealed an anti-phase pattern (with respect to *BMAL1*) with high amplitude changes in both WT and -/- cells (Fig 4.8b). Collectively, these data reveal that the positive (*BMAL1*) and negative feedback (*Per2)* arms of the clock show predicted changes at 2 circadian time points in both Cav-1 WT and -/- cells.

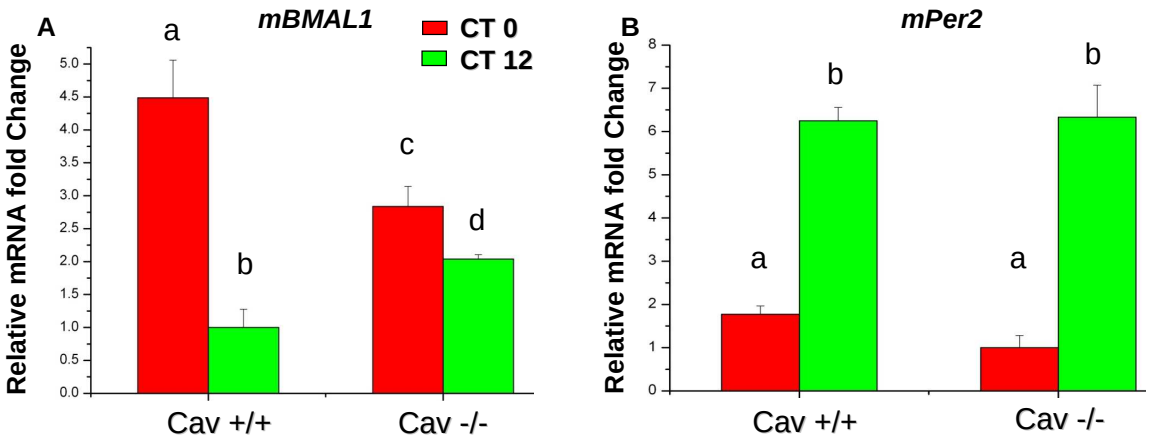

Figure 4.8: Temporal *mBMAL1* and *mPer2* expression in WT and *Cav-1* -/- cells: Relative transcript expression is shown at CT0 (red) and CT12 (green) for *BMAL1* (A), *Per2* (B). Expression is represented as fold change relative to the lowest expression. Error bars are SEM of n=3, *Per2* and *BMAL1* expression was quantified from the same samples. Statistical analysis was by Two-way ANOVA (in both A and B: P<0.0001 for time interaction and in A: P=0.17 and in B: P=0.21 for genotype interaction) with a Bonferroni post-hoc analysis, (a) versus (b) and (c) P<0.001, (c) versus (d), (b) versus (d) P<0.05, (e) versus (f) P<0.01.

4.3.4.2 CAV-1 effects on the CORT mediated expression of *mPer2*

In order to compare a time course of response to CORT and 67a, *Cav-1* WT and -/- cells were treated for 1h, 2h and 4h and 2h and 4h with CORT and 67a. Treatment with CORT induced a small but significant increase in *mPer2* expression at 1h in WT cells and the response was further augmented at 2h and 4h (Fig 4.9). The treatment with 67a also strongly induced *mPer2* expression at 2h and 4h (Fig 4.9). (The data shown in both Fig 4.9 and 4.10 are CORT or 67a treated samples as a fold induction over their respective DMSO control in order to show a time effect. These data are re-plotted in appendix J along with their respective DMSO control to show time point induction over control values).

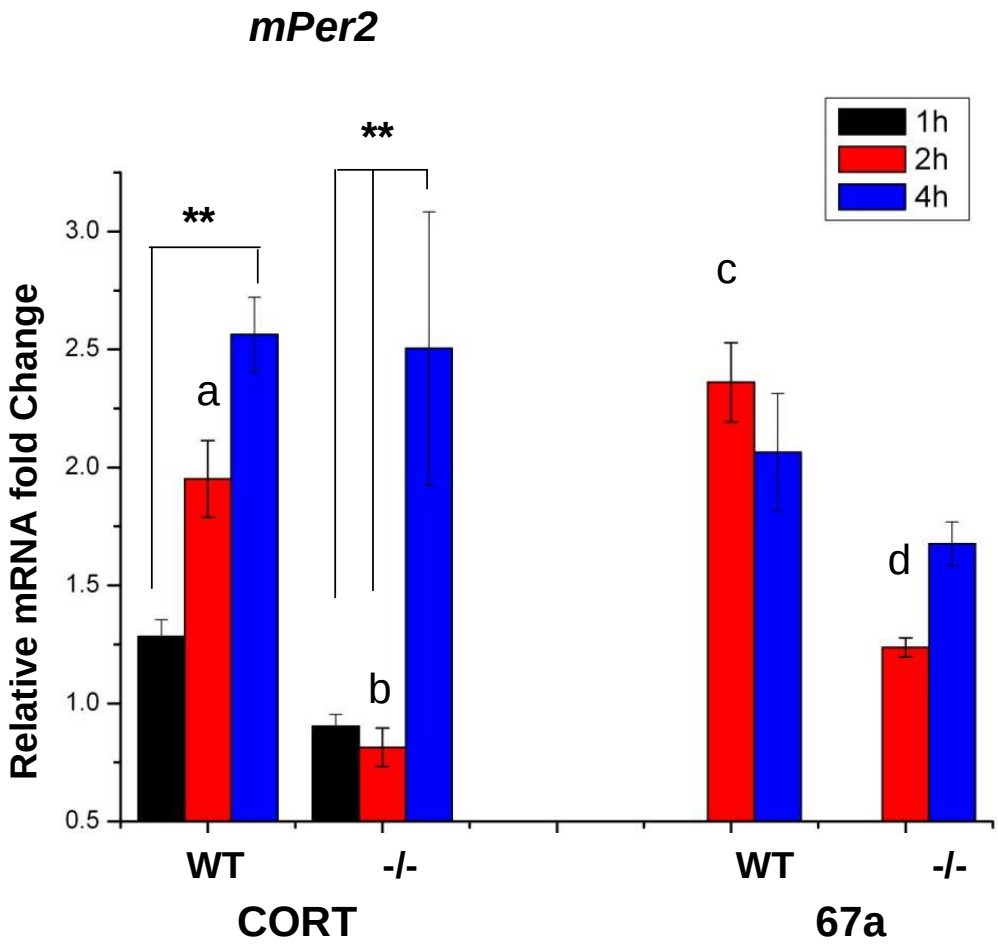

Figure 4.9: CORT and non-steroidal GR agonist-induced *mPer2* expression over 1h, 2h and 4h in *Cav-1* WT and -/- cells: *Cav-1* WT or -/- cells were treated with either CORT or the non-steroidal agonist (67a) for 1h (CORT only, black), 2h (red) or 4h (blue) prior to RNA extraction and qPCR analysis for *mPer2*. Data are shown relative to their respective DMSO control (fold change of 1) in order to show time effects of CORT; a further representation is shown in appendix J. Statistical analysis was by two-way ANOVA ($P<0.05$ for time and genotype with CORT and 67a) with a Bonferroni post-hoc analysis and error bars are SEM of n=3. ** $P< 0.01$, (a) versus (b) $P< 0.05$; (c) versus (d) $P< 0.001$.

In contrast, *Cav-1* -/- cells showed no significant responses at 1h and 2h for CORT but similar increase in amplitude responses at 4h to those seen in WT cells (Fig 4.9 and appendix J). The 2h response to CORT was significantly reduced in the *Cav-1* -/- cells with respect to the WT (Fig 4.9, red bars) but no difference was observed a either 1h or 4h. Similarly the 2h expression of *mPer2* was significantly reduced in the presence of 67a (Fig 4.9, red bars) but no difference was observed at 4h. In contrast, *mGILZ* showed significant responses to CORT at 1h, 2h and 4h in WT cells and to 67a at 2h and 4h (Fig 4.10). Responses of *Cav-1* -/- cells to CORT and 67a were significantly augmented at 2h and 4h but overall the amplitude of response was significantly lower than the WT cells at 4h

(blue) for CORT and 2h (red) and 4h (blue) for 67a. (Again the data in Fig 4.10 are re-plotted in appendix J for a direct comparison to DMSO).

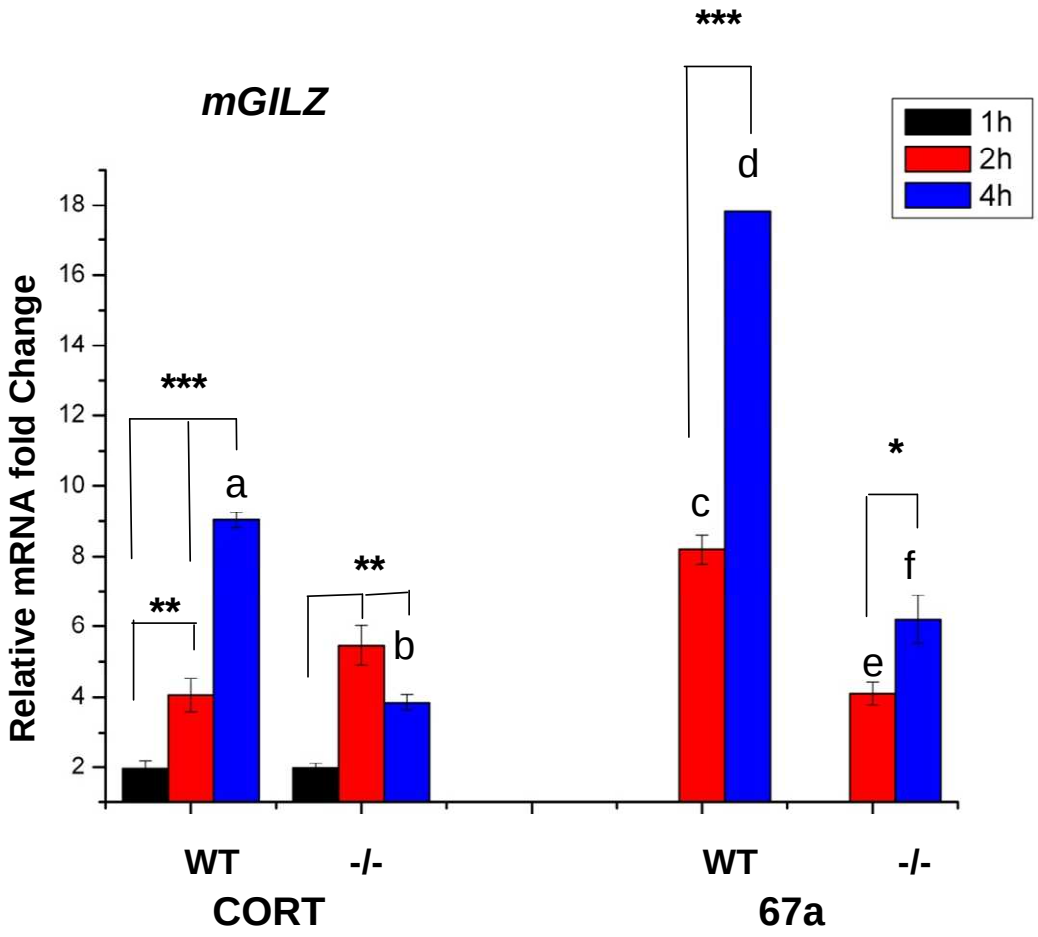

Figure 4.10: CORT and non-steroidal GR agonist-induced *mGILZ* expression over 1h, 2h and 4h in *Cav-1* WT and -/- cells: *Cav-1* WT or -/- cells were treated with either CORT or the non-steroidal agonist (67a) for 1h (CORT only, black), 2h (red) or 4h (blue) prior to RNA extraction and qPCR analysis for *mGILZ.* Data are shown relative to their respective DMSO control (fold change of 1) in order to show time effects of CORT; a further representation is shown in appendix J. Statistical analysis was by two-way ANOVA ($P<0.0001$ for time and genotype with CORT and 67a) with a Bonferroni post-hoc analysis and error bars are SEM of n=3. * $P< 0.05$, ** $P< 0.01$, ***$P< 0.001$; (a) versus (b) $P< 0.001$; (c) versus (d) $P< 0.001$ and (e) versus (f) $P< 0.001$.

4.3.5 Inhibition of phosphatidylinositol 3 (PI3) kinase pathway

To attempt to further investigate the signalling pathway behind the *Cav-1* mediated attenuation of CORT induction of *mPer2* transcription (Fig 4.9), the signalling molecule PI3 kinase was inhibited and the action of CORT on *mPer2* transcription was assayed. This pathway was chosen specifically due to previous work characterising the relationship between rapid Gc signalling and PI3 kinase (Matthews et al. 2008). PI3 kinase plays an

important role in mediating *Cav-1* signalling and lies up-stream of the kinase PKB, which is involved in *Cav-1* signalling. A prediction would be that inhibition of PI3 kinase should block or attenuate the induction of *mPer2* following CORT administration. Cells were pre-treated with LY294002 (section 2.3.9.2) for 1h prior to administration of 100nM CORT, in the *Cav-1* WT cells. Cells were sampled at 2h and 4h. There was a significant induction in *mPer2* expression after CORT administration after 2h and 4h (1.9-fold and 3-fold respectively). PI3 kinase inhibition had a weak inhibitory effect, in the presence of CORT, at 2h, but a weak inductive effect at 4h. However, PI3 kinase inhibition did not significantly alter the responses to CORT at either 2h or 4h (Fig 4.11a and b).

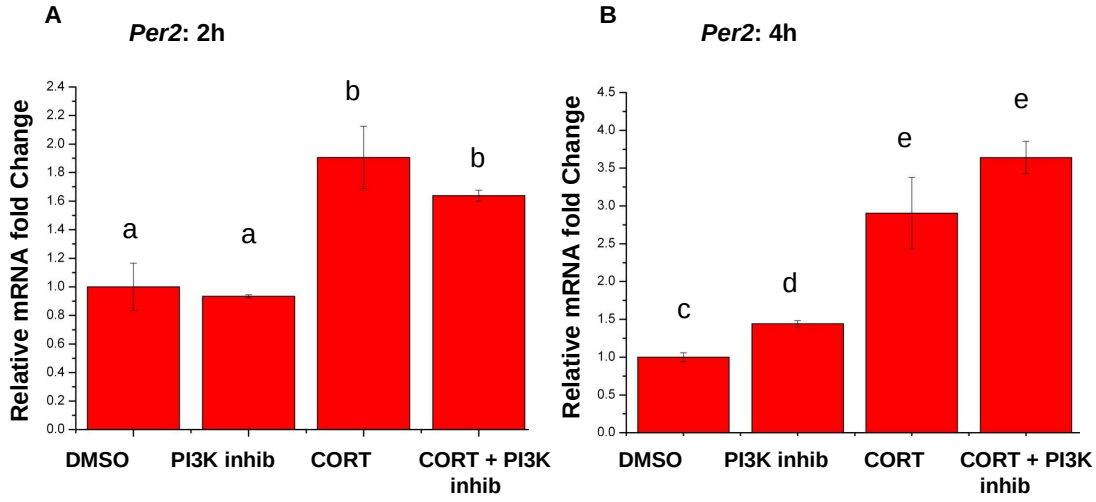

Figure 4.11: PI3 kinase inhibition of *mPer2* transcript induction after CORT: CORT or 50µM PI3 kinase inhibitor were added to *Cav-1* WT cells for 2h (A) or 4h (B) prior to RNA extraction and the *mPer2* transcript was measured. Transcript expression is fold change relative to the DMSO control. Error bars are SEM of n=3. Statistical analysis is by One-way ANOVA ($P<0.0026$ for both A and B) with a Bonferroni post-hoc analysis, (a) versus (b) $P<0.001$; (c) versus (e) $P<0.001$, (c) versus (d) $P< 0.01$ and (d) versus (e) $P<0.001$.

4.3.6 Mechanistic overview of known genomic and non-genomic CORT inducted interactions

The classical genomic pathway shows CORT passing through the bilayer and into the cytoplasm where it binds to the GR and the chaperone proteins are released, the GR becomes active, by phosphorylation, and translocates to the nucleus where it will induce transcription of GRE containing genes, *Per1* and *2* are the circadian genes. This generally takes between 2 and 4h to produce a maximal response. Coupled to this CORT can induce rapid amplitude effect on the PER2 protein, seen as a surrogate luciferase signal (Fig 3.18), which are too rapid to be genomic based. This effect has been proposed here to act

through membrane bound CAV-1 and associated GR (Matthews et al., 2008) and a kinase signalling cascade which involves the phosphorylation and subsequent activation of PKB; the phosphorylation and inactivation of GSK3β (Beaulieu et al. 2004) and the activation of MAPK p42/44 (Goold and Gordon-Weeks 2005). The inactivation of GSK3β results in the stabilisation and subsequent retention of PER2 in the cytoplasm, which is then phosphorylated by other kinases such as CK1ε, δ and CK2 for subsequent but delayed protein turnover (Gallego and Virshup 2007; Iitaka et al. 2005; Lowrey et al. 2000). CAV-1 has a direct transcriptional effect on *Per2* too, which is different to that seen with *GILZ* induction (Fig 4.9 and 4.10), hence it is a *Per2* specific effect. In this Chapter, I have shown that disruption of Caveolae signalling using methyl cyclodextrin inhibits the rapid rise in the PER2::luc signal and inhibits the phase change induced by CORT, and that knocking out Cav-1 has a direct effect on *mPer2* mRNA expression, as shown by the red dashed arrows in Fig 4.12.

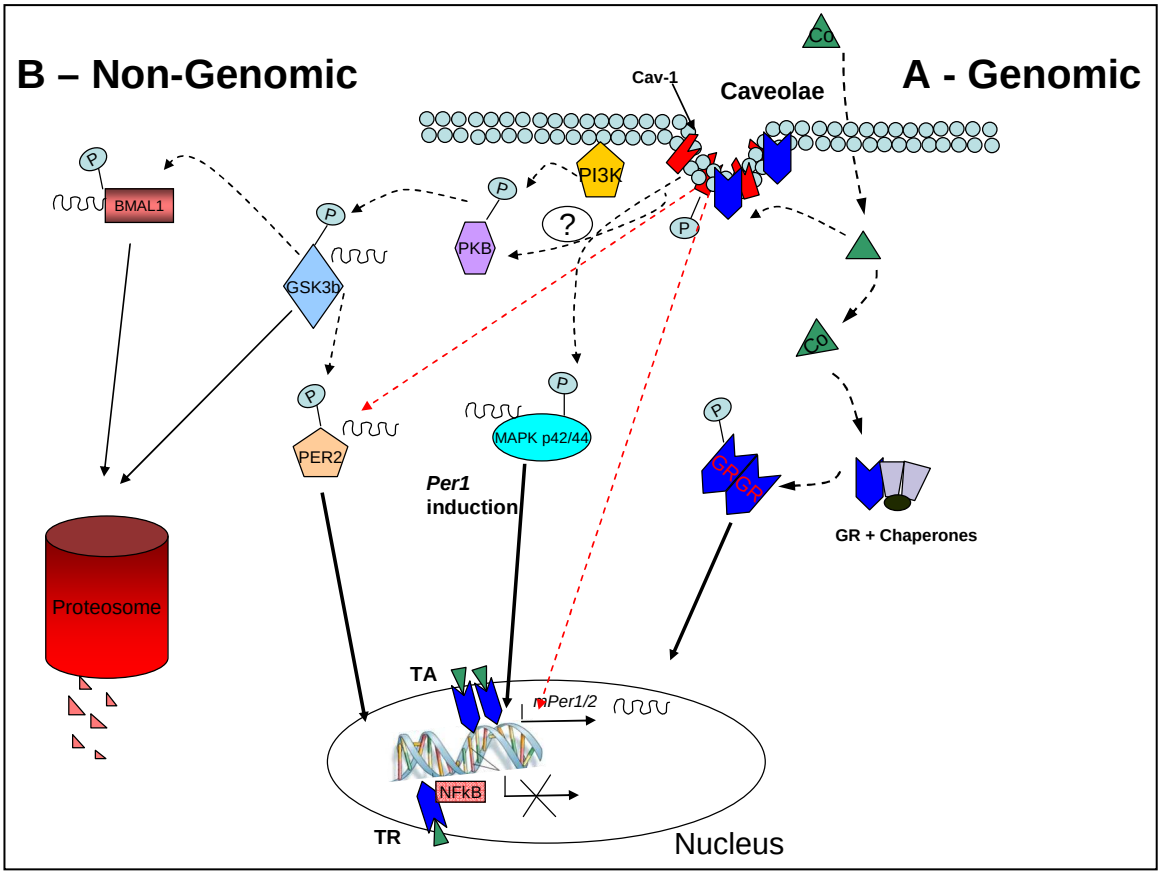

Figure 4.12: Diagram of rapid (non-genomic) and slow (genomic) actions of CORT: The black-dashed curly arrows depict known phosphorylation events. The solid black arrow shows translocation events and the red-dashed arrow shows the events described in this thesis. All proteins are labelled, glucocorticoid receptor (GR), CORT (co), transactivation (TA) and transrepression (TR). The oscillation next to BMAL1, PER2, p-MAPKp42/44 and p-GSK3β show that they are rhythmic.

4.4 Discussion

4.4.1 PER2::luc protein amplitude response

It is becoming more evident that Gcs have a rapid, non-genomic, action on the cell (Croxtall 2002) as well as the more established 'classical' genomic action (Nishi et al. 2001). In this thesis I have shown a clear, rapid amplitude effect of CORT on the clock protein PER2 (Fig 3.3) and that this rapid effect is not seen in the presence of novel non-steroidal GR specific agonists (Fig 3.18). Furthermore, this rapid amplitude rise can be inhibited by GR and MR antagonism (Fig 3.9 and 3.10 respectively) suggesting that the effect is mediated through these two receptors. There is less published evidence to support a role for MR-mediated induction of the clock by CORT. There is some evidence that the MR is involved in non-genomic signalling, at lease in the brain (Karst et al. 2005). However, further work on the non-genomic re-setting effects of either receptor was not investigated in this thesis.

The rapid amplitude rise of PER2 after CORT treatment was not a product of immediate post-translational modification events, since CORT treatment after protein synthesis inhibition did not result in altered degradation rates (Fig 4.1). However, it is known that active GSK3β can phosphorylate PER2 and induce nuclear translocation (Iitaka et al. 2005), resulting in the inhibition of CLOCK/BMAL1 mediated transcription (Sato et al. 2006a). If GSK3β function is increased, this can induce a phase advance, whereas a decrease in function results in a phase delay (Iitaka et al. 2005). So the lack of an apparent increase in protein stability, after protein synthesis inhibition, in the presence of CORT may be due to the complexity of post-translational interactions; as with GSK3β mediated phosphorylation of PER2, this essentially results in nuclear localisation and subsequent degradation, rather than stabilisation. It must be considered here that the degradation assays are just a direct measure of luciferase degradation and not PER2 itself. The degradation of PER2 is inferred as the luciferase protein is fused to the C-terminal of the PER2 protein (Yoo et al. 2004). Furthermore, as predicted from the PER2::luc protein oscillations (Fig 3.18) the presence of the GR agonist had no stabilisation effects on PER2, after protein synthesis inhibition. Despite similar degradation rates following CORT or agonist treatment there was a clear difference in the pattern of PER2::luc induction. Since effects of CORT after transcriptional inhibition (Fig 4.3) caused no difference in PER2::luc degradation suggesting that the CORT effects on PER2::luc are likely to be a

transcriptional-dependent process. At a transcript level, there was no difference between the *Per2* induction by CORT or the agonist 67a, treated at either the peak or trough of the PER2::luc rhythm, in the lung, or treated at the peak of the *rPer2* transcript rhythm in the Rat-1 cells. There was perhaps a small attenuation of induction by 67a, at the peak in the lung, but this was not replicated at the trough or in the Rat-1 model nor did it reach significance. In summary, the different immediate temporal events observed after CORT or GR agonist treatment could not be readily explained by either post-translational modification events or transcriptional differences in *mPer2*. There must yet be a further explanation.

4.4.2 Non-genomic Caveolae signalling on the clock

Evidence had suggested that the GR could associate with the membrane bilayer with a larger molecular mass to the more common cytoplasmic form (145kDa and 95kDa respectively), and with a separate function to the cytoplasmic/nuclear GR (Gametchu et al. 1993). The GR has been shown to co-precipitate with CAV-1 (Matthews et al. 2008), a major protein component of a sub-set of lipid rafts which are involved in rapid kinase signalling events (Matthews et al. 2008; Okamoto et al. 1998). The compound methyl cyclodextrin is part of a family of compounds which are used to regulate cholesterol insertion into the membrane bilayer. This compound is very non-specific but will indicate lipid raft-mediated membrane signalling and was used to indicate the potential involvement in the immediate amplitude effects of CORT on PER2. Methyl cyclodextrin had three effects on the PER2::luc rhythm; it inhibited the CORT-induced acute amplitude rise, similar to that seen with the GR and MR antagonists (Fig 3.9 and 3.10); it inhibited the phase shifting effects of CORT, reducing it to a level relative to the vehicle control (Fig 4.6) and it inhibited the CORT-induced chronic amplitude rise (Fig 4.6). Period effects were not seen in the PER2::luc pulmonary reporter. The phase and chronic amplitude inhibition were also seen with the Rat-1-*Per2* promoter reporter (Fig 4.7), again similar to that seen with the GR and MR antagonists (Fig 3.9 and 3.10). Interestingly in the Rat-1-*Per2::luc* reporter CORT significantly shortened the period of the resulting oscillation (Fig 4.7c), yet this was not inhibited by methyl. This was in direct contrast to the inhibition of the period shortening effects by GR blockade with RU (Fig 3.11), suggesting independent actions on period clock function between membrane based and classical genomic action of CORT. However, the rapid kinase signalling events induced by DEX were inhibited by RU mediated GR blockade (Matthews et al. 2008). It is also known that inhibition, or

knock down of GSK3β, results in a period shortening of approximately 2h, using a *BMAL1::luc* reporter (Hirota et al. 2008), which complements the effects seen with CORT and subsequent RU blockade. This cannot yet explain the lack of attenuation of period shortening by methyl cyclodextrin, as this disrupts PKB inactivation of GSK3β, by phosphorylation; therefore GSK3β theoretically remains active in the presence of methyl.

The *Cav-1* -/- cells were used to enhance the studies using methyl cyclodextrin. A significant draw back to using this model was that the cells did not have a circadian reporter, transcript or protein, to study and analyse. Instead, the endogenous *mPer2* transcript was used to report the effect of CORT on the *Cav-1* WT and -/- cells. The CORT induction of *mPer2* transcript was not observed in the *Cav-1* -/- cells at 1h and 2h compared to the WT construct but induction was equivalent at 4h. This suggested that CAV-1 is involved in the immediate induction of *mPer2* transcription. Responses seen at 4h suggest that CORT was functioning through a classical genomic mechanism. Blockade of GR signalling, by RU, would block predicted rapid non-genomic and slower genomic mediated pathways. Recent studies have shown this is the case (Matthew (2008) and Fig 3.9). In contrast to the *mPer2*, *mGILZ* induction was relatively unaffected by the loss of CAV-1 although the *Cav-1* -/- cells did exhibit a reduced amplitude of response to CORT (Fig 4.10). A further prediction from these studies is that the non-steroidal agonist (67a) may be expected to act exclusively through genomic mediated pathways. However, data shown here suggest that loss of CAV-1 also significantly alters the response to the agonist with greatly attenuated amplitudes of response at 2h (Fig 4.9 and appendix J). However, conclusions over the origin of the protein expression (Fig 3.18) differences cannot be fully made form these results.

The loss of CAV-1 did not alter rhythmic expression of *mPer2* but there was a significant dampening of *mBMAL1* expression over the two time points studied, yet to fully characterise the response CT6 and CT18 time points should be analysed (Fig 4.8). This may be a consequence of GSK3β mediated phosphorylation. GSK3β can phosphorylate BMAL1, which targets it for proteasomal degradation (Sahar et al. 2010). One model is that in the *Cav-1* -/- cells, GSK3β is not inactivated by PKB (Akt) phosphorylation, and could therefore phosphorylate BMAL1 at all times resulting in degradation; hence reducing the amplitude of the protein rhythm and feeding back to result in an almost constitutive transcript rhythm (Fig 4.8). Further to this, GSK3β is known to phosphorylate

and stabilise REV-ERBα protein (Yin et al. 2006), which acts to repress *BMAL1,* resulting in much lower transcript expression. However, it is known that BMAL1 does not need to oscillate to induce a functional *Per2* rhythm (Liu et al. 2008). This may explain why despite dampened *mBMAL1* expression in the *Cav-1* -/- cells *mPer2* expression is as robust as in the WT cells (Fig 4.8b). Other clock genes such as *mCry1* were not analysed, so the expression pattern of *mPer2* does not necessarily mean that the entire circadian complex was rhythmic, as *Per2* is known to oscillate independent, of a functional clock (Kornmann et al. 2007).

4.4.3 PI3 kinase inhibition

In an attempt to try and identify an individual signalling pathway, downstream of the membrane based CAV-1 signalling, the effect of CORT on *mPer2* induction after PI3 kinase inhibition was addressed. This pathway had been shown to mediate the Gc induced rapid signalling events resulting in PKB and GSK3β phosphorylation (Hafezi-Moghadam et al. 2002; Matthews et al. 2008), which has reported downstream effects on clock protein (Iitaka et al. 2005; Sahar et al. 2010). The inhibition of PI3 kinase directly inhibits the phosphorylation of PKB (Wu et al. 2008) which therefore remains inactive, and results in an unphosphorylated, active GSK3β, which can phosphorylate PER2. PI3 kinase inhibition had direct opposite effects depending on the length of its exposure and the presence of CORT did not affect this. After 2h PI3 kinase inhibitor alone had no effect on *mPer2* induction yet together with CORT resulted in a reduced *mPer2* expression, although this was not shown to be significant (Fig 4.11). This result complies with the hypothesis that CORT is working through CAV-1 and via the PI3 kinase pathway as the same net result is reached with *Cav-1* -/- cells and PI3 kinase inhibition after 2h, that is *mPer2* expression is reduced, however, firm conclusions cannot be drawn. A greater response may be gained with other, more potent PI3 kinase inhibitors, such as wortmannin, which has an IC_{50} value of 7nM compared to 10μM of LY294002, used in this study (Wu et al. 2008). However, after 4h incubation with CORT and the PI3 kinase inhibitor, the opposite effect was seen. The PI3 kinase inhibitor alone significantly induced *mPer2* over basal levels (Fig 4.11), which was augmented by the presence of CORT, yet did not significantly induce *mPer2* over CORT alone. This result is not coincident with the earlier model proposed for 2h induction, suggesting that either other pathways are causing the effect, or that the inhibitor is no longer working as the half life of LY294002 is 3.5h (Balciunaite and Kazlauskas 2001), which means at the 4h time point the inhibitor has

been exposed to the cell for 5h (added 1h before CORT) and well over a half life has passed.

4.4.4 The proposed genomic and non-genomic model

CORT has been shown to induce rapid non-genomic signalling events mediated through *Cav-1* and PI3 kinase cascade (Matthews et al. 2008), which includes PKB phosphorylation and inactivation of GSK3β (Cross et al. 1995) (drawn up on diagram, Fig 4.12). In accordance with previous work, disruption of lipid raft (CAV-1 signalling) via methyl cyclodextrin or *Cav-1* -/- resulted in the disruption to this described cascade, which affected down stream targets. In the case of the work reported here, the target measured was the *mPer2* transcript and the luciferase surrogate of the mPER2 protein. Active GSK3β is known to have a significant role in the post-translational modification of clock proteins with its phosphorylation of PER2 (Iitaka et al. 2005), BMAL1 (Sahar et al. 2010), CRY1 (Harada et al. 2005) and REV-ERB α (Yin et al. 2006). GSK3β exhibits a circadian phosphorylation rhythm, peaking in phase with PER2 expression (Iitaka et al. 2005), and as such this may implicate PKB as a rhythmic component directly upstream of GSK3β. Direct involvement of kinsase PKB, GSK3β, MAPKp42/44 was not shown in this thesis but it would be an interesting to hypothesise that they are involved in mediating the immediate response of PER2::luc to CORT administration similar to that shown by Matthews et al. (2008), and that the agonists 67a and 69a do not activate such pathways. *Cav-1* -/- cells were also shown to have a direct, although transient, effect on *mPer2* transcription, which has not been shown, or even implicated, previously in the literature. As a result it is unknown how this is mediated, whether it is via constitutive inactivation of GSK3β, as shown by Matthews et al. (2008) or some other pathway.

4.5 Conclusions

It has been shown here, and in Chapter 3, that there is a rapid change in PER2::luc signal by CORT, but not with the GR agonist, which could point to a non-genomic mechanism via post-translational modifications such as phosphorylation. However, this appears not to be the case as CORT had no effect on PER2::luc decay rates after protein synthesis inhibition. The transcript responses to CORT and the novel agonist were identical. These are novel findings and are currently under further investigation.

For the first time here, a direct link between the circadian clock, *Per2* transcript and respective protein product, has been made with membrane bound caveolae (lipid raft) and CAV-1 based signalling. Disruption of the lipid raft with methyl cyclodextrin can inhibit both phase and amplitude effects of CORT in the lung and Rat-1 cell models. Furthermore, knocking out *Cav-1* in the cell has a transient effect on CORT induction of *mPer2* transcription, as seen at 2h but not at 4h. Also the GR agonist failed to induce a significant *mPer2* induction at either time, leading to potential mechanistic differences. For the first time *Cav-1* knock out cells were shown to have robust *mPer2* rhythm but a dampened *mBMAL1* rhythm, potentially leading to clock based perturbations.

The CAV-1 mediated CORT induction on *mPer2* was not shown to be mediated via the PI3 kinase signalling pathway, as small molecule inhibition of PI3 kinase did not replicate the effects seen with the *Cav-1* knock out cells. At 2h there was a small, yet insignificant, reduction but this turned into a small induction after 4h. This suggests either that PI3 kinase signalling is not involved or that the inhibitor is ineffective.

4.6 Future directions

- What is the extent of the CAV-1 influence on the circadian clock? Will the clock continue to oscillate in *Cav-1* -/- cells, and if so is it dampened or induced?

- In *Cav-1* -/- cells how is the cytoplasmic/nuclear distribution of PER2 affected, is turnover increased or is it stabilised, perhaps a parallel with the tau mouse/hamster?

- To further investigate the signalling pathway through which CORT is signalling. It is not through the PI3 kinase pathway, so it may well be acting through the MAPK pathway and therefore such inhibitors should be used. Also this pathway should be tested in relation to the difference between CORT and the GR agonist as there is a clear difference in action, between the two compounds, at the protein level.

- The activation of kinases such as GSK3β, Akt and MAPK p42/44 also needs to be investigated after CORT and agonist treatment of lung slices/primary cells to identify differences between CORT and agonist activation within the first few hours following treatment.

- Recent work by Wang et al. (2009) has provided an association between TLR4 and CAV-1, where the presence of CAV-1 reduces the LPS induction of IL-6 and TNFa, in macrophages. Does this association contribute to the CORT induced inflammatory suppression? What would happen in *Cav-1* -/- cells, would the gating be lost? Does this CAV-1 association have any part to play in the increased expression in *Rev-erbα* knock out cells?

- What effect does the loss of *Cav-1* have on the whole animal circadian phenotype and what would the response to LPS i.p. challenge be? Increased mortality?

Chapter 5: The gated inflammatory response

5.1 Introduction

The innate (or non-specific) immune system is the organism's initial defence against invading pathogens, chemical or physical attack where a rapid response is needed to prevent further widespread damage to the body and to initiate the repair process. Inflammation, a by-product of innate immunity, is characterised by swelling, heat, redness and pain around the compromised area followed by a gradual loss of function within the affected organ (Pickup 2004). Innate immunity involves several cell types but most important are those derived from the mononuclear phagocyte lineage, such as macrophages (Hansson et al. 2002). Macrophages have membrane receptors, such as TLRs, that recognise invading pathogens and respond by triggering signalling cascades that activate, amongst others, NF-κB and MAPK pathways (Guha and Mackman 2001), leading to the production of many cytokines (IL-6 and TNF α), chemokines (IL-8 and CCL2/MCP1) and transcription factors (i.e. p50 and c-Rel) (Guha and Mackman 2001).

Several elements of the innate immune response are thought to be under endogenous circadian control, even in the absence of pathogen attack, including serum levels of cytokines and chemokines (Cutolo et al. 2003; Guan et al. 2005) but also lymphocyte proliferation (T and B cells), part of the adaptive immune response (Hiemke et al. 1995). Isolated macrophages, in mice and man, have been shown to express all the core clock genes and oscillate with a robust rhythm (Guan et al. 2005; Hayashi et al. 2007; Keller et al. 2009) as well as other major tissues of the immune system, such as the spleen and lymph nodes (Keller et al. 2009).

The majority of studies in this field have looked *in-vivo* and *in-vitro* at responses to LPS challenge. LPS is a component of the gram negative bacterial cell wall which activates TLR4, which then induces a potent immune, and therefore, inflammatory response. LPS can induce rhythmic secretion of both IL-6 and TNF-α which is independent of systemic cues, like glucocorticoids, suggesting that secretion is under endogenous circadian control (Keller et al. 2009). Furthermore, direct links have been made between individual clock genes and cytokine production. Knock down of *BMAL1* inhibited *CCL2*/*MCP1* and *NFκB* production at the transcript level, whereas CLOCK/BMAL1 expression vectors induced its expression, albeit weakly (Hayashi et al. 2007). PER2 has also been shown to regulate IFN-γ and IL-1β after LPS administration. Both are down regulated in *Per2* deficient mice (Liu et al. 2006b), which has the effect of increasing LPS induced mortality, suggesting

that PER2 has immune stimulatory properties. REV-ERBα has also been shown to be involved in the secretion of MCP-1 and TNF-α, in an LXRα dependent manner (a transcription factor involved in *Rev-erbα* transcript induction) (Fontaine et al. 2008). Interestingly LPS can also induce a circadian phase delay, by approximately 43 min, if administered in the early part of the subjective night (CT15) (Marpegan et al. 2005), which is not augmented by a simultaneous light pulse. Individual clock gene, such as *Per1* transcription can be transiently induced after LPS challenge, in specific brain regions, like the PVN (Takahashi et al. 2001), which is a region that releases CRF in response to stress (section 1.6). This acute induction, however, does not occur in the SCN or periphery, as seen in the liver, although other reports show that transient suppression in the SCN does occur after LPS challenge (Okada et al. 2008). This shows a direct regulation of the circadian clock by LPS and that the clock has an important role in cytokine synthesis and secretion.

A number of inflammatory diseases, such as asthma, (Martin et al. 1991) and rheumatoid arthritis (Cutolo et al. 2008), are known to have a circadian component to their pathology symptoms with the hypothalamic-pituitary-adrenal axis and cortisol being implicated in the circadian phenotypes of their respective symptoms. Rheumatoid arthritis is generally worse in the morning (Cutolo et al. 2008) and nocturnal asthma exacerbations are associated with the early morning nadir of cortisol (Fujitaka et al. 2000). These observations connect the core circadian clock with the inflammatory response in serious, often debilitating, disease states.

Glucocorticoids are potent anti-inflammatory drugs and are often used in the treatment of inflammatory diseases (Barnes 1998). These steroids inhibit the production of pro-inflammatory cytokines (such as TNFα, and IL-6) (Wang et al. 2008), to reduce the side effects of inflammation and to ultimately prevent shock and even death. Glucocorticoids are also potent regulators of the circadian clock (Chapter 3 and Balsalobre et al, (2000)), suggesting potential circadian effects on the suppression of the inflammatory response.

5.2 Aims

- To investigate the circadian variation of cytokine and chemokine secretion after LPS challenge (the gated response) in both the whole animal and isolated peritoneal exudate cells (PECs).

- To characterise the effects of timed administration of Gc on the suppression of the inflammatory response.

- To identify a mechanism by which the gated response occurred, using a *Rev-erbα* -/- mice mouse line.

- To address the effect of LPS on clock gene transcription, using both PER2::luc lung slices and individual tissues from WT and *Rev-erbα* -/- mice.

5.3 Results

5.3.1 LPS dosing effects on serum cytokine levels

Prior to any large scale *in vivo* experiments, using LPS, a suitable dose had to established in order to produce cytokine secretion that was well below reported doses at which mortality occurs, within the detectable range and at a non-saturating dose. Mice were injected i.p. with saline control, 1mg/kg or 10mg/kg LPS for 4h before sacrifice and serum cytokine quantification. IL-6, TNF-α, CXCL1/KC and CCL2/MCP1 were all assayed (Fig 5.1) and all, except CXCL1/KC (due to high variation), showed a significant release in response to 1mg/kg LPS with a much higher release to 10mg/kg, which were almost above detectable levels. A 1mg/kg dose of LPS produced IL-6, CCL2/MCP1 and TNF-α expression that was within a previously reported range (Bhattacharyya et al. 2007; Renckens et al. 2006), with CXCL1/KC expression being higher. The mice showed no obvious behavioural response to the 1mg/kg dose, whereas with the 10mg/kg does, animals were immobile, possibly due to a high body temperature although temperature analysis was not undertaken, and a fever like response. On this basis 1mg/kg was chosen as an effective does to use for future studies as the cytokine expression at 1mg/kg lies well within the dose response curve (Fig 5.1).

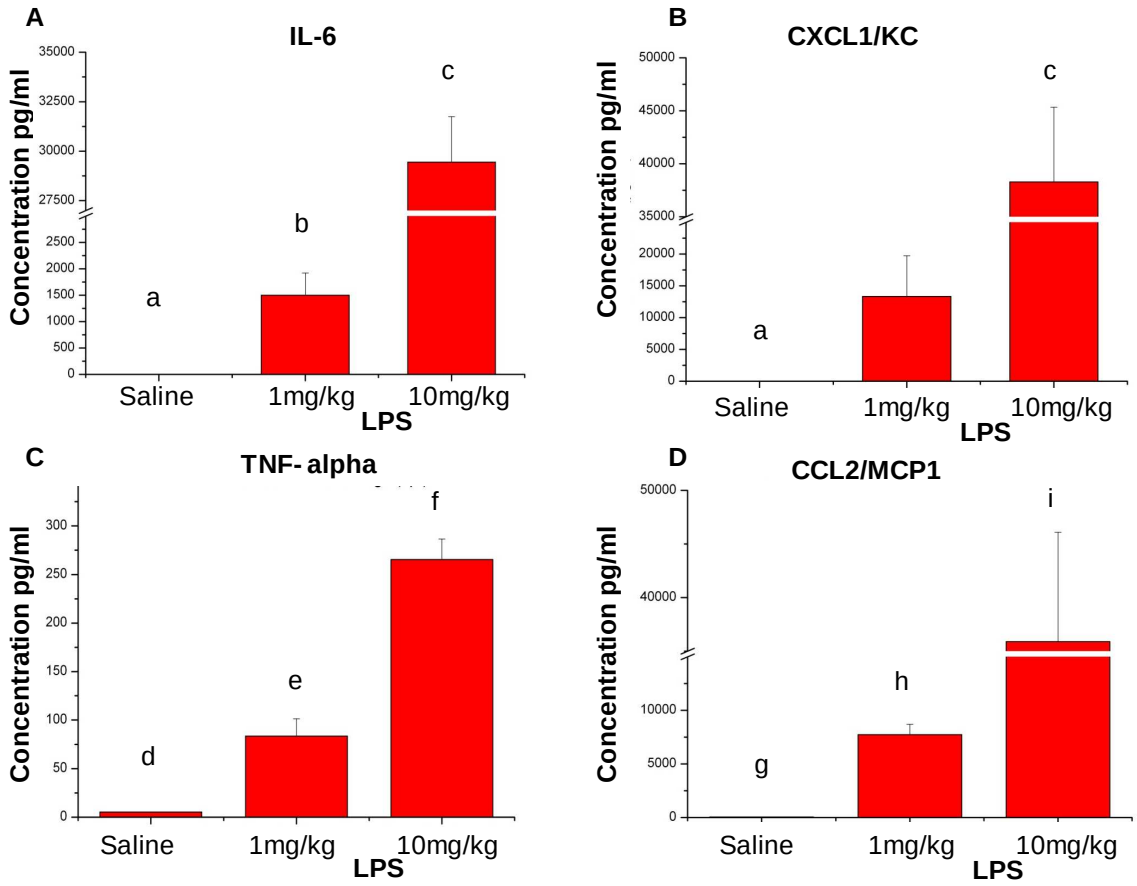

Figure 5.1: Dose dependent release of inflammatory mediators after LPS challenge: Saline, 1mg/kg or 10mg/kg LPS was injected i.p. 4h before sacrifice and quantification of serum expression of IL-6 (A), CXCL1/KC (B), TNFα (C) and CCL2/MCP1 (D). The serum concentration is measured in pg/ml. Statistical analysis is by One-Way ANOVA (in all graphs $P<0.0118$) with a Bonferroni post-hoc analysis. Error bars are SEM. With regard (A) and (B): (a) versus (b) $P< 0.05$, (a) versus (c) and (b) versus (c) $P< 0.01$. With regard (C): (d) versus (e) $P< 0.05$ and (d) versus (f) and (e) versus (f) $P<0.001$. With regard (D): (g) versus (h), (g) versus (i) and (h) versus (i) $P<0.05$.

5.3.2 Gated inflammatory response

5.3.2.1 Testing the gated response in WT mice

Animal treatments: 6-8 week old male mice were entrained to a 12h light: 12h dark cycle (lights on 0800: lights off 2000) for 10 days and then released into constant darkness for 24h prior to challenge. Saline or 1mg/kg LPS was administered, at either subjective CT0 or CT12 (section 2.2.1). Animals were killed by decapitation 4h post challenge, when blood and liver samples were taken. A panel of 23 inflammatory mediators (cytokines and chemokines) were assayed for each condition using the BioPlex assay system (section 2.5.4). Of the 23 inflammatory mediators screened, 8 did not respond to the LPS challenge; and 12 mediators increased after LPS challenge but with an equivalent response

at both times, such as TNFα and G-CSF (Fig 5.2a and b). Interestingly, only 3 of the 23 mediators were induced by LPS with a time of day, gated, response, (Fig 5.2c, d and e). In contrast, none of the mediators showed a temporal difference in response to the saline only control, such that there was no endogenous, unstimulated expressed gated response, (see table 5.1 for a full breakdown of the results).

Chemokine/Cytokine	**CT 0 (Saline - LPS difference)**	**CT 12 (Saline-LPS difference)**	**CT 0 LPS-CT12 LPS difference**	**CT0 Saline-CT 12 saline**
IL-1 a	***	**		NO
IL-1 b	***	***		NO
IL- 2	**			NO
IL- 3				NO
IL- 4				NO
IL- 5				NO
IL- 6	*	***	**	NO
IL- 9				NO
IL- 10	***	**		NO
IL- 12 p40	***	***	*	NO
IL- 12 p70				NO
IL- 13				NO
IL- 17				NO
Eotaxin	**	***		NO
G-CSF	*	**		NO
GM-CSF				NO
IFN-g	*			NO
CXCL1/KC	***	***		NO
CCL2/MCP1	***	***		NO
MIP1 alpha	**	*		NO
MIP1 beta	***	***		NO
CCL5/RANTES	***	***	*	NO
TNFα	***	***		NO

Table 5.1: Serum release of 23 inflammatory mediators 4h after LPS challenge: A panel of 23 inflammatory mediators were screened in mouse serum 4h after i.p. 1mg/kg LPS challenge. The table shows if there was an LPS induced increase in release after

challenge at either projected ZT0 or ZT12, relative to the saline control. The table also shows which mediators showed a time of day, gated, response to LPS (bold). Statistical analysis was by Two-way ANOVA with a Bonferroni post-hoc analysis, * P<0.05, ** P<0.01, *** P<0.001, n>7 for each condition.

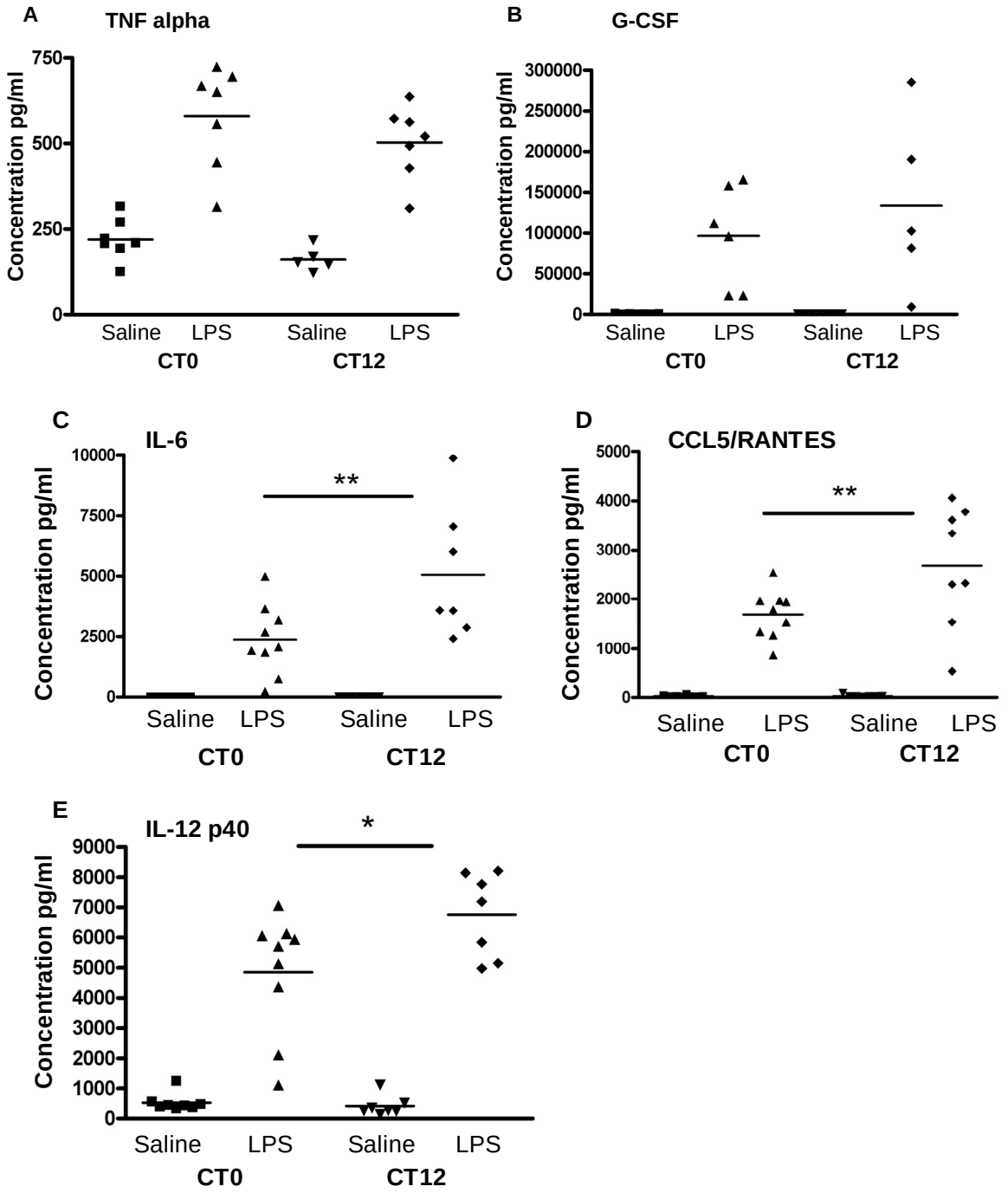

Figure 5.2: Timed LPS challenge on inflammatory mediator release in WT mouse serum: WT mice were injected with 1mg/kg LPS i.p. for 4h prior to serum quantification of inflammatory mediators. TNFα (A) and G-CSF (B) are shown here with no difference between the LPS challenge (non-responders). IL-6 (C), CCL5/RANTES (D) and IL-12 p40 (E) all showed a time of day response (responders) to 1mg/kg LPS challenge given at CT0 and CT12. Dots represent individual mice, n=7-9. Statistical analysis is by Two-way ANOVA (in all cases P<0.0001 for treatment interaction and in A: P=0.119 in B: P=0.41,

in C: P=0.020, in D: P=0.046 and in E: P=0.065 for time interaction) with a Bonferroni post-hoc analysis; * P<0.05, ** P<0.01.

IL-6, CCL5/RANTES and IL-12 p40 showed gated responses to LPS challenge, with greater expression at ZT12, (Fig 5.2). This differential expression conforms nicely with the peak expression of the *Per2* rhythm (Fig 5.3). The difference in mean cytokine secretion 4h after treatment between ZT0 and ZT12 treatment times is approximately 2-fold with respect to IL-6 and 1.5-fold difference for CCL5/RANTES and IL-12 p40 (Fig 5.2).

5.3.2.2 LPS effect on clock gene transcript expression in WT liver

In order confirm that the mice were entrained to the L:D cycle (lights on ZT0: lights off ZT12) qPCR validation of tissue was performed (*mPer2*, *mBMAL1*, *mCry1* and *mRev-erb α*). Furthermore, as LPS has been reported to alter clock gene expression, comparisons were made between the LPS and saline treated groups, (Okada et al. 2008), (Fig 5.3). As expected each gene exhibited a significant time of day difference in saline controls with *mPer2* having a 40- fold difference between CT0 and CT12 expression (*mBMAL1* with a 3.5-fold; *mRev-erbα* with a 3-fold and *mCry1* with a 1.5-fold change). There was differential expression between *mPer2* and *mBMAL1*, which showed that they were in anti-phase. Furthermore, *mCry1* was expressed in phase with *mPer2* and *mBMAL1* and *mRev-erbα* were also in phase with each other and in anti-phase to *mPer2/Cry1* (Fig 5.3).

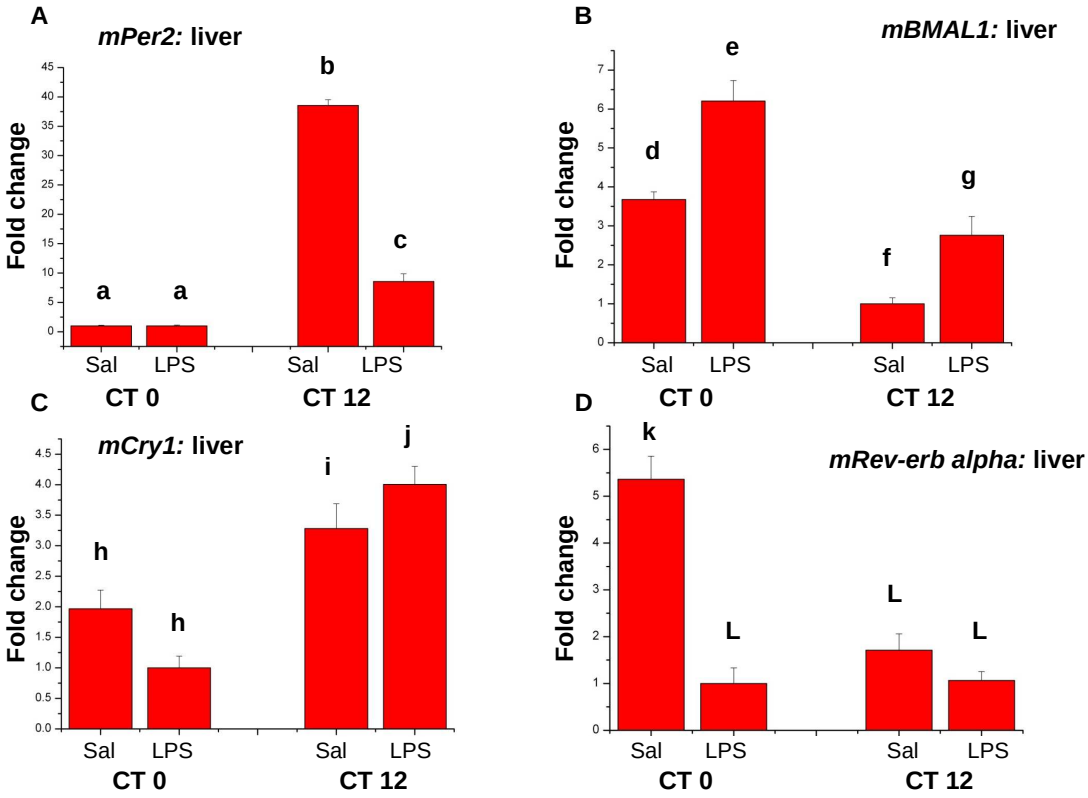

Figure 5.3: Clock gene transcript expression in the murine liver post LPS challenge: *mPer2* (A), *mBMAL1* (B), *mCry1* (C) and *mRev-erbα* (D) transcript expression was quantified after saline or LPS challenge at ZT0 and ZT12 with the samples collected 4h later (ZT4 and ZT16). Statistical analysis was by Two-way ANOVA (in A and B: P<0.0001 for time and treatment interaction, in C: P<0.0001 for time and P=0.79 for treatment interaction, in D: P<0.0047 for time and treatment interaction) with a Bonferroni post-hoc analysis. With regard to (A): (a) versus (b) and (b) versus (c) P< 0.001. With regard to (B): (d) versus (e), (d) versus (f) and (e) versus (g) P< 0.001, (f) versus (g) P< 0.05. With regard to (C): (h) versus (i) P<0.05, (h) versus (j) P< 0.001, (i) versus (j) not significant (n.s). Error bars are SEM with n=6-8 mice per challenge.

At CT0 LPS had no effect on *mPer2* relative to the saline control, but at CT12 LPS significantly reduced *mPer2* transcript expression by approximately 4-fold (Fig 5.3a), whereas *mCry1* exhibited no significant suppression at either CT0 or CT12. *Rev-erbα* expression was significantly suppressed at CT0 but not at CT12. In summary, LPS significantly reduced the expression of known transcriptional repressors *Per2* and *Rev-erbα* at CT0 (*Rev-erb α*) and ZT12 (*Per2*) (Fig 5.3a and d). Contrary to this, *mBMAL1* was significantly induced after LPS at CT0 and CT12, (Fig 5.3b).

5.3.2.3 The loss of gating with *Rev-erb α* -/- mice

Previous studies have implicated REV-ERBα, an output from the clock, in regulating downstream physiology (Duez and Staels 2008). The loss of *Rev-erbα* in mice does not

prevent expression of normal circadian rhythmic behaviour (Preitner et al. 2002) and these observations led to the proposition that *Rev-erbα* was part of the stabilisation loop rather than an essential clock component. Earlier studies, in the Chapter (Fig 5.2), showed significant circadian gating for a subset of cytokines (IL-6, CCL5/RANTES and IL-12p40). In order to test whether *Rev-erbα* was part of that gating mechanism the LPS response in WT and *Rev-erbα* -/- mice was compared at different time points.

Using the same protocols as described above (section 5.3.2.1) mice were treated with LPS at two circadian time points and cytokines responses were measured. The data is summarised in Fig 5.4 and table 5.2. It can be seen that a time of day dependence of LPS response for IL-6, G-CSF and TNFα were lost in the *Rev-erbα* -/- mice. For CCL5/RANTES the earlier comparison revealed a significant circadian component to its LPS response (Fig 5.2), augmented at ZT12. But in this second study no significant responses were observed for WT or *Rev-erbα*-/- mice (Fig 5.4b).

Chemokine/Cytokine	WT ZT0 vs ZT12	KO ZT0 vs ZT12
IL 6	*	ns
G-CSF	*	ns
KC	ns	ns
MIP1 alpha	ns	ns
MIP1 beta	ns	ns
RANTES	ns	ns
TNF alpha	**	ns

Table 5.2: Summary of inflammatory mediator release 4hr after LPS challenge in WT and *Rev-erbα* -/- mice: A panel of 7 inflammatory mediators were screened in mouse serum 4hr after i.p. 1mg/kg LPS challenge in either Wild type (WT) or *Rev-erbα* -/- (KO) mice. The table shows if there was gated response to LPS administration in either WT or KO mice. Mediators in bold showed a gated response in the WT that was lost in the KO mice. Representative data are shown in Fig 5.4. Statistical analysis was by Two-way ANOVA with a Bonferroni post-hoc analysis, * $P<0.05$, ** $P<0.01$, non significant (n.s), n=6-8 for each genotype.

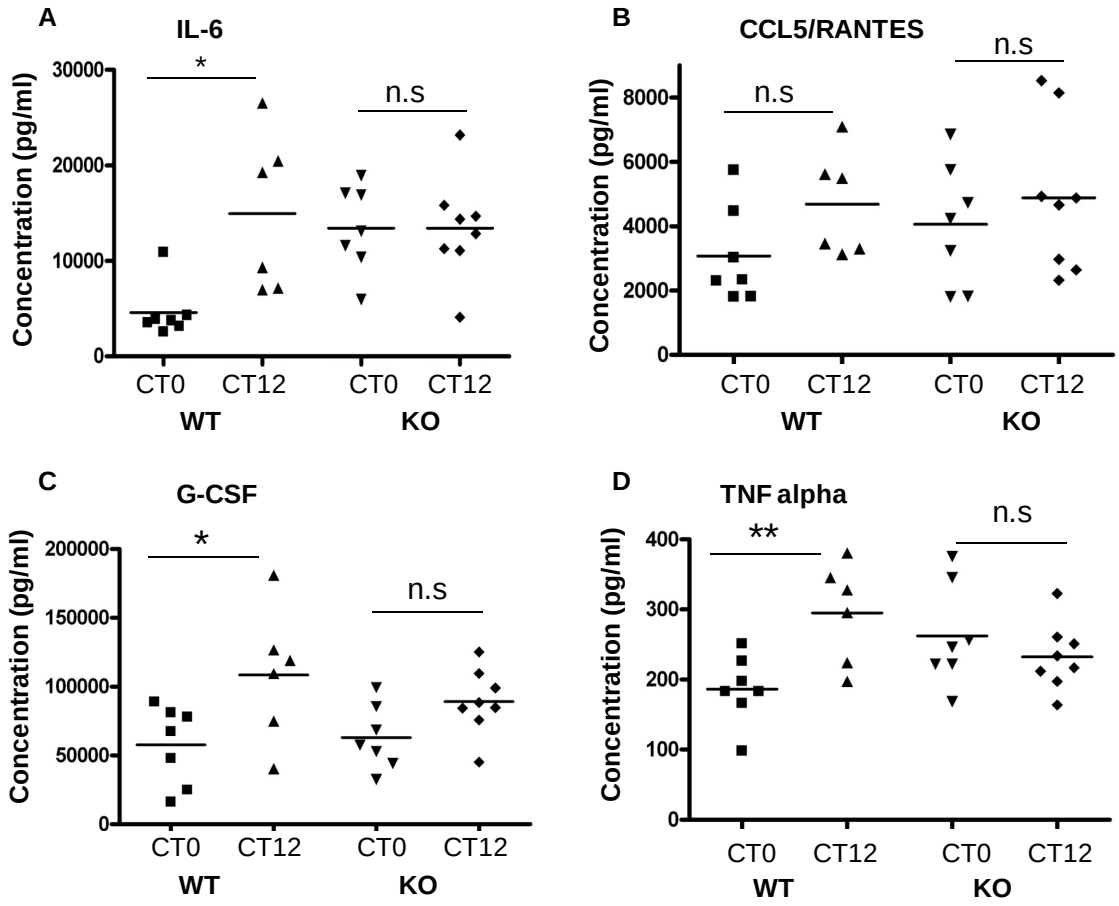

Figure 5.4: Loss of gating in *Rev-erbα* -/- mice: WT or *Rev-erb α* -/- mice (KO) were treated with 1mg/kg LPS at CT0 or CT12 for 4hr prior to analysis. IL-6 (A), CCL5/RANTES (B), G-CSF (C) and TNF-α (D) were all analysed. Dots represent individual animals and statistical analysis was by Two-way ANOVA (A: P<0.078 for time and genotype interaction, in B: P=0.1 for genotype and P=0.42 for time interaction, in C: P=0.55 for genotype and P=0.036 for time interaction and in D: P=0.79 for genotype and P=0.099 for time interaction) with a Bonferroni post-hoc analysis; * P<0.05, ** P<0.01, not significant (n.s). There was not significant difference between genotype, n=6-8.

5.3.2.4 LPS affect on clock gene transcript expression in *Rev-erbα* -/- peripheral tissues.

As in section 5.3.2.2, samples of peripheral tissues were taken, along with the serum, for clock gene analysis. Previously only liver samples were taken but this time both liver and lung samples were removed. The lung was to act as a control tissue to ensure that the results obtained with the liver were a reflection of the periphery and not individual to the liver. The two clock genes *mPer2* and *mCry1* were analysed in both WT and *Rev-erbα* -/- liver and lung (Fig 5.5). In general both *mPer2* and *mCry1* were expressed in a similar pattern to the previous study (Fig 5.3) in both liver and lung. The amplitude of response was less in *Rev-erbα* -/- lung compared to the WT for *mPer2* (5-fold versus 3-fold) and *mCry1* (5-fold versus 2-fold), and in the liver, *mCry1* (5-fold versus 2-fold) and *mPer2* (7-

fold versus 4-fold) (Fig 5.5). The LPS also suppressed *mPer2* and *mCry1* mRNA expression, in both tissues, at CT12. Together these data (Fig 5.5) showed that the previous effects seen with the WT mice (Fig 5.3) were reproducible, in both the WT litter mates and the *Rev-erbα* -/- mice and that data obtained with the KO mice would be an accurate biological representation.

Clock gene expression in the *Rev-erbα* -/- mice showed a very similar response to LPS in both liver and lung, to their WT counterparts i.e. suppression (Fig 5.5). Both *mCry1* and *mPer2* expression was maintained in the *Rev-erbα* -/- tissue with a significant induction at ZT12, with regard to the saline control, yet overall expression was reduced. LPS significantly suppressed *mPer2* expression at ZT12 in both liver and lung of the *Rev-erbα* -/- genotype. However, LPS only significantly suppressed *mCry1* expression in the *Rev-erbα* -/- liver, but not the lung, (Fig 5.5).

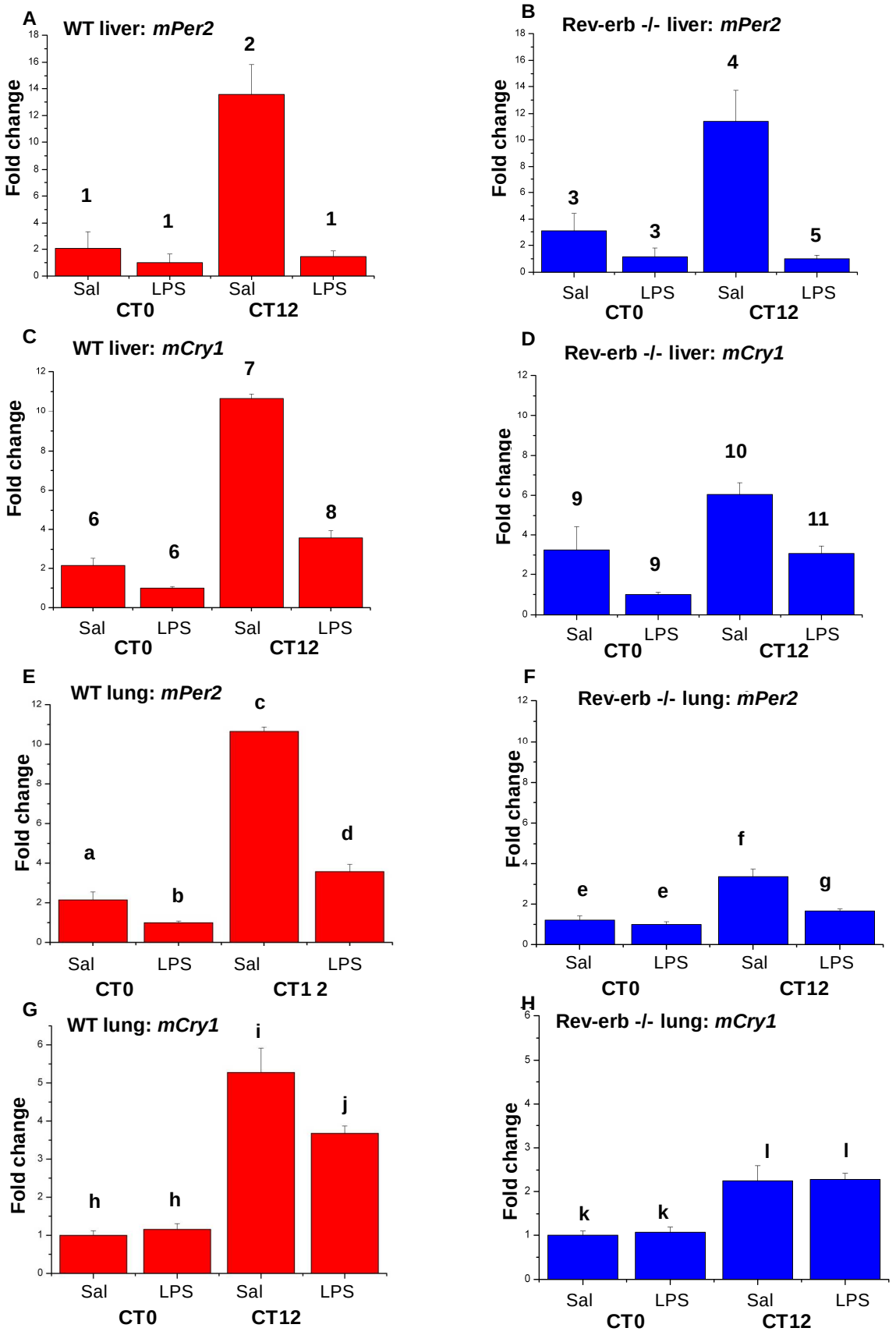

Figure 5.5: Clock gene transcript expression in WT litter mates and *Rev-erb α* -/- mice after LPS challenge: Transcript induction is shown *mPer2* and *mCry1* for liver (A-D) and lung (E-H) with WT responses (red) and *mRev-erbα* -/- responses (blue). Induction

is fold change over the lowest value (CT0 saline (sal) or LPS). Statistical analysis is by two-way ANOVA (with regard to *Per2* P<0.004 for time and treatment interaction and for *Cry1* P<0.0036 for time and for treatment P=0.0059 (C), P=0.0023 (D), P=0.15 (G), P=0.82 (H)) with a Bonferroni post-hoc analysis. With regard to (A): (1) versus (2) P< 0.001. With regard to (B): (3) versus (4) P< 0.01, (4) versus (5) P< 0.001, (3) versus (5) n.s. With regard to (C): (6) versus (7) P< 0.001, (6) versus (8) P<0.001, (7) versus (8) P< 0.01. With regard to (D): (9) versus (10) P<0.05, (10) versus (11) P< 0.05, (9) versus (11) not significant. With regard to (E): (a) versus (b) P< 0.05, (a) versus (c) P< 0.001, (b) versus (d) P< 0.001, (c) versus (d) P< 0.001. With regard to (F): (f) versus (g) P< 0.001, (e) versus (f) P< 0.001, (e) versus (g) not significant. With regard to (G): (h) versus (i) P< 0.001, (h) versus (j) P< 0.01, (i) versus (j) not significant. With regard to (H): (k) versus (l) P< 0.01.

5.3.3 LPS effects on the phase and period of the pulmonary oscillator

The action of LPS on both the phase and period of the lung circadian clock was investigated, using the PER2::luc pulmonary model. Following LPS administration at the peak of the PER2::luc rhythm the phase of the subsequent PER2 rhythm was delayed, relative to vehicle controls, by 3.12 ± 0.75h (n=3, student t-test P< 0.037). This treatment had no effect on the subsequent period of the oscillation (Fig 5.6). These data showed that LPS has and acute re-setting effect on the pulmonary oscillator but not on the subsequent period. Furthermore, LPS does not induce an amplitude response (acute or chronic), unlike CORT (Chapter 3), just a phase re-setting response.

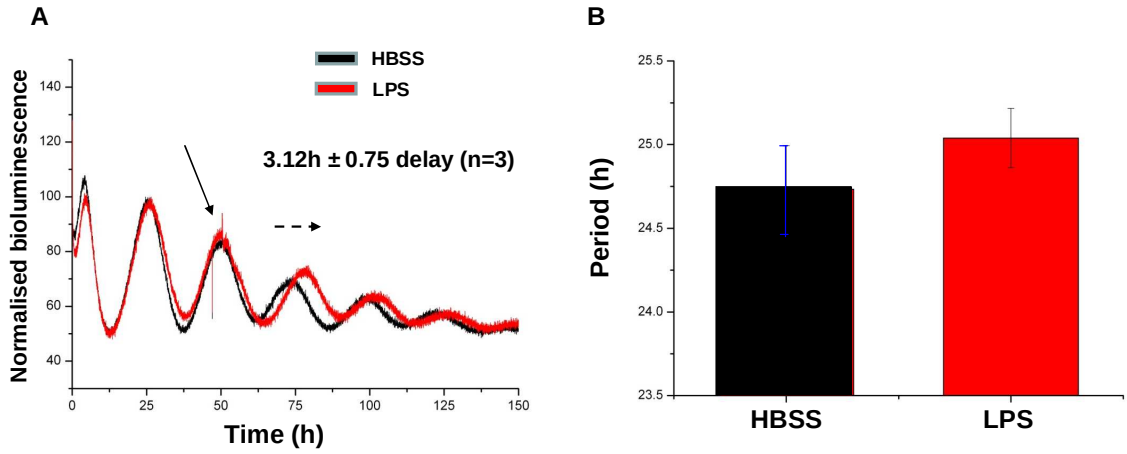

Figure 5.6: Phase and period effects of LPS on the lung: A representative oscillation (A) shows LPS (red) and HBSS (black) with the time of treatment (full arrow). The subsequent phase delay is shown relative to the HBSS control (dashed arrow). The respective periods after treatment are shown (B). Error bars are SEM of n=3.

5.3.4 The response of peritoneal exudate cells (PECs) to LPS challenge

5.3.4.1 WT PECs and LPS

PECs were extracted from the peritoneal cavity of WT mice (section 2.2.6) and cultured for experimental use (an example of the cells extracted from the exudate mix is shown in appendix I). As stated in section 2.2.6, some extracted cells were used as a temporal reference for the phase of the oscillation, as they contained the PER2::luc transgene. These reporter cells meant that an average period of the PECs oscillation could be derived (Fig 5.7), which was 23.24 ± 0.7h (n=5). This period is slightly shorter than that stated in Fig 5.6, but the cells used here were isolated PECs not lung slices. The representative oscillation (Fig 5.7) also depicts the time at which the experimental cells were treated with LPS at either the peak or trough of the PER2::luc oscillation, assuming that both the reporter and experimental cells maintained constant phase with one another. Cells were treated with LPS and 4h later the serum was collected for cytokine analysis. Total protein was also extracted from the adherent cells and the concentration quantified. Although the protein concentrations showed a large degree of variation, there was no statistical variation between treatment groups (Fig 5.7b), perhaps due to the sensitivity of the assay.

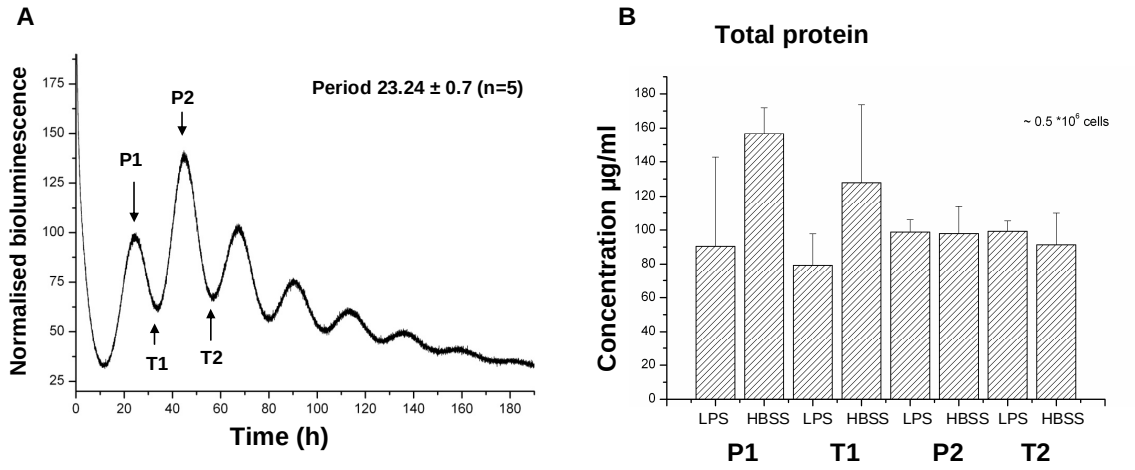

Figure 5.7: Representative PEC oscillation and protein concentration from WT PER2::luc mouse: PECs were cultured from the WT PER2::luc mouse and a representative oscillation is shown (A), with the post treatment protein concentrations shown in (B). The times of challenge were either (A); peak 1 (P1), trough 1 (T1), peak 2 (P2) or trough 2 (T2). The average period was based on n=3 biological repeats using a pool of 5 male mice and the protein was based on n=3 of 1 experiment using a pool of 5 male mice. Statistical analysis was by One-Way ANOVA (P=0.48) but no significance was found. Error bars are SEM.

Initially PECs were treated with either LPS or vehicle at the first two peaks and troughs, and the resulting media collected 4h later. This was in order characterise any prolonged temporal effects on the release of inflammatory mediators, as potentially release may alter after 48h (compared to 24h) as the amplitude of the oscillation changed. The results showed (Fig 5.8) that challenging the cells at the first peak and trough after

synchronisation; that is 24h and 36h after the onset of the experiment (P1 Fig 5.7), was the more appropriate treatment time, as IL-6 showed a greater amplitude response between first peak and trough treatment, as against the second peak and trough treatment. Also CCL2/MCP1 showed much less variation in expression when treated at the first peak and trough, (Fig 5.8). LPS treatment induced IL-6, CCL2/MCP1 and CXCL1/KC when administered at the peak of the PER2::luc rhythm, but less induction was observed when administered at the trough, (Fig 5.8). Similarly, both IL-6 and CCL2/MCP1 showed a significant gated response to LPS when challenged at either the first peak or trough, but significance was lost with CCL2/MCP1 when challenged at the second peak or trough. IL-6 expression was also dampened when challenged at the second peak and trough (Fig 5.8a). CXCL1/KC and TNFα showed no gated response when treated at the first peak and trough but TNFα was gated when treated at the second peak and trough. Therefore, further challenges were carried out at either the first peak or trough of the PER2::luc rhythm. Saline controls exhibited very low to non-detectable cytokine release but these values have been subtracted to provide normalisation.

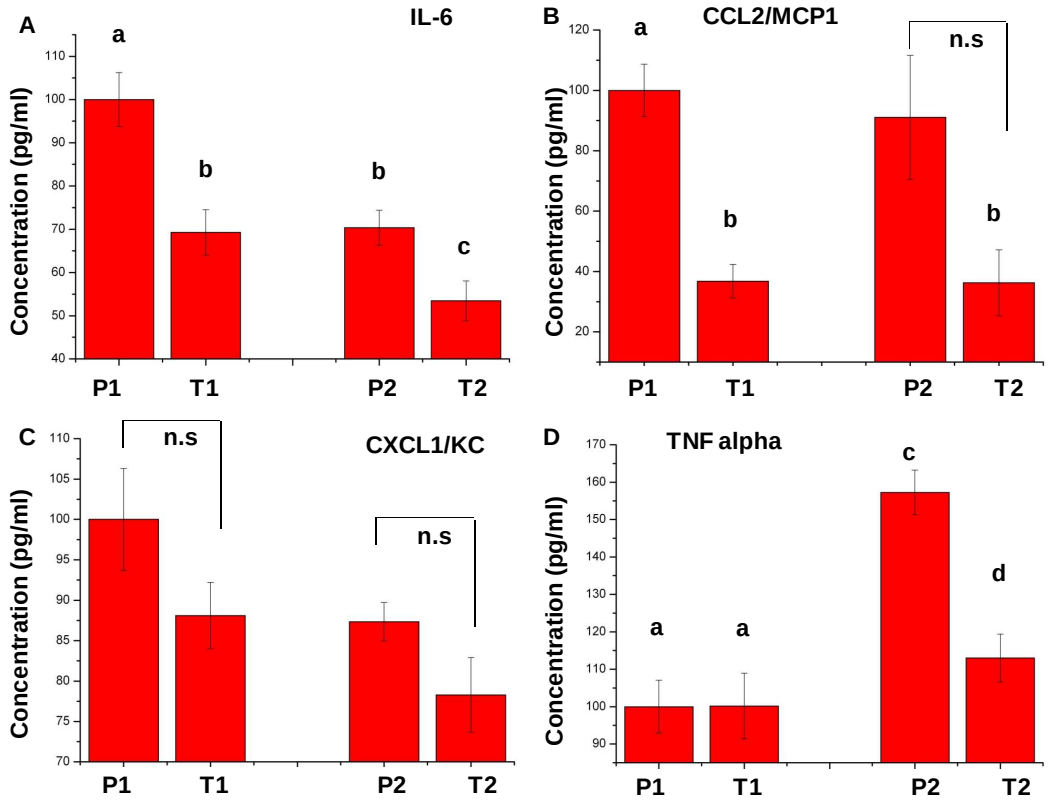

Figure 5.8: Expression of inflammatory mediators after LPS challenge at the peak and trough of the PER2::luc rhythm: PECs were treated at subsequent peak (P) and trough (T) of the PER2::luc rhythm with LPS and expression of IL-6 (A), CCL2/MCP1 (B) and CXCL1/KC (C) were measured. Results were normalised to peak expression. Statistical analysis was by One-way ANOVA (in A and D: $P<0.0014$, in B: $P=0.012$ and in C: $P=0.059$) with a Bonferroni post-hoc analysis, (a) versus (b) $P<0.05$, (a) versus (c) $P<$

0.01, (c) versus (d) P< 0.05, (a) versus (d) n.s. Error bars are SEM and are taken from 3 internal replicates from pooled cells from 5 male mice.

5.3.4.2 WT PECs: LPS and CORT

In order to test Gc mediated suppression of cytokine response, PECs were treated with LPS or LPS plus CORT (co-administered), at both the peak and trough of the PER2::luc rhythm. A representative PER2::luc oscillation is shown with a representative protein concentration from LPS and LPS plus CORT treated PECs (Fig 5.9), with no statistical difference between treatment groups. 4h after treatment the medium was analysed for cytokine release. In confirmation of earlier studies (Fig 5.8) LPS administration at the trough of the PER2::luc rhythm resulted in significantly reduced expression of all 4 cytokines (CXCL1/KC, IL-6, TNFα and CCL2/MCP1) compared to the peak administration. Co-administration of CORT with LPS reduced cytokine responses, when administered at both peak and trough of the PER2::luc cycle, for all 4 cytokines measured (Fig 5.10). The strongest suppression, by CORT, was seen for CXCL1/KC, TNFα and IL-6 with greater than 50% suppression. However, there was no difference in the magnitude of suppression when CORT was co-administered at either the peak or trough. CORT significantly suppressed CCL2/MCP1 when co-administered at the peak but not when administered at the trough (Fig 5.10 b).

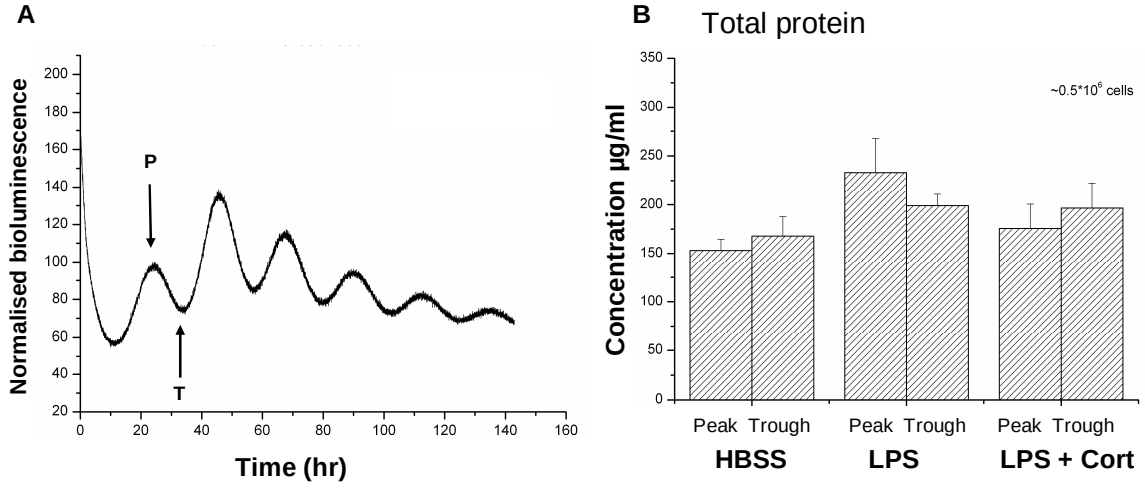

Figure 5.9: Representative PEC oscillation and protein expression from the WT PER2::luc mouse: PECs were cultured from the WT PER2::luc mouse and a representative oscillation is shown (A), with the post treatment protein concentrations shown in (B). The time of challenge is shown in (A); peak (P, CT12), trough (T, CT0). The oscillation was based on a pool of 5 male mice and the protein was based on n=3 from within that pool. Statistical analysis was by One-Way ANOVA (P=0.17) but no significance was observed. Error bars are SEM.

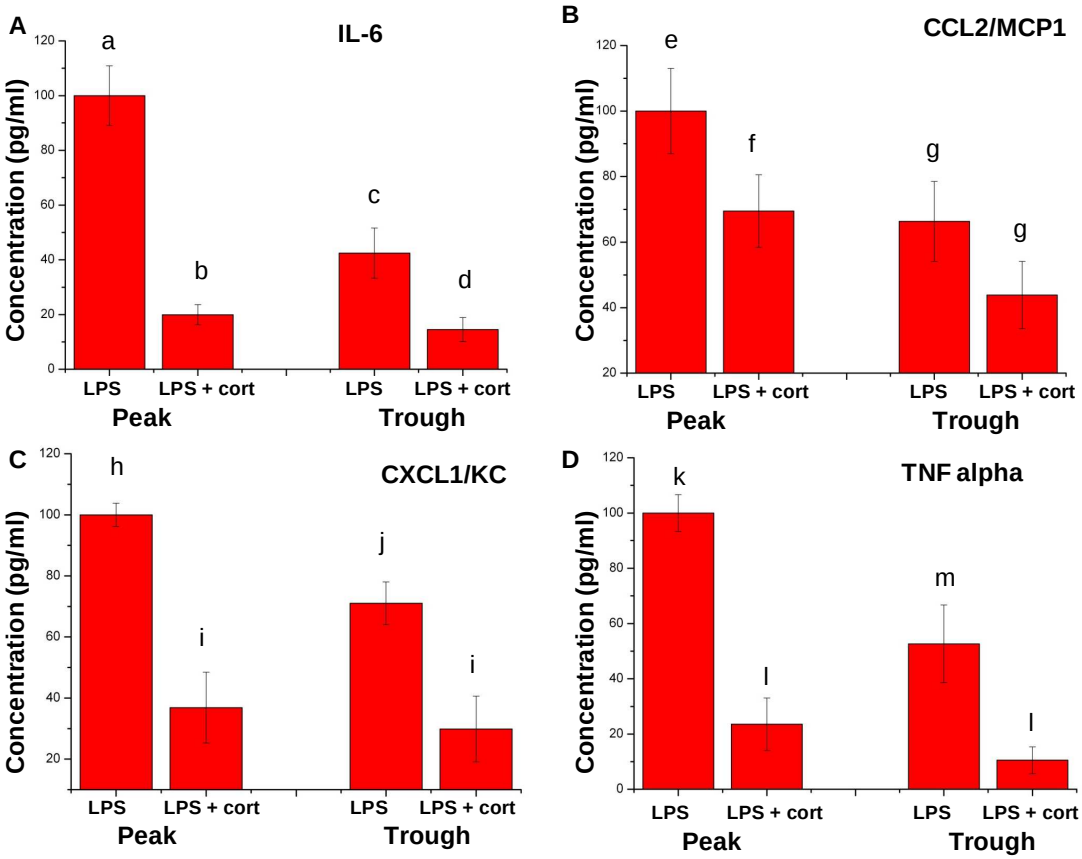

Figure 5.10: CORT suppression of LPS induced inflammatory mediators after PEC challenge at the peak and trough of the PER2::luc rhythm: PECs were treated at the first peak and trough (Fig 5.9) with LPS only, or combined with cort, and expression of IL-6 (A), CCL2/MCP1 (B) CXCL1/KC (C) and TNFα (D) were measured. Results were normalised to the LPS peak expression. Statistical analysis was by One-way ANOVA ($P<0.0001$ for each cytokine) with a Bonferroni post-hoc analysis. Error bars are SEM of n=3 (taken from 3 separate experiments using cells from a pool of 5 male mice). With regard to (A): (a) versus (b), (a) versus (c) and (c) versus (d) $P< 0.001$, (b) versus (d) not significant. With regard to (B): (e) versus (g) $P< 0.01$, (e) versus (f) $P< 0.05$, (f) versus (g) not significant. With regard to (C): (h) versus (i) and (j) versus (i) $P< 0.001$, (h) versus (j) $P< 0.01$. With regard to (D): (k) versus (l), (k) versus (m) and (m) (l) $P< 0.001$.

5.3.5 Responses to LPS in *Rev-erb α* -/- PECs

PECs from the PER2::luc mouse were also cultured in parallel to the *Rev-erbα* WT and -/- mice to act as a reporter for the phase of the PER2 oscillation, as neither the *Rev-erbα* WT or -/- mice carried the luciferase reporter. *Rev-erbα* -/- mice have a reported free-running circadian period of 23.28h ± 0.45 (Preitner et al. 2002) and mPER2::luc mice have a period of 23.69h ± 0.07 (Yoo et al. 2004) so using the PER2::luc reporter as a circadian reference is justified. WT or *Rev-erbα* -/- PECs were treated with vehicle or LPS at the peak or trough of the PER2::luc rhythm and 4h later the medium was removed and analysed for cytokine release. As previously described, IL-6, CCL2/MCP1, CXCL1/KC

and TNFα were each quantified after LPS treatment in WT or -/- PECs. IL-6, CCL2/MCP1 and TNFα each showed a significant gated response, with reduced expression at the trough, relative to the peak, in WT cells (Fig 5.11). The amplitude of response seen in the WT peak to trough comparison was greatly diminished in the *Rev-erb α* -/- mice with CCL2/MCP1, IL-6 and TNFα, but with respect to IL-6 and TNFα significant differences were still observed (Fig 5.11). CXCL1/KC showed no significant difference in peak to trough expression in either genotype but a significant increase in expression was observed between WT and *Rev-erbα* -/- cells (Fig 5.11c). Similarly, IL-6 expression in the *Rev-erbα* -/- cells was significantly increased yet TNFα expression was significantly diminished (Fig 5.11a and d).

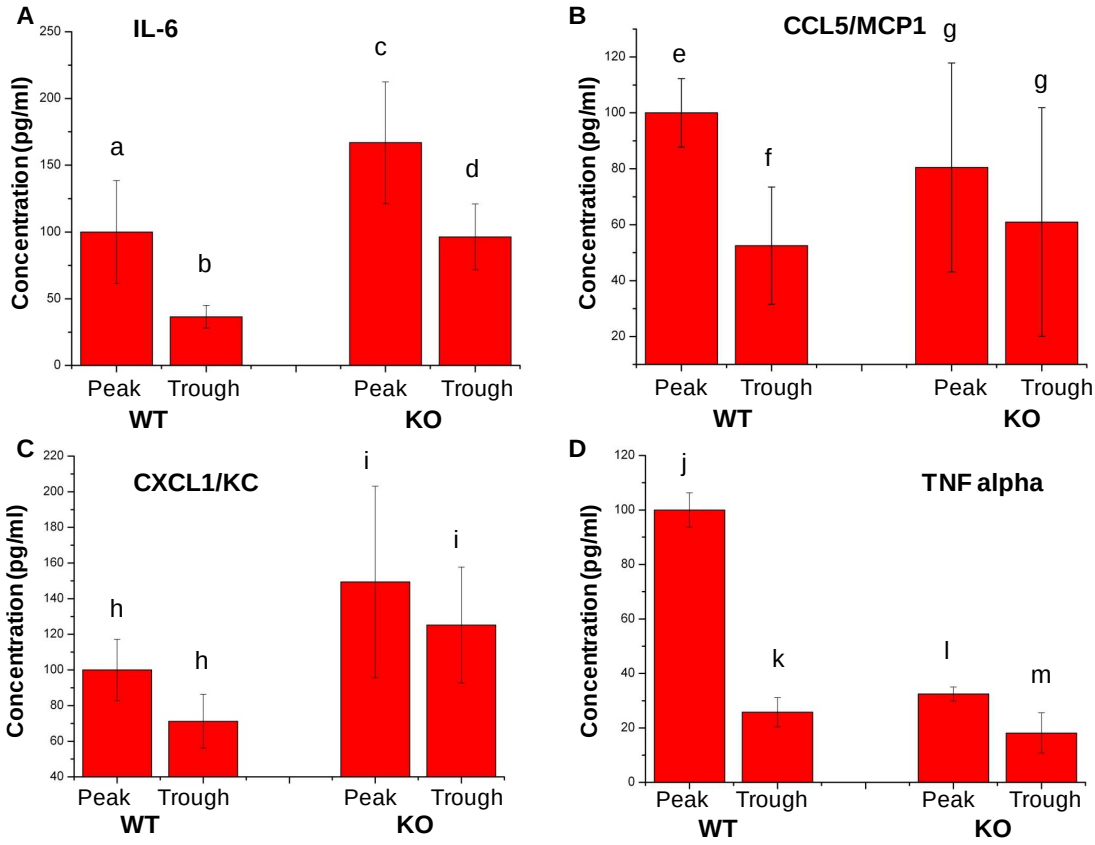

Figure 5.11: Expression of inflammatory mediators after LPS challenge in WT and *Rev-erb α* -/- PECs at the peak and trough of the PER2::luc rhythm: IL-6 (A), CCL2/MCP1 (B), CXCL1/KC (C) and TNFα (D) were treated with LPS at the peak and trough of the PER2::luc rhythm in either *Rev-erb α* -/- or WT litter mates PECs. Expression was normalised to the WT peak values. Statistical analysis was by Two-way ANOVA (in A: P<0.0001 for time and genotype, in B: P=0.66 for genotype and 0.013 for time, in C: P=0.012 for genotype and 0.067 for time and in D: P<0.0001) with a Bonferroni correction. Error bars are standard deviation of an n=2 (TNFα was n=1) experiment each consisting of pooled cells from 3 mice of each genotype. With regard to (A): (a) versus (b), (a) versus (c), (c) versus (d) and (b) versus (d) P< 0.01. With regard to

(B): (e) versus (f) $P< 0.01$, (e) versus (g) and (f) versus (g) are not significant. With regard to (C): (h) versus (i) $P< 0.05$. With regard to (D): (j) versus (k) and (j) versus (l) $P< 0.001$, (l) versus (m) $P< 0.05$, (k) versus (m) not significant.

5.4 Discussion

5.4.1 The LPS induced gated response in WT (C57/BL6) mice and PECs

Although the innate immune system has an immediate response to an external challenge, this work demonstrates that only a small number of inflammatory mediators are gated by the circadian clock, the response to LPS (table 5.1).

In each experimental protocol in this chapter, be it *in-vivo* or *in-vitro,* IL-6 showed a gated response to LPS challenge, confirming the observations previously reported, (Keller et al. 2009). However, the circadian element to IL-6 protein secretion has been well documented, with expression highest during times of sleep compared to times of wakefulness, in humans (Redwine et al. 2000; Vgontzas et al. 2002) but IL-6 has also been shown to oscillate in various rat tissues, such as the pituitary (Seres et al. 2004) hypothalamus and periphery (Guan et al. 2005). The IL-6 transcript itself has also been shown to be under circadian control with 3 conserved canonical E-boxes in its promoter, which have been shown to oscillate, albeit weakly, in luciferase reporter studies (PhD thesis of Andrew McMaster, 2007, University of Manchester) and at the endogenous transcript level (Hayashi et al. 2007). Therefore, IL-6 was used more as an internal control in theses studies, as its circadian regulation has been well characterised.

Like IL-6, TNFα has been shown to freely oscillate at both the transcript (Hayashi et al. 2007) and protein level (Arjona and Sarkar 2005), even in the absence of any immune challenge and has two conserved E-boxes within its promoter (PhD thesis of Andrew McMaster, 2007). Upon immune challenge with LPS, TNFα still showed a robust oscillation at the protein level, like that of IL-6 (Keller et al. 2009). However, in the studies shown here the results were different. In the WT mouse *in-vivo* LPS challenge TNF-α did not show a gated response (Fig 5.2), yet in the WT litter mates to the *Rev-erbα* -/- mice a robust gated response was seen (Fig 5.4), likewise in the *in-vitro* PEC studies there was a peak/trough gated response to LPS (Fig 5.10 and 5.11). Interestingly, in the studies presented here (Fig 5.2 and 5.4) that there is no unstimulated rhythm (saline controls) in any of the cytokines in the WT mice used (table 5.1); this is of note

considering that IL-6 is known to by rhythmic in plasma in the naive animal (Guan et al., 2005). In this series of experiments (table 5.1), saline controls were at undetectable levels i.e. below 5pg/ml (IL-6, RANTES and G-CSF) via BioPlex analysis. This is perhaps due to the 1 in 4 dilution factor used in order to ensure that the LPS stimulated samples were within the detectable range of the assay. Guan et al., 2005 reported serum samples of IL-6 in the upper range of 40pg/ml so if my samples were diluted 1 in 4 this would take the working concentration to 2.5pg/ml and below detectable levels. If there was sufficient time and funding available assaying the neat saline controls would no doubt show differential expression. Together these data suggest that TNFα, like IL-6, is under circadian regulation yet the experimental model used to characterise these effects needs to be considered carefully, as the underlying circadian regulation is not as robust as IL-6.

Interestingly two other inflammatory mediators, CCL5/RANTES and IL-12p40 (Fig 5.2) showed a time of day, gated, response to LPS challenge, yet as with IL-6 and TNFα a gated response was not seen under baseline conditions. CCL5/RANTES has not been shown previously to be under circadian control, although it is documented that both its mRNA and protein expression are increased in response to TNFα (itself circadian, Keller (2010)) and melatonin (Luo et al. 2004). However, TNFα did not show a gated response in the study with WT mice (Fig 5.2) nor do the mice used in the study (C57/BL6) express melatonin, so there must be yet another source of the gated response, perhaps direct E-box regulation. A search of 3kbp 5' to the transcriptional start site of the CCL5/RANTES promoter revealed no canonical, or non-canonical, E-box elements at described by Ueda (2005), yet 10 CACATG motifs were present, which fits the generic CANNTG (where N is any base) E-box motif (appendix G), and therefore, may contribute to the gated response to LPS. CCL5/RANTES showed a mixed gated response dependent on the model and conditions that were investigated. CCL5/RANTES was gated in the WT mouse LPS study (Fig 5.2) but not in the WT litter mates to the *Rev-erbα* -/- mice (Fig 5.4), and there was an equal discordance of results with the isolated PECs (Appendix H). A gated response was shown in the WT and *Rev-erbα* -/- cells, with an approximate 50% reduced expression with treatment at the trough, but in the WT LPS with and without CORT a gated response was not shown between peak and trough LPS and the CORT showed no significant suppression of release. This variability in the gated response between the two WT strains (C57/BL6 and WT litter mates to the *Rev-erbα* -/- mice) could be due to sex differences in the experimental set up used. For the WT experiments (Figs 5.2, 5.8 and table 5.1) male mice

were exclusively used, whereas in the WT versus *Rev-erbα* -/- experiments (Fig 5.4, 5.11 and table 5.2) mixed sex mice were used due to breeding and number constraints of the *Rev-erbα* -/- mice, and it was thought better to maintain matched sex experiments for the WT versus *Rev-erbα* -/- studies rather than unequal matching.

The response to LPS in the WT PECs was very similar to the whole animal response where the greater expression coincided with the peak of the PER2::luc oscillation (Fig 5.7 and 5.9). The mediators involved were a little different though. IL-6, as expected, showed a robust gated response but so did JE/MCP1 and TNFα, previously not seen in the whole animal study (table 5.1), yet Keller et al. (2009) has shown TNFα to oscillate in the response to LPS in *ex-vivo* macrophages. Furthermore, CCL5/RANTES was shown to be gated (Fig 5.2) at the whole animal level but conflicting results were seen at the cellular level, (appendix H), as previously described. Also mediators such as CXCL1/KC, even though they were expressed in response to LPS in both whole animal and at the cellular level, did not produce a gated response in either model. Together these data suggested that the response seen may well be a product of the model system used and that there is not just a global systemic inflammatory response, more individual to a particular area or organ.

5.4.2 Loss of gated response in the *Rev-erbα* -/- mice and PECs

LPS has been previously shown to modulate a bi-phasic response of *Rev-erbβ* expression in bone marrow derived macrophages, with an immediate decrease in expression at 4-8h followed by an increase at 12-16h (Barish et al. 2005). Furthermore, the over expression of *Rev-erbα* in vascular smooth muscle cells induced NF-κB transactivation and an increase in the pro-inflammatory response, with factors such as IL-6 (Migita et al. 2004). Furthermore, the reverse occurs with the over expression of RORα in human cells, in this instance IL-6 and IL-8 (human homologue of CXCL1/KC) expression were suppressed (Delerive et al. 2001). Together these data suggested an important role of REV-ERB and ROR in mediating the magnitude of the inflammatory response. The data shown in Fig 5.4 revealed, for the first time, that the loss of *Rev-erbα* can result in the loss of the gated response to LPS and that basal expression is increased relative to the WT counterparts. These data are in contrast to the work of Migita et al. (2004) as the authors saw an increase in mediator expression after *Rev-erbα* over expression, whereas in Fig 5.4, a loss of *Rev-erb α* resulted in an increased expression, yet the reduced TNFα expression in the *Rev-erbα* -/- cells does correlate with the Migita et al. (2004) study (Fig 5.11). Furthermore, the

work here takes the analysis much further and includes the loss of a gated response with G-CSF and TNFα in the *Rev-erbα* -/- genotype, relative to the WT mice. However, other mediators such as CCL5/RANTES, previously shown to be gated, in WT mice, in response to LPS challenge (Fig 5.2) did not show a similar gated response in the WT counterparts to the *Rev-erbα* -/- mice, although a similar trend was still apparent and expression was increased in the *Rev-erbα* -/- genotype. Little work has been carried out into the role of the REV-ERBs, and RORs, in the inflammatory response particularly with respect to the gating effects, but *Rev-erbα* -/- mice do show a shorter activity cycle that their WT counterparts in both DD and LD conditions, approximately by 30 min (Preitner et al. 2002). However, inflammatory mediators are not reported to have RORE sites in their promoters, although this does not rule out a direct effect at other locations of the genetic locus. If this were the case, then this could account for the increased expression in the *Rev-erbα* -/- model, as the repressive effects of REV-ERB would have been lifted, in particular subjective day repression when *Rev-erbα* (Fig 5.3) expression peaks, followed by its protein product. If there is no direct REV/ROR effect then this suggests that an intermediate protein may be involved, potentially plasminogen activator inhibitor 1 (PAI-1), which has a circadian component to its transcript cycle, it is suppressed by REV-ERBα and has been shown to play a role in the inflammatory response (Wang et al. 2006). Inflammation has been associated with a strong rise in circulating PAI-1 levels, and PAI-1 -/- mice show an increased expression of IFN-γ, IL-6, IL-12 p70, (incidentally, IL-12p70 was not gated in this study nor did was it induced by LPS and IFN-γ only showed a weak induction at CT0, table 5.1) (Renckens et al. 2006). Indeed, further work needs to be carried out before the contribution of PAI1 can be fully concluded.

The *in-vitro* cellular (PECs) study presented similar findings to the *in-vivo* whole animal study, where the gated response was lost in the *Rev-erbα* -/- cells. However, the mediators involved were slightly different. In the whole animal study (Fig 5.4) both IL-6 and TNFα lost the gated response in the *Rev-erbα* -/- genotype, (G-CSF also but this was not replicated in the PECs study), yet in the PECs study (Fig 5.11) gating was lost with CCL2/MCP1 but not lost with either TNFα or IL-6, the amplitude of response was just diminished. Furthermore, in whole animal study (Fig 5.4), the trend with IL-6 and TNFα expression in the *Rev-erbα* -/- mice was increased but with the cells, although IL-6 expression did increase, TNFα expression significantly decreased. Again this suggests differential regulation in the different model systems used. The overall loss of the gated

response is perhaps largely due to the effects of *Rev-erbα* on core clock gene expression, at both transcript and protein level. In the *Rev-erbα* -/- genotype both CLOCK and BMAL1 are expressed at higher, constitutive levels than in the WT, no longer showing a circadian rhythm (Preitner et al. 2002). This explains why the gated response is lost and why basal levels are higher, particularly in IL-6, however, fails to explain the loss of expression in the TNFα response in the PECs.

5.4.3 CORT and its immunosuppressive activity

Although Gcs have been known for many decades to suppress the inflammatory response (Berry and Smythe 1964; Spink and Anderson 1954), little characterisation on the circadian component to their action has been carried out. It has been shown here (Fig 5.10) that, as predicted, CORT inhibited the pro-inflammatory effects of LPS but this action was not itself circadian gated. Previous work by Yamamura et al. (2010) has shown that mouse TNF α serum protein levels were suppressed 2h after LPS/DEX co-administration i.p., but *TNFα* transcript was not. However, a circadian component to its expression at either transcript or protein level was not investigated in their study nor were other inflammatory mediators characterised. Furthermore, contrary to previous reports neither *Per1* nor *Per2* transcription was induced by DEX, in the heart, over control levels nor could DEX counteract LPS suppression of either *Per1* or *Per2* (Yamamura et al. 2010), suggesting that LPS is much more potent in its effects on clock gene transcription than DEX is. Furthermore, the GR (which induces *Per1* and *Per2* transcription) is negatively regulated, by clock transcription factor, CLOCK, through the acetylation of lysine residues on its hinge region. When the HAT containing CLOCK acetylates GR, it is no longer able to induce transcription (Nader et al. 2009). This could explain a gated response to CORT treatment, however, this was not seen (Fig 5.10), suggesting that CORT acts equally irrespective of the time of day.

5.4.4 LPS and clock gene transcription

It has been shown here that 1mg/kg LPS can both suppress *Per2*, *Cry1* and *Rev-erbα* and enhance *BMAL1* clock gene transcription (Fig 5.4) in the WT mouse liver. The suppression of *Per2* compares well with previous studies with suppression in both mouse heart and liver 4-6h after 1mg/kg LPS challenge (Yamamura et al. 2010). The data presented here (Fig 5.4) also aligns with previous reports that LPS suppresses *Per2* in the

rat SCN (Okada et al. 2008), and the suppression of *Per2*, *Cry1* and *Rev-erbα* in human blood leukocytes to 2ng/kg of LPS (Haimovich et al. 2010). However, the data regarding *BMAL1* expression contradicts that shown by Haimovich B et al., (2010) in leukocytes, yet is in agreement with data from male horses in which a 0.045μg/kg of LPS was injected (Murphy et al. 2007); where *BMAL1* transcript expression was increased 4h after LPS, although so was *Per2* in this model system. It is perhaps for the fist time here that differential expression has been documented with regard to LPS and it action on the core clock in the liver. Although the direct effect of LPS on the clock gene transcription cannot be ruled out, mediated through transcription factors such as NF-κB, it seems unlikely as it would have to cause simultaneous up and down regulation. Recent evidence suggested that the regulation is likely to be mediated through TNFα and the suppression of E-box mediated transcription (Cavadini et al. 2007). In their work Cavadini et al. (2007) showed that TNFα, and IL-1β (both induced by LPS, table 5.1) could suppress transcription of *Per1, 2* and *Rev-erbα*, in both liver and cell line studies (but this suppression could not be replicated by IL-6 or IFN α). In this study *Cry1* was not investigated. The reason for *Per2* suppression appears to be protective, as *Per2* KO mice are far more resistant to LPS-induced death than WT counterparts (Liu et al. 2006b), suggested to be due to reduced IFN-γ and IL-10 and as such may provide evidence for PER2 regulation. Cavadini et al. (2007) also showed that *BMAL1* expression was not affected, in either cell line or whole mouse study, as it is not under direct E-box regulation. However, this does not explain why *BMAL1* was up regulated in this study, which leads back to a direct mechanism, perhaps through NF-κB but the specific promoter based elements have not yet been shown. Overall, this relationship between LPS, TNFα/IL-1β and clock gene suppression could have important implications in the development of effective chronotherapeutics.

It was also shown here, for the first time, (Fig 5.5) that *Per2* transcription was largely unaffected by the loss of *Rev-erb α* in both the liver and lung and expression in both tissues showed significant suppression after LPS challenge. This, however, could be expected given that TNFα is the likely mechanism though LPS mediated suppression, and TNFα was still expressed in the *Rev-erbα* -/- mice (Fig 5.4) and that *mPer2* itself is known to oscillate even in the absence of a functional clock (Kornmann et al. 2007), perhaps taking it cues from systemic signals, such as glucocorticoids. *Cry1* expression was very much the same as *Per2*, (Fig 5.5) with time dependent expression in both liver and lung but with a much dampened expression in the *Rev-erbα* -/- tissue and no LPS induced suppression in

the lung. The dampened expression can, in part, be explained by the loss of the repressive effects of REV-ERBα resulting in constitutive *BMAL1* expression at both transcript and protein level (Preitner et al. 2002). This has been shown to maintain high level of *Cry1* transcript throughout the circadian day, in the liver, and thereby raising basal expression and reducing the magnitude of the amplitude change. *mPer2* expression does not change in the event of the loss of *Rev-erbα* (Preitner et al. 2002) and so explains why the amplitude of the response was largely unaffected in both WT and *Rev-erbα* -/- liver. Data for the lung has not been determined but a similar mechanism of action is assumed as the results between the liver and lung were largely consistent for *Per2* and *Cry1*. Greater attenuation of the amplitude response was not seen, most likely due to the compensatory effects of *Rev-erbβ*, which has been shown to play a redundant role in the absence of *Rev-erbα* (Liu et al. 2008).

5.5 Conclusions

LPS has been shown here to induce a gated inflammatory response in both inflammatory cells and in a whole animal model, (through i.p. administration and serum analysis). IL-6 was shown to be gated in both model systems yet other mediators such as TNFα and CCL5/RANTES showed differential regulation. Likewise CXCL1/KC and CCL2/MCP1 showed a gated response to LPS, alongside IL-6 and TNFα, in the PECs but were not gated in the whole mouse study. The different observations with the two different models most likely come from the different cell types involved. The PECs, although not 100% pure, are largely resident peritoneal macrophages and are therefore just one cell type, whereas in the *in-vivo* challenge all the organs of the mouse are being stimulated by LPS. In this case resident macrophages from all cell types containing the TLR-4 receptor are being stimulated and are contributing to the observation, and as such contribute to the differential effects. Furthermore, the gated response appears to be REV-ERBα mediated as the loss in REV-ERBα resulted in the loss (or attenuation IL-6 and TNFα PEC study) of the gated response where it was present in the WT.

CORT significantly reduced the expression of the inflammatory mediators, although interestingly the reduction was equivalent at both phases of the circadian day (peak and trough), and although CORT is itself under circadian regulation, this is not transferred to the suppressive effects on the innate immune response.

LPS attenuated transcription of the core clock genes *Per2* and *Rev-erbα* at their relative peak expression, which has been shown by Cavadini et al. (2007) to be TNFα mediated, but with an increase in *BMAL1* transcription at both phases of treatment, which has currently no mechanistic answer to the observations. The effects of LPS on *Per2* and *Cry1* transcription were generally maintained in the absence of *Rev-erbα* although small differences were seen, *Per2* still maintained a response to LPS, as in the WT, and *Cry1* expression was maintained in the WT and *Rev-erbα* -/- liver but some minor differences in the lung. Ultimately these data lead to the conclusion that there is not a global response to immune challenge via LPS, and that the mediators and underlying genetic response vary slightly depending on the tissues and models that are used in the experimental design.

5.6 Future directions

- To further investigate the role of REV-ERBα on circadian gating, by using cell line studies with siRNA knock down of *Rev-er β* on its own and in conjunction with a *Rev-erbα* -/- genotype, to address and redundancy issues described by Liu et al. (2008).

- To look at whether the phenotype seen in the *Rev-erbα* -/- genotype is a direct result of the loss of REV-ERBα or as a result of constitutive BMAL1 expression, due to the loss of REV-ERBα repression (Preitner 2002). To address this siRNA knock down of BMAL1, and possibly over expression of REV-ERBα (as in Kornmann er al. (2007)), would be used in LPS stimulated macrophages.

- A further validation of the mechanism behind *Per2* suppression and *BMAL1* induction after LPS challenge. Cavadini et al. (2007) showed that both TNFα and IL-1β could suppress E-box mediated transcription but not IL-6 or IFNγ, explaining both *Per2* and *Rev-erb,* findings, but what is the mechanism behind BMAL1 induction and how rapid is this regulation. Could the *BMAL1* induction be NFκB mediated? Also can *Per2* expression be induced, if so by what mediator?

- What is the basis behind the protective role of *Per2* suppression after LPS challenge, seemingly TNFα and IL-1 β mediated? What does the PER2 protein do, what does it induce? On a similar thought will BMAL1 over expression reduce

LPS mortality and will it reduce the inflammatory response and will BMAL1 knock out induce a greater response and increase mortality?

- CORT is known to suppress the inflammatory response but also induce *Per1* and *Per2* transcripts. Why then does LPS suppress *Per2*, via TNFα, and that this suppression is protective against LPS induced mortality, but CORT also being immuno suppressive has the effect of inducing *Per2*. Yamamura et al. (2010) showed that DEX cannot override LPS induced *Per2* suppression. How does this occur? Why is it better to suppress *Per2* in the anti-inflammatory response than inducing it? Perhaps *Per2* is the basis behind the undesirable effects of CORT treatment.

Chapter 6: The circadian regulation of C/EBP alpha and delta

6.1 Introduction

The C/EBP proteins are a family comprising six transcription factors, all of which contain a highly conserved bZIP domain, for DNA binding and dimerisation, located at the C-terminal. As a result all members of the family, except C/EBP ζ, can heterodimerise and bind to DNA (Ramji and Foka 2002). Two members of this family have been shown to be potently regulated by Gcs; C/EBPα which is down regulated and C/EBPδ which is up regulated. However, a consensus GRE has not been located within either promoter (MacDougald et al. 1994). C/EBPα and δ are both highly expressed in adipose tissue, intestine and in the lung, with C/EBPα also being expressed in the liver (Ramji and Foka 2002). However, knockout of the α isoform in mice causes death soon after birth (Wang et al. 1995), indicating the vital importance of this protein, whereas if δ is knocked out this just impairs the ability of BAT to take up lipids and express the protein uncoupling protein-1 (UCP-1) (Tanaka et al. 1997). Furthermore, C/EBPα is thought to be regulated by itself and the β and δ isoforms (Cao et al. 1991), which are induced early in cellular differentiation.

C/EBPα has been shown to have a direct link to the clock through activation of *Per2* and an inhibitory effect on *Rev-erbα* (Gery et al. 2005). The *Per2* promoter contains a C/EBP binding site between -900 and -1500 bp, 5' to the transcriptional start site, to which C/EBPα and ε can bind to induce *Per2* transcription by 7 fold and 5 fold respectively (Gery et al. 2005). However, the inhibitory mechanism behind *Rev-erbα* regulation is unknown. Furthermore, the C/EBP homologue in the California sea slug, *Aplysia californica*, (ApC/EBP) has a circadian variation in expression (Lyons et al. 2006), suggesting that the mammalian homologues too could be under circadian regulation, in particular C/EBPα. In addition to this, C/EBPα is known to be activated by GSK3β phosphorylation (Datta et al. 2007; Ross et al. 1999). The activity of which is under circadian control (Iitaka et al. 2005).

C/EBPα and δ are known to induce transcription of the Clara cell secretory protein (CCSP) gene (Cassel et al. 2000), which is specific to the Clara cells that surround the bronchioles in the lung. Clara cells are known to show a robust oscillation when isolated in culture (Gibbs et al. 2009). There are further links between the C/EBP family and circadian output pathways such as the inflammatory response (discussed previously in Chapter 5). The

anti-inflammatory effects of TNFα, shown to be under circadian regulation, Chapter 5 and (Keller et al. 2009), have been shown to be in part mediated through the induction of 11β hydroxysteroid dehydrogenase type 1, an enzyme that converts inactive cortisol or corticosterone into its active form. This Gc conversion enzyme is induced by C/EBPβ with basal regulation mediated by C/EBPα (Ignatova et al. 2009). Furthermore, *C/EBPα -/-* mice cannot undergo full myeloid differentiation and as a result fail to produce mature neutrophils, leading to a lack of receptors for G-CSF and IL-6, (Zhang et al. 1997). IL-6 is a known inflammatory cytokine under circadian control (Guan et al. 2005) and G-CSF was further discussed in Chapter 5. C/EBPα has also been shown to directly up regulate both IL-6 and G-CSF transcription, (Zhang et al. 1998), as well as TNFα receptor production (Bristol et al. 2009), therefore, having a direct effect on the inflammatory response.

6.2 Aims

- To define whether C/EBPα has a circadian component to its expression at either transcript or protein level.

- To show that C/EBPα was directly regulated by the clock.

- To characterise its response to glucocorticoids at both transcript (luciferase reporters) and protein level.

- To characterise the effects of glucocorticoids on C/EBP δ transcript expression (using a luciferase reporter).

6.3 Results

6.3.1 Validation of the C/EBP α antibody

Previous commercial antibodies (Santa Cruz: code Sc-61 (Wolins et al. 2006)) proved to be somewhat misleading in the model systems used within this thesis. In both the Rat-1 cell line and murine lung tissue, a band which was larger than the predicted (43kDa) was observed. Because of this, I employed a different antibody (Epitomics: code 1074-1 (Bristol et al. 2009)) to characterise C/EBPα. This was first validated with the use of an over expression plasmid (Fig 6.1). Putative C/EBPα expression was confirmed in HEK293

and HeLa cells, and as a result of this HEK293 cells were used for future over expression studies, as expression was shown to be much greater in this cell line (Fig 6.1a). Furthermore, the over expression plasmid contained a His6 tag which was used to validate the over expressed band shown with the C/EBPα antibody (Fig 6.1b). This antibody, however, would only recognise one of the two C/EBPα isoforms, the p42 and not the p30. This did not pose a great problem as the latter is just a truncation of the former. The over expressed (upper band) is approximately 4kDa larger than the putative endogenous lower band, due to the His6 tag at the C-terminal end.

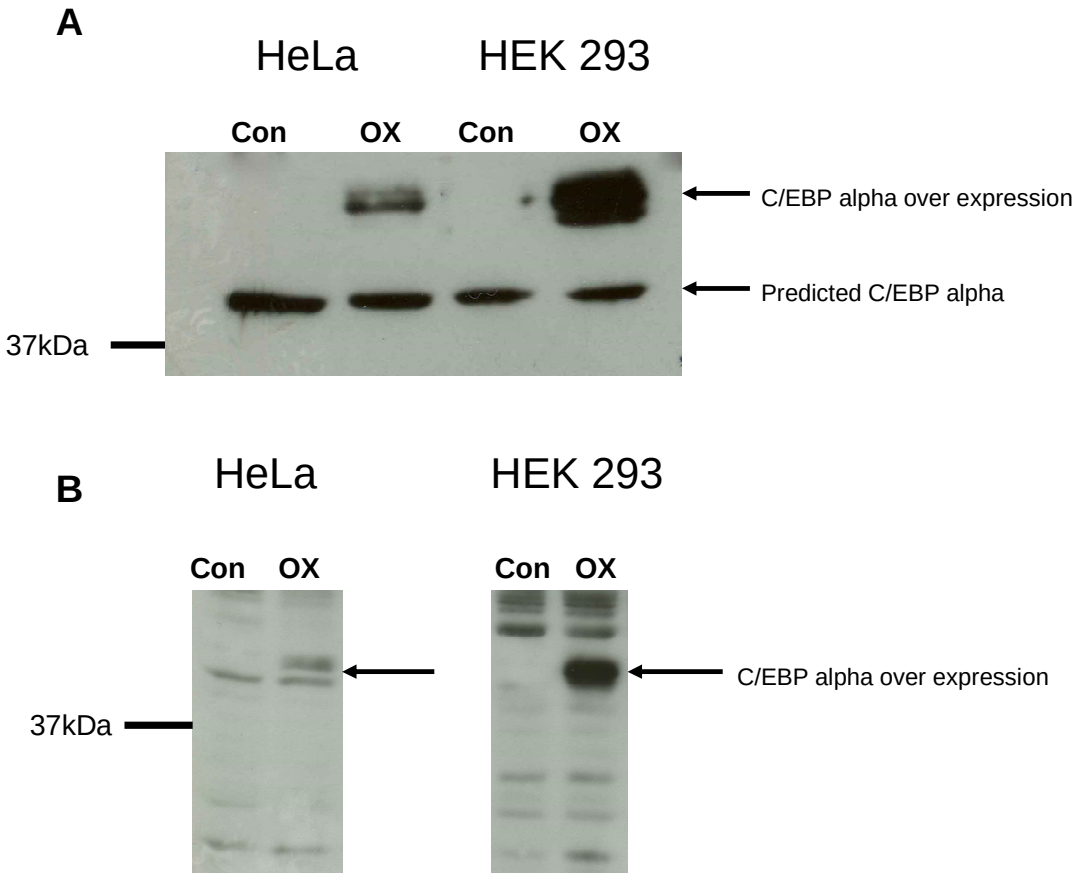

Figure 6.1: Validation of the C/EBPα antibody: C/EBPα was immuno blotted in either HeLa or HEK293 cells using an anti-C/EBPα antibody (A), which showed putative (lower band) or C/EBPα over expression (upper band). C/EBPα expression was confirmed by immuno blotting against the His6 tag, over expressed with C/EBPα (B), in both HeLa and HEK293 cells. Control (con) and over expressed (OX).

6.3.2 Circadian expression of C/EBP α mRNA and protein.

To detect whether C/EBPα was circadian in expression, protein was collected, again from the Rat-1 cells over the circadian day with a 4h temporal resolution and immuno blotted

for C/EBPα. There was no overall effect of time on protein expression (using One-Way ANOVA), nor did a pair-wise comparison between peak (CT4 or CT20) and trough (CT12) values reveal any significant difference (P= 0.07) (Fig 6.2b).

The expression of *C/EBPα* was also characterised at the transcriptional, mRNA, level using the Rat-1 cell line, with a 2h resolution throughout the circadian day using Taqman qPCR. The clock gene *Per2* was assayed in parallel as a marker of circadian phase, using cells samples 24h post-synchronisation (CT0). *Per2* expression showed clear circadian expression, as expected, with minimal expression between 8h and 12h and maximal expression between 20h and 22h, approximately a 4.5-fold increase between the two time points (Fig 6.2c). However, *C/EBPα* expression was minimal at 0h and steadily increased as time progressed, which resulted in a 6-fold increase between 0h and 22h (Fig 6.2c) with no apparent circadian variation. These data suggested that *C/EBPα* gene expression was not under circadian control in the Rat-1 cell line. The C/EBP*α* protein expression does, to some extent, conform to *Per2* transcript expression, with a 2-4h phase lag (Fig 6.2c versus b), as minimal *rPer2* mRNA expression is reached at 8 to 12h, and 12h showed minimal protein expression. The *Per2* transcript then increased at 16h and the C/EBPα protein showed maximal expression between 20h and 22h. However, the small amplitude oscillation in C/EBPα protein expression did not correlate with the respective transcript expression, and therefore, must be a result of post-translational modification.

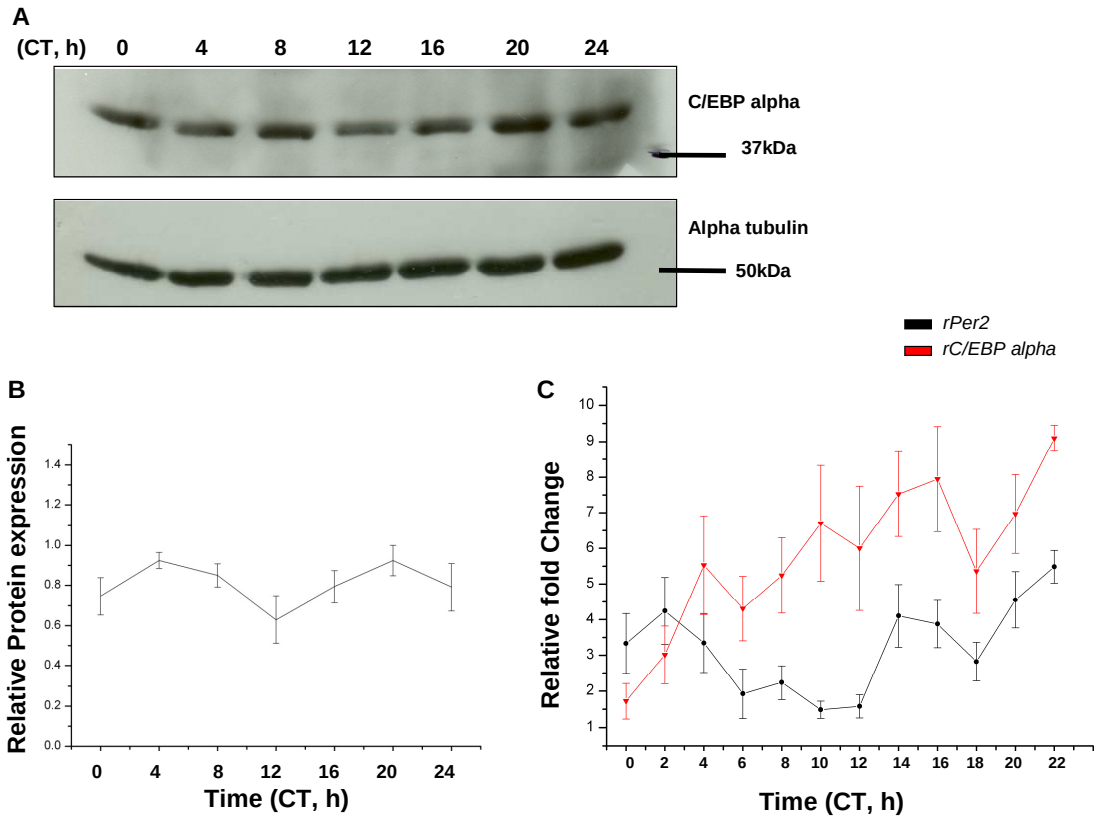

Figure 6.2: Putative C/EBPα protein and endogenous transcript expression in the Rat-1 cells over 24h: Putative protein expression was characterised for C/EBP α (A; representative blot, upper panel) and α-tubulin (A; representative blot, lower panel) in the Rat-1 cells every 4h over the circadian day by immuno blot. Quantified expression is relative to α-tubulin (B). *C/EBPα* transcript expression was characterised for *rC/EBPα* (red) and *rPer2* (black) in the Rat-1 cells every 2h, over the circadian day, by Taqman qPCR. Expression is fold change over basal expression. Error bars are SEM of n=3.

To complement the expression in the Rat-1 cells, lung protein was extracted from mice collected over the circadian cycle, (section 2.3.2.2). The results from the immuno blot (Fig 6.3) showed that there was no defined C/EBPα protein rhythm in the lung. The results from Fig 6.3 are based on only 2 replicates per time point due to limited sample availability. The representative blot (Fig 6.3a, upper panel) showed constitutive expression over the time course. Together with rhythm in the Rat-1 cells (Fig 6.2) this suggested that the C/EBPα protein was only weakly rhythmic and if so, perhaps only in certain cell/tissue types.

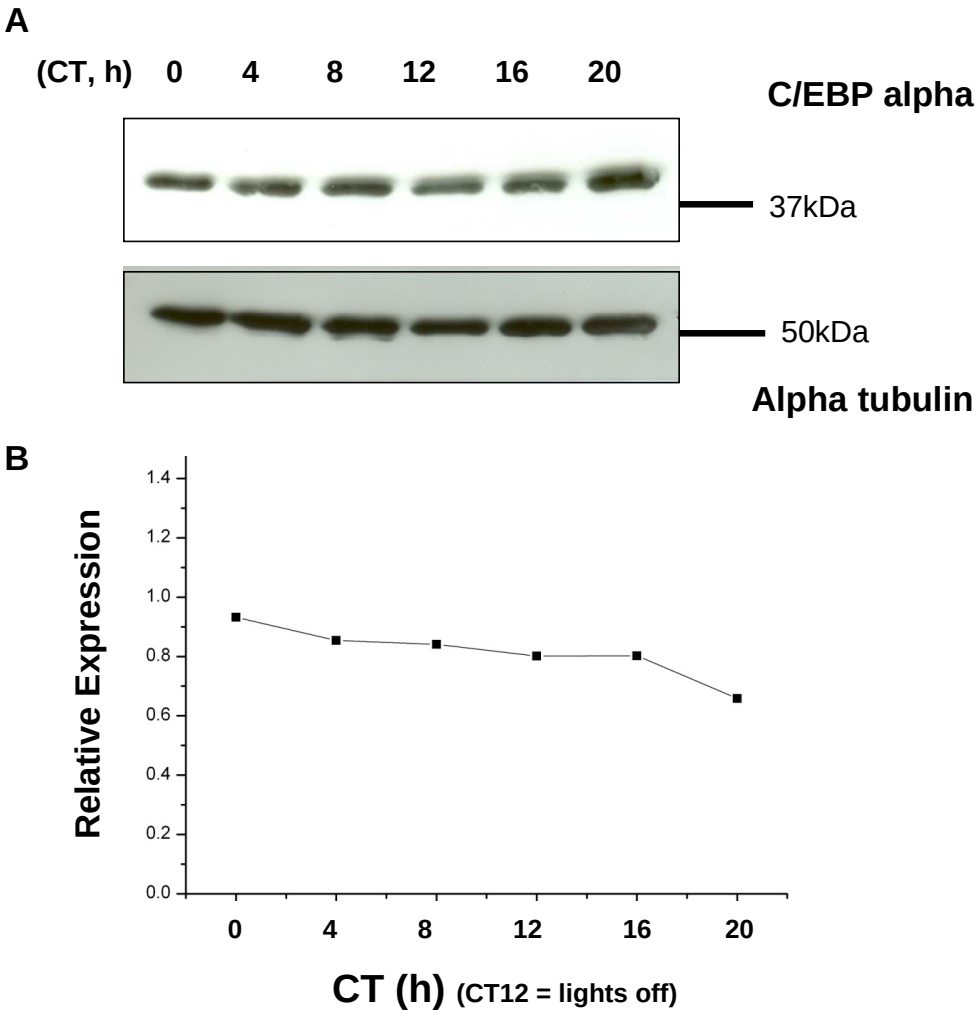

Figure 6.3: C/EBP α protein expression in mouse lung over 24h: Protein expression was characterised for C/EBP α (A; representative blot, upper panel) and α-tubulin (A; representative blot, lower panel) in the WT mouse lungs every 4h over the circadian day, by immuno blot. Expression is relative to α-tubulin (B). There are no error bars due to n=2.

6.3.3 CLOCK/BMAL1 mediated E-box regulation of C/EBP α

Previous sequence searches of the *C/EBPα* promoter, carried out by Dr. Qing-Jun Meng, had revealed there to be two classic E-boxes (CACGTG) and three non-canonical ones (CACATG) within 5kb of the transcriptional start site (refer to schematic Fig 6.7a). Thus, CLOCK/BMAL1 regulation of the C/EBPα protein was characterised. The resulting immuno blots showed that a co-transfection of CLOCK/BMAL1 had no effect on C/EBPα protein expression, above CLOCK or BMAL1 single transfection or the empty vector control, pcDNA3.1 (One-Way ANOVA P=0.66, Fig 6.4), although in work appendix E suggests that CLOCK/BMAL1 can induce a C/EBPα::luc promoter. These data suggest

that the E-boxes were either non-functional, although endogenous transcript was not analysed, or that C/EBPα protein expression is constitutive and regulation, circadian or otherwise, is controlled at a post-translational level.

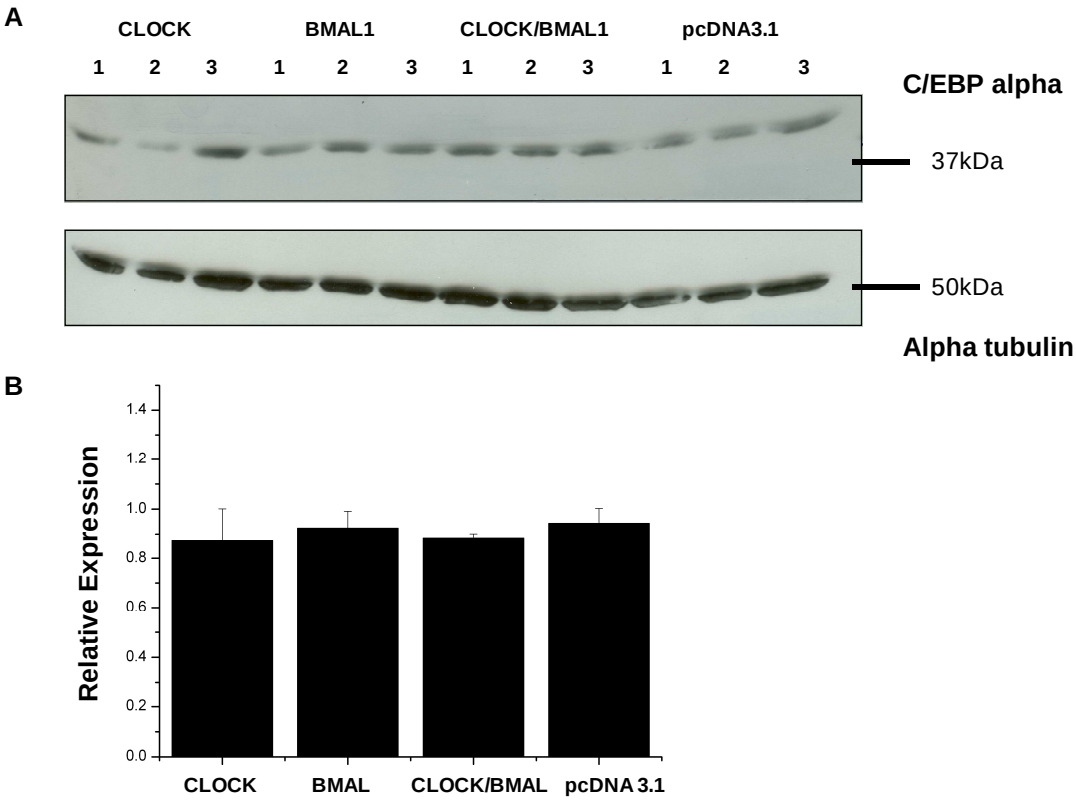

Figure 6.4: C/EBPα protein expression after CLOCK/BMAL1 over expression: Protein expression was characterised for C/EBPα (A; immuno blot, upper panel) and α-tubulin (A; immuno blot, lower panel) 24h after CLOCK, BMAL1 or CLOCK/BMAL1 was over expressed. Expression is relative to α-tubulin (B). Error bars are SEM of n=3 with all replicas shown (1-3).

6.3.4 Glucocorticoid regulation of C/EBPα

6.3.4.1 DEX regulation of C/EBPα protein

Previous published work had shown that DEX can repress C/EBPα protein expression after 4h, yet at the same time increase C/EBPδ expression. To confirm this report, synchronised Rat-1 cells were treated with 100nM DEX and protein was extracted over the following 8h (Fig 6.5). It was clear that DEX did not repress C/EBPα protein at any of the time points studied, relative to the vehicle control (One-Way ANOVA P=0.097). However, protein expression was reduced after 8h but this also occurred in the presence of the vehicle, which suggested that this was a result of the endogenous decline in expression towards the trough

of the relatively weak circadian rhythm, previously shown in the Rat-1 cells (Fig 6.2) and not a result of DEX regulation.

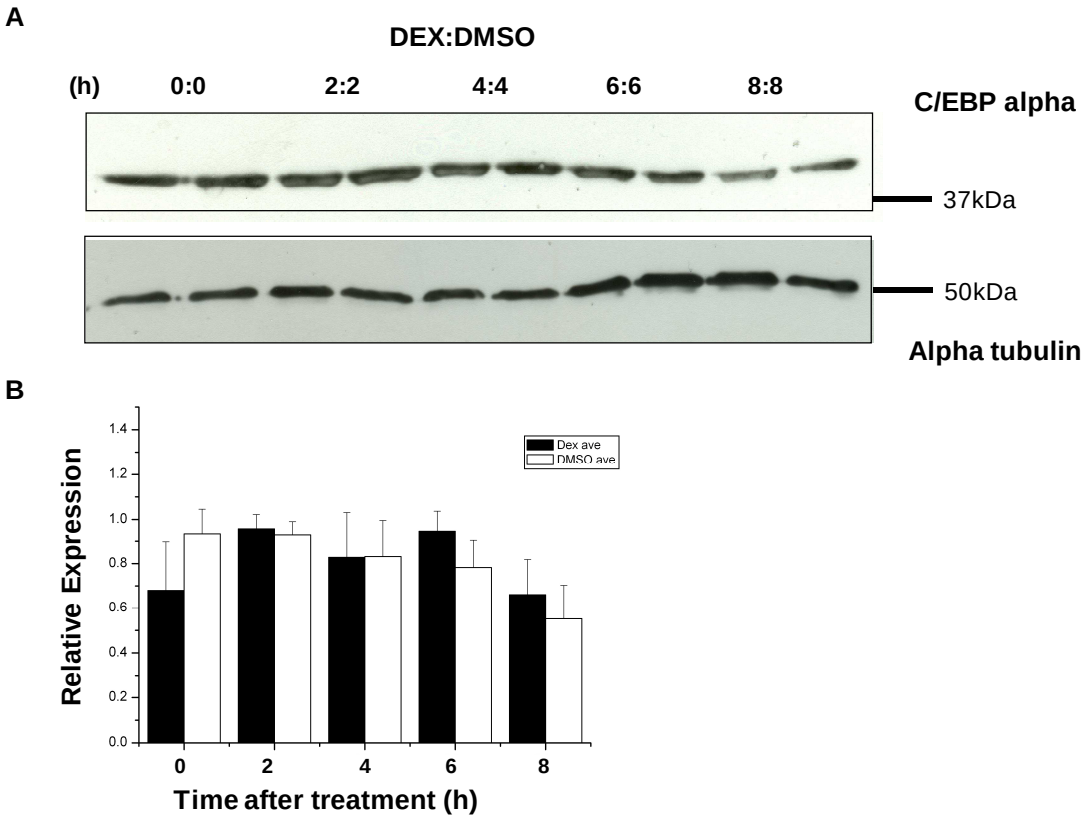

Figure 6.5: C/EBPα protein expression after DEX: Protein expression was characterised for C/EBPα (A; representative blot, upper panel) and α-tubulin (A; representative blot, lower panel) over 8h following 100nM DEX. Expression is relative to α-tubulin (B). Error bars are SEM of n=3.

6.3.4.2 DEX regulation of C/EBPα and δ promoter reporters

As DEX did not appear to regulate C/EBPα at the protein level, at least in the Rat-1 cells, the effect of DEX on the various promoters constructs of both α and δ was characterised. There were 4 α constructs (1300bp, 650bp, 280bp and 240bp) and 1 δ construct (1700bp) each 5' to the transcriptional start site (section 2.3.2) supplied by Dr Qing-Jun Meng. DEX was added to the *C/EBPα* 1300bp and *δ* construct for either 4h or 24h, using a range of concentrations (Fig 6.6), and its affects analysed by luciferase assay. DEX had significant repressive effects on both constructs, after 4h, with all the concentrations of DEX used. DEX had a greater repressive effect on *C/EBPδ* resulting in an approximate 40% reduction, relative to the DMSO control, compared to a 27% reduction with *C/EBPα* shown with 100nM DEX (Fig 6.6a and b) but both constructs showed significant

reduction. Importantly DMSO alone had no significant effects on the constructs as there was no difference between DMSO treated cells and untreated cells (labelled as cells, Fig 6.6).

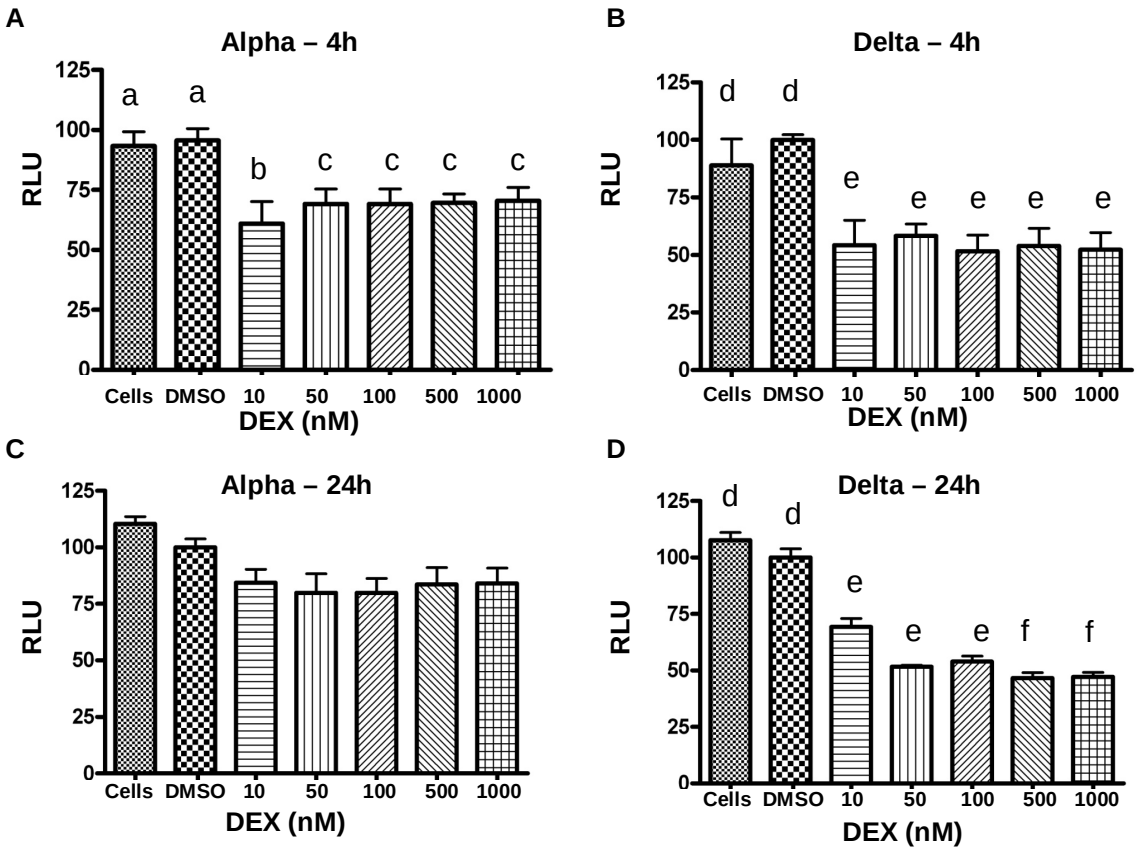

Figure 6.6: DEX effects on C/EBPα 1300bp and C/EBPδ 1700bp promoter constructs: DEX was added to Rat-1 cells expressing either C/EBPα or δ reporter constructs for 4h (A and B respectively) or 24h (C and D respectively), then analysed using a luciferase assay. DEX concentrations are shown with DMSO as the vehicle control, cell refers to untreated cells. Units are RLU, normalised to DMSO. Error bars are SEM of n=3 repeated 3 times. Statistical analysis was by Two-Way ANOVA ($P<0.0014$ for DEX interaction with both constructs and $P=0.9$ for time interaction with the delta construct, and $P=0.0008$ for a time interaction with the alpha construct) with a Bonferroni post-hoc analysis. With regard (A): (a) versus (b) $P< 0.001$, (a) versus (c) $P< 0.01$, (b) versus (c) not significant. With regard to (B) and (D): (d) versus (e) and (d) versus (f) $P< 0.001$, (e) versus (f) $P<0.05$. There is no significant specific interaction with regard time for either the alpha or delta construct after post-hoc analysis.

However, the repressive effects of DEX on the α construct were only transient, relative to the δ construct, as the DEX suppression was still observed after 24h with the δ construct but was not detectable using the α construct (Fig 6.6c and d). Taking the 100nM DEX treatment as an example, after 24h there was a significant 50% reduction, relative to the DMSO control, with the δ construct, but only a 20% reduction with the α construct, which

was not shown to be significant. This suggested that although DEX was able to repress both constructs, up to 4h, the effects of DEX were much more sustained with regard to the δ construct, with repression up to at least 24h after DEX treatment.

As different truncated constructs of *C/EBPα* were available, the repressive effects of DEX were further investigated, to try and isolate a particular region of the promoter responsible for the observed effects and to relate it to a known mechanism. DEX was added to the 4 *C/EBPα* constructs (1300bp, 650bp, 280bp and 240bp) and the effects were analysed, by luciferase assay after 4h, based on the earlier work (Fig 6.6). As a control the 1300bp construct showed significant inhibition in the presence of DEX, relative to DMSO, with an approximate 22% reduction (Fig 6.7b), as seen in Fig 6.6. Further to this the 650bp construct also showed significant repression in the presence of DEX, with a significant 20% reduction in luciferase signal. However, with the 280bp construct no significant DEX induced suppression was shown (Fig 6.7b). Basal expression, as seen by DMSO, was increased relative to the 650bp construct, by approximately 10%, but this did not reach significance with DEX expression being significantly increased by approximately 20% (relative to 650bp construct), but still much less than the 1300bp construct. The 240bp construct showed only background expression in the presence of either DMSO or DEX. Together these data suggest that in the 370bp between the 280 and 650bp constructs there is an element that can mediate the effects of DEX via the GR, yet within this region there is also a repressive element, or simply the loss of further inducing elements, that results in the overall reduction of basal expression.

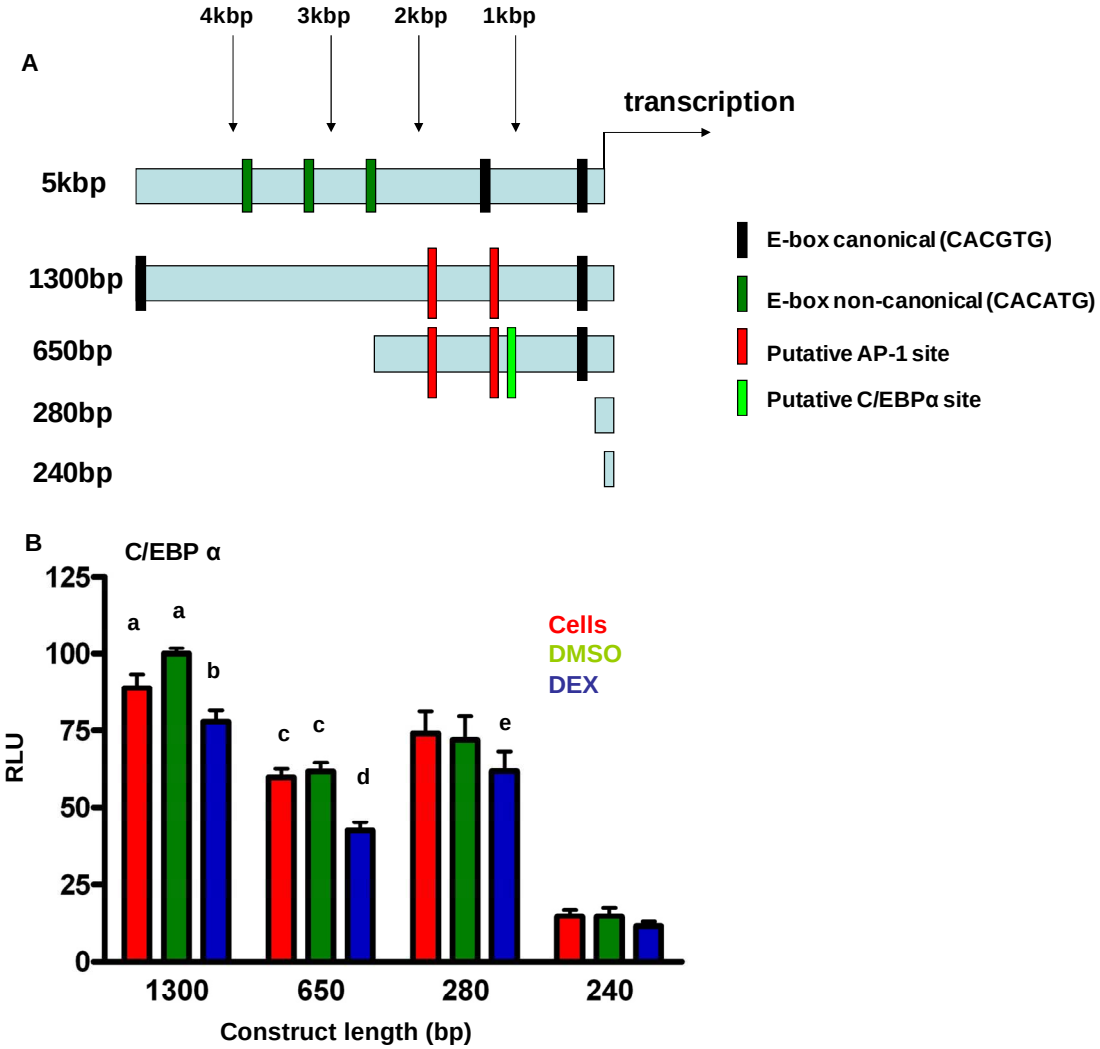

Figure 6.7: DEX mediated repression of various C/EBP α constructs: A cartoon diagram of the 4 C/EBP α constructs (1300, 650, 280 and 240bp) with canonical E-boxes (402bp and 1290, black); non-canonical E-boxes (2589bp, 3012bp and 3856bp, dark green); putative AP-1 sites (331bp and 441bp, red) and the putative C/EBP α binding site (316bp, light green). Each construct was treated with either 100nM DEX (blue), DMSO (green) or untreated (cells, red) for 4h before lysis and quantification by luciferase assay (B). Expression is relative to the 1300bp construct treated with DMSO. Error bars are SEM of n=3 repeated 3 times. Statistical analysis was by Two-Way ANOVA ($P<0.0001$ for treatment and construct length) with a Bonferroni correction, (a) versus (b) and (c) versus (d) $P<0.001$, (d) versus (e) $P<0.05$, (a) versus (c) and (b) versus (d) $P<0.001$.

6.4 Discussion

6.4.1 Rational for the C/EBP studies

At an early phase in this study I started out to test whether C/EBP (α and δ) proteins were a key link between Gc and the circadian clock. This hypothesis was supported by early literature which reported strong circadian control of C/EBP at both the RNA and protein level in Aplysia (Hattar et al. 2002; Lyons et al. 2006). C/EBPα had also been shown to

induce *Per2* transcription (Gery et al. 2005). Earlier studies had reported significant action of DEX on both C/EBPα and δ isoforms (MacDougald et al. 1994), thereby providing a mechanism where Gc could re-set the clock through the intermediary action of C/EBP. Unexpectedly, this hypothesis proved not to be the case as the reported effect of DEX could not be repeated nor was the transcript or protein overtly rhythmic.

6.4.2 Circadian expression of C/EBPα transcript and respective protein

The antibody used to detect C/EBPα protein was a rabbit monoclonal antibody, which even though very specific, shown by the data here (Fig 6.1) and Bristol et al. 2009, only recognised the larger, p42, isoform and not the p30. Furthermore, the qPCR data (Fig 6.2) was non specific, and as such, did not distinguish between either isoform. However, both isoforms are a product of the same genetic locus and are therefore subject to the same promoter and enhancer effects (Ramji and Foka 2002), which suggest that what is seen with one will be the same as the other, at least at the transcript level, but has also been shown at the protein level, with respect to DEX repression (MacDougald et al. 1994). The *C/EBPα* transcript does not oscillate in phase with *Per2* expression, even though there is a time of day difference, where 0h showed minimal expression and 22h showed maximal expression, however, these data do not suggest purely E-box mediated circadian regulation. It is perhaps possible that *C/EBPα* is under circadian regulation via multiple mechanisms as there are three elements that are known to regulate circadian transcription; E/E' boxes (so called canonical and non-canonical respectively), D-boxes and RORE (Ueda et al. 2005). As described by Ueda et al. (2005) a combination of these three transcriptional regulatory elements determines the phase of expression within the circadian day. There are two canonical E-boxes within the *hC/EBPα* promoter region (used in the luciferase assay studies), at -402bp and -1290bp and a potential RORE at -706bp. The human and mouse promoters also have 2 canonical E-boxes within 2kb of the transcriptional start site and potential RORE. There may also be further regulatory elements upstream or, as in the case of the GRE in the *Per2* gene (So et al. 2009), downstream within the intronic region. If this hypothesis is correct and there is both E-box and RORE regulation of *C/EBPα* then this will put transcription in phase with *Cry1* and phase delayed relative to *Per2* (Etchegaray et al. 2003; Ueda et al. 2005). Although more recent studies have suggested this delay can be achieved through the E-box alone (Fustin et al. 2009). *rCry1* expression was not analysed in this model system,

The protein product was shown to be weakly rhythmic with the peak at 0h and a trough at 12h (Fig 6.2) but only in the Rat-1 cell model, and expression in the lung was constitutive across the circadian day. Absence of circadian changes in the lung may be a reflection of the complications of using the tissue explant with multiple cell types, only some of which appear to be under circadian control (Gibbs et al. 2009). The only other previously reported circadian oscillation of C/EBP has been in the *A. californica* (Hattar et al. 2002; Lyons et al. 2006). Taken together, C/EBPα is at best only weakly circadian and only in specific cell types. However, there could be a post-translational oscillation, like that of GSK3β (Iitaka et al. 2005), as C/EBPα is a target protein of GSK3β (Ross et al. 1999).

6.4.3 Gc regulation of the *C/EBPα* transcript and respective protein

In the Rat-1 model DEX failed to repress C/EBPα protein expression, as shown previously by MacDougald et al. (1994) but a DEX mediated repression in promoter expression was shown (Fig 6.6a) similar to that of MacDougald et al. (1994), that reached a minimum at 2h post DEX (4h shown in this study) and returned to baseline expression after 6h (24h in this study). However, opposing results were seen with respect of *C/EBPδ*, since MacDougald et al. (1994) showed an immediate increase in δ mRNA expression after DEX, that reached a maximum at 4h, and then returned to basal expression after 24h. In the study presented above, sustained δ repression was seen up to 24h in the presence of DEX (Fig 6.6b and d) but this was not reported by MacDougald et al. (1994). Taken together the repressive effects of DEX on *C/EBPα* are not observed at the protein level and as such do not conform to the previous literature; likewise opposing effects on the δ isoform were seen. These conflicting results can only be explained by differences in cell models and DEX concentration. Endogenous RNA from 3T3-Ll adipocytes were used by MacDougald et al. (1994) with 1μM DEX, whereas Rat-1 cells were used with 100nM DEX, in this study presented above. This also means that the use of limited size promoter constructs will no doubt exclude other regulatory sites and as a result could produce conflicting outcomes compared to an endogenous readout.

The inhibitory effect of DEX on the *C/EBPα* promoter fragments was of some interest as there was significant inhibition regarding the two larger fragments, 1300bp and 650bp, but no inhibition with the smaller fragments, 280bp and 240bp (Fig 6.7). These results suggest that there is an inhibitory element in the promoter between the 650bp and the 280bp region upstream of the transcriptional start site. A simple promoter search for transcription factor

binding sites, using the bioinformatics software, Genomatix, revealed an AP1 binding element was located within 370bp region. AP1 is transcription factor made up of cJun homodimers or c-Jun/c-Fos heterodimers and the subsequent interactions determines whether glucocorticoids have positive, negative or no effect on transcription. No effect means that there is a high cJun:cFos ratio and repression is a result of a high cFos:cJun ratio (Imai et al. 1993). However, following on from these findings no further work was carried out on the *C/EBP* promoter fragments, as these results conflicted with published work and the endogenous protein no rhythmic.

6.4.4 E-box mediated regulation of C/EBPα protein

The CLOCK/BMAL1 mediated induced expression was first shown with the *C/EBPα* 1300 and 650bp, at the construct level by Dr. Qing-Jun Meng (appendix E), but not with *C/EBPδ* 1700bp, perhaps due to there being only one non-canonical E-box (CACATG) within the promoter region at -1184bp. There was significant induction over the empty vector control by CLOCK/BMAL1 co-transfection with the alpha constructs but no induction with the delta construct. This E-box induced expression was similar to work with a *Per1::luc* construct where CLOCK/BMAL1 transfection significantly induced expression (Motzkus et al. 2007). However, the expression shown at the promoter level, with regard to alpha, was not replicated at the protein level, where CLOCK/BMAL1 over expression failed to induce significant expression over the empty vector control. This suggested that although C/EBPα transcription was under E-box, CLOCK/BMAL1, mediated circadian regulation, the protein product was not (Fig 6.4), and coupled with the weak endogenous protein rhythm (Fig 6.2) suggested that there was no post-translational modification coupled to degradation that defined a circadian oscillation.

6.4.5 Post-translational modification of C/EBPα

However, there could be a phosphorylated C/EBPα rhythm as GSK3β, known to be under circadian regulation (Iitaka et al. 2005), has been shown to phosphorylate C/EBPα on amino acids T222 and T226 and is involved in adipocyte differentiation (Ross et al. 1999). Although others have reported that C/EBPα is a very poor substrate for GSK3β in *in-vitro* cells line studies of hepatic gene expression (Liu et al. 2006a). This perhaps points to tissue specific regulation of C/EBPα and why more promising results were not seen in this study, as cell lines and lung tissue were used. Furthermore, phosphorylation of C/EBPα,

by MAPKP42/44 at S21, has also been shown induce conformational changes causing the amino termini of the C/EBPα homodimer to move apart and consequently lose transcriptional activity (Ross et al. 2004). Sumolyation, a small ubiquitin-related modifier like protein, is another reported post-translational modification of C/EBPα. Sumolyation itself adds approximately 20kDa to the protein, producing a 64kDa product which inhibits the transcriptional potential of C/EBPα, as seen in the differentiation of hepatocytes (Sato et al. 2006b). However, there is little evidence to support extensive post-translational modification and regulation of C/EBPδ

6.6 Conclusions

C/EBP α has been shown here to be weakly rhythmic at the protein level, in the Rat-1 cell line, but with constitutive expression in the lung. There is also no obvious transcript rhythm in the Rat-1 cells, although differential expression was shown it is not in phase with *Per2*. At the promoter level CLOCK and BMAL1 were shown to induce expression of *C/EBPα* but not *δ*, likely to have been through the multiple E-boxes, however, this did not carry through to a direct protein regulation of *C/EBP α*. Furthermore, direct regulation of C/EBPα by the glucocorticoid analogue, DEX, was not shown although this has been established in the literature. However, at the promoter level DEX did repress both *C/EBPα* and *δ* expression, which confirms that seen in the literature with regard to *C/EBPα* at the protein level but was opposite to the induction seen at the protein level with respect to *C/EBPδ*. The Gc regulation of *C/EBPα* was further narrowed down to a 370bp region between -280bp and -650bp, which is potentially an AP-1 binding site. However, a more thorough investigation of *C/EBPδ* was not undertaken, nor was this initial work with C/EBPα progressed due to it lack of circadian and Gc regulation in the model systems used here.

6.7 Future directions

- C/EBPα did not appear to oscillate at the transcript level and only with a weak rhythm at the protein level but a phospho-protein rhythm was not investigated and therefore would be useful, if only to rule it out.

- A transcript rhythm of C/EBP delta was not investigated here and neither was the protein rhythm, so these too should be investigated, if nothing else than for

completeness. However, there was no CLOCK/BMAL1 induction of the delta construct (appendix E) so perhaps there is no rhythm to be found.

- There is literature linking C/EBPβ and the induction of HSD11β type 1 (the enzyme converting inactive CORT to active CORT) in association with TNFα, also C/EBPα and beta directly bind to and induce the TNFα receptor. Therefore, it would be interesting to further characterise these inflammatory associations, particularly with respect to the circadian component of TNFα both under basal and stimulated conditions. Is the *TNFα* gene regulated by C/EBP α and β, how does this link in with the circadian characteristics of TNFα, is IL-6 regulation similar?

- To take a C/EBPα expression vector and added it to both clock promoter reporters and primary protein reporters, such as the lung, to characterise direct C/EBPα input effects on the clock. Does C/EBPα change the phase of the clock? Does it induce, as suggested by Gery et al. (2005), or suppress the oscillation?

Chapter 7: General Discussion

7.1 Thesis overview

There have been two major themes throughout this thesis, one being circadian timing and the other being its regulation by the steroid hormones, glucocorticoids. The study began by investigating the effects of the endogenous glucocorticoid, CORT, on the core clock genes *Per*1 and *2* and the PER2 protein itself; with the aid of transgenic luciferase reporters using both cell lines, a reductionist approach, and using tissue reporters, primarily the lung. Use of the lung as a peripheral model is novel, as most work to date had focussed on the liver (Kornmann et al. 2007; Reddy et al. 2007). However, the lung has been shown to have a functional oscillator using *ex-vivo* slice preparations, and isolated pulmonary cell types such as the Clara cells, which are robustly rhythmic (Gibbs et al. 2009; Yoo et al. 2004). Further to this, respiratory diseases such as asthma and COPD have been shown to have a circadian component to their symptoms, while Gcs are one of the main treatments of choice in the suppression of inflammatory responses, used specifically to alleviate airway tightness in asthma (Barnes and Adcock 2003). Glucocorticoid resistance is a problem in COPD and these hormonal agents are often ineffective (Barnes et al. 2004). The mechanisms underpinning this are poorly understood. Lung tissue has provided a robust and reproducible model to study circadian timing, using luciferase transgenes and the PMT system. Lung slices have also been a much better peripheral reporter than other tissues such as the liver or kidney, most likely due to the nature of the lung being a very light, open structure compared to the dense nature of the liver or kidney making it difficult for photons of light to be emitted and detected.

CORT has been shown to be a potent regulator of circadian timing, especially in the periphery of the mammalian system. It has been proposed that this may act as one of the key systemic signalling molecules that confer the phase of the central clock, located in the SCN, to the rest of the brain and peripheral oscillators (Balsalobre et al. 2000a), allowing individual oscillators throughout the organism to maintain and co-ordinate time. The nature of glucocorticoid regulation on the peripheral clock, using the lung as a model, was investigated in Chapter 3, with particular attention paid to the roles of the closely related nuclear hormone receptors the GR and MR. It is clear from these studies that GR and MR act differently on the CORT-mediating re-setting of the circadian clockwork. These repeatable observations formed the basis of studies reported in Chapter 4 and the concept of non-genomic glucocorticoid regulation of the pulmonary clock, mediated through CAV-1 signalling. This is novel and has not previously been reported in the literature.

In addition to studies regarding the central clock and glucocorticoid regulation, the influence of the circadian clock on the innate inflammatory response was characterised. Many inflammatory cell types and mediators, such as IL-6 and TNFα have been shown to exhibit a circadian rhythm and, perhaps more interestingly, in anti-phase to that of the circulating glucocorticoids (Haus and Smolensky 1999; Petrovsky et al. 1998). This background led to the work in Chapter 5 where the *in–vivo* gated inflammatory response was investigated in relation to LPS challenge, revealing that a small number of mediators had a circadian-gated response to LPS. This work was repeated in a primary cell culture model using PECs, which revealed a slightly different response in terms of mediators involved. However, effects on IL-6 remained consistent throughout the different experimental protocols. It was also shown, in Chapter 5, that REV-ERBα has a significant role on the gated inflammatory response, particularly with regard to IL-6, where gating was lost in a *Rev-erbα* -/- genetic background. This is currently uncharacterised in the literature, and the mechanism previously only hypothesised (Chapter 5 discussion). A recent study has provided a link between the inflammatory response and CAV-1 (Wang et al, 2009), and CAV-1 was discussed with regard to the non-genomic action of CORT on the clock in Chapter 4. These authors provided evidence that CAV-1 signalling is associated with TLR4, and that this association reduced inflammatory mediator expression after LPS challenge, mediators such as IL-6, CCL5/RANTES, TNFα and IL-12p40, amongst others, all of which were shown to be gated in response to LPS challenge. Together theses data provide a link between the clock and a clock output process (the inflammatory response), in terms of Cav-1 signalling.

C/EBPα was hypothesised to be under circadian regulation at both the transcript and protein level, as such acting as a clock output gene, but also C/EBPα was shown by Gery et al. (2005) to induce *Per2* transcription and, as such, acting as a clock input. Therefore, C/EBPα potentially has a dual role in relation to the clock. Lyons et al. (2006) had shown the *C/EBP* homologue in the sea slug *Aplasia* to have a circadian expression pattern, and MacDougald et al. (2004) had provided evidence that DEX suppressed C/EBPα and induced C/EBPδ protein expression. However, these observations on C/EBPα could not be confirmed in this study (Chapter 6), nor a robust transcript or endogenous protein rhythm detected in either cell lines or lung tissue. Therefore, although C/EBPα had began with potential as clock regulator and itself regulated by the clock, this did not materialise and

further experiments were not undertaken. However, more recent evidence has now provided a link between C/EBPα and the pro-inflammatory response and the anti-inflammatory effects of CORT. C/EBPα acts as a transcription factor that forms part of the pathway involved in TNFα receptor transcription (Bristol et al. 2009), and in this way C/EBPα regulates pro-inflammatory responses. Also C/EBPα is involved, along with C/EBPβ, in the basal regulation of 11-β HSD type1, which is the enzyme that converts inactive CORT to active CORT (Ignatova et al. 2009), and has been shown to have circadian rhythm in expression at the mRNA level in the hippocampus (Buren et al. 2007). Therefore, C/EBPα has both pro-inflammatory and anti-inflammatory properties, with significant circadian links. It is possible that the reason for not detecting a role in the studies reported in this thesis is that the wrong cell types were used, and in this regard an inflammatory cell mediator (i.e. macrophage) may prove more reliable.

7.2 The complexity of Gc signalling: integrating circadian and ultradian pulses

Plasma CORT levels are known to oscillate in a circadian manner, in both rodents and humans, peaking prior to the onset of activity and in phase with *Per1* and *Per2* mRNA expression (Oster et al. 2006; Takimoto et al. 2005; Yang et al. 2009b). However, CORT also oscillates in an ultradian manner, as shown in the rat, peaking approximately every 50 min (Windle et al. 1998a; Windle et al. 1998b), which has been proposed to be in response to pulsatile ACTH and CRF release, yet the origin of the pulsatile release is still unknown (Carnes et al. 1988; Mershon et al. 1992).

It has been shown from the work in Chapters 3 and 4 of this thesis, and from other published work, that Gcs can reset the circadian clock and stimulate rhythmic gene expression, yet have no direct effect on the SCN, as the GR is not expressed (Balsalobre et al. 2000a). The effect of the ultradian oscillation has been shown to be biologically relevant at both the behavioural and transcriptional level. At the behavioural level the ultradian pulses have been shown to be vital in the animals' response to stress (Lightman et al. 2008; Windle et al. 1998b). If the animal (female rat in this study) was exposed to noise stress, which coincided with a rise in the endogenous ultradian rhythm, then plasma CORT increased rapidly. However, if the stress coincided with the decline in the ultradian rhythm then no CORT induction was observed, and therefore the animal failed to respond to the stress situation (Windle et al. 1998b). At the transcriptional level the cell and GR

has been shown to respond effectively to rapid pulsing of CORT, but not other long lasting steroid agonists such as DEX or prednisolone (Stavreva et al. 2009). Through pulsing experiments the authors showed that 10 min after removal of CORT (wash out) the GR will dissociate from the GRE within a target gene, yet endogenous GR levels remained constant, showing that the GR-CORT interaction is highly dynamic. Furthermore, the un-liganded GR must re-associate with chaperone proteins (HSP90) prior to new ligand binding, which provides an inbuilt delay mechanism (Stavreva et al. 2009). Of particular interest in this study was the clock gene *Per1*. *Per1*, as well as other genes, responded differently when exposed to pulsatile and continuous CORT treatment. The nascent *Per1* transcript (unspliced or pre-mRNA) was rapidly induced by the CORT pulse and expression quickly fell after washout, whereas continuous CORT treatment gave constitutively high levels. Further to this, when the mature *Per1* transcript (spliced and prepared for translation) was quantified, CORT pulsing resulted in significantly lower levels of expression relative to continuous treatment but showed no pulsing effects, which was mirrored by two other genes and at the protein level (Stavreva et al. 2009). Together these data provide evidence that CORT pulsing is biologically relevant and is interpreted by the animal at a behavioural and gene transcript level, and has a direct effect on the circadian clock (*Per1*).

So how then does the body integrate an ultradian and circadian signal, which can entrain the peripheral clock (including brain regions except the SCN) but whose signal can also be ignored at times other than when required, so that the peripheral body is not reset every 50 min? The answer to this question remains undetermined, but it is likely to be mediated through a gating mechanism in the adrenal clock and the effects of light directly on the adrenal gland (Ishida et al. 2005; Oster et al. 2006).

The canonical clock genes (*Per1*, *Per3*, *Cry1*, *BMAL1* and *Rev-erbα*) are all preferentially expressed in the cortical layers, rather than the medulla, of the adrenal gland (ZF and ZG), which are the areas from where CORT is synthesised and released (Simpson and Waterman 1988). Interestingly, *Per2*, *Cry2* and *Clock* mRNA were not preferentially expressed in the cortex, but PER2 protein expression did show a defined temporal difference in the ZF and ZG compared to the medulla (CRY2 and CLOCK protein was not tested) (Oster et al. 2006). Using WT and *Per2/Cry1* knock out, arrhythmic mice Oster et al. (2006) showed that in WT mouse the ACTH rhythm peaks 2h prior to the peak of the

CORT rhythm. They also showed that both rhythms are lost in the arrhythmic mouse, in both LD and DD conditions, with consistent low levels of expression. Together these data suggest that in the absence of a functional clock, the adrenal gland cannot properly respond to ACTH and fails to integrate a rhythmic output (CORT). Furthermore, Oster et al (2006), using a series of transplant experiments involving *Per2/Cry1* knock out mice being transplanted with WT adrenals and vice versa, concluded that the adrenal clock can be light entrained, even in the absence of a rhythmic SCN clock. Also, that there is no apparent feedback from the adrenal clock to the SCN (most likely due to the absence of the GR), but the adrenal gland does affect CORT rhythms by gating sensitivity to ACTH, as CORT rhythms were observed in arrhythmic mice (constitutive ACTH levels) implanted with WT adrenals in LD, but not DD. Incidentally, a WT adrenal transplanted into an arrhythmic mouse did not restore differential expression in *Per1* and *Rev-erbα* transcript in the kidney in LD or DD; rather expression reflected the genotype of the host animal. Taken together these data suggest that rhythmic CORT release requires a functional adrenal clock yet only requires constitutive ACTH release under LD conditions.

In addition to ACTH stimulation of CORT release from the adrenal glands, the adrenals are innervated through multiple synapses, resulting in the innervation of the adrenal with a branch of the splanchnic nerve (Buijs et al. 1999; Ishida et al. 2005). Ishida et al (2005) showed that a 30 min light pulse can induce *Per1*::luc expression in the adrenal gland, with specific *Per1* mRNA expression observed in the ZF, ZG and zona reticularlis (ZR). The 30 min light pulse induced a rapid, yet transient rise in plasma CORT levels which were maximal 30 min after the light exposure and had declined to basal levels by 180 min. Importantly, this light induced release of CORT was independent of ACTH changes. Denervation of the adrenal gland resulted in the loss of light-induced *Per1* expression, in a similar manner to lesion of the SCN itself. Interestingly, Ishida et al (2005) showed that light intensity had a dose dependent effect on serum CORT release, leading to maximal release in response to an intensity of 40 lux roughly equal to sunset and sunrise (beginning and end of activity phase). Also serum CORT levels and *Per1* mRNA expression only responded to light in the subjective night under constant darkness, similar to how the SCN responds to a light pulse (section 1.9.2.1).

Therefore, in the rodent, the adrenal release of CORT is gated by the clock, which follows ACTH release, and peaks prior to the onset of activity. Light is also able to induce *Per1*

expression and CORT release, with a maximal response at intensity similar to that observed at dawn and dusk, but does not induce expression during the subjective day; therefore light at dusk is likely to be the first light the animal can respond to at a molecular level. The pulsing of CORT occurs every 50 min and is involved in the stress response, but does not result in an ultradian *Per1* transcript rhythm. So the animals response to CORT is two fold. First the circadian response and induction of *Per1* and rhythmic clock gene expression, perhaps being an integration of multiple zeitgebers such as CORT, light (indirectly via the SCN) and other entraining factors such as melatonin and ghrelin (sections 1.9.3.1 and 1.9.3.4 respectively), which are induced in response to darkness (melatonin) and as an appetite stimulant (ghrelin). Second to the circadian expression is the ultradian CORT pulses, which will induce *Per1* transcription but perhaps due to the lack of integration with other zeitgebers these pulses do not constantly reset the peripheral clock and only become relevant under a stress response and then only when the stress coincides with a rise in the pulse.

7.3 Why is there a rapid re-setting of the clock and amplitude response of PER2?

Work in this thesis (Chapter 3) has shown that CORT can not only alter the phase, period and sustained (chronic) amplitude of the PER2::luc rhythm, but can induce an immediate (acute) amplitude rise (as seen through the luciferase signal). It is understandable that a stimulus, like CORT, should induce a transcriptional response (i.e. *Per1* and *Per2*) which in turn produces more protein, and results in a phase advance or delay. This entrainment process (one of many) is in place in order to synchronise peripheral clocks with the SCN, and therefore the external environment. It is also advantageous to be a slower, transcriptional based response so that the peripheral clocks are not constantly re-set every time a stimulus passes by via the circulation (melatonin, serotonin, CORT and food signals), as this would easily lead to internal de-synchrony. However, why would it be necessary to produce a CORT-induce rapid protein response?

In Chapter 3, I showed only a CORT-induced acute amplitude response of PER2::luc (Fig 3.3 and 3.9), a response to other stimuli was not shown nor was the response of other clock proteins tested (CRY1, PER1 or BMAL1). It is therefore currently unknown whether this effect is specific to PER2 and CORT, or a general response to re-setting stimuli. However, work with the non-steroidal agonists (Fig 3.18) did not induce an acute amplitude rise, even though it induced a phase shift. Also in Chapter 5 (Fig 5.6), LPS induced a phase

shift but, like the non-steroidal agonists, did not result in an acute amplitude rise. This suggests that the amplitude effects seen with CORT are specific and not just a general re-setting effect.

Presumably CORT does not re-set all peripheral clocks every time it is released, or in this case high amplitude ultradian pulsatility would certainly cause de-synchrony (Windle et al. 1998b). However, CORT no doubt has a synchronising effect due to its circadian oscillation, with peak levels of *Per1, 2* and *3* mRNA in the human aligning with the morning peak of the cortisol rhythm (Bjarnason et al. 2001; Takimoto et al. 2005) with the same occurring in the mouse but in the evening (Lee et al. 2001; Yang et al. 2009b). The clock has also been shown to gate CORT production in response to ACTH in the adrenal gland (Oster et al. 2006), and therefore there is a reciprocal interaction between CORT and the clock. So then, what is the advantage of a rapid PER2 protein induction? In an attempt to answer this question it is worth thinking about why CORT is released, one major reason is in response to stress (Windle et al. 1998b), which could result in the 'fight or flight' response. Work with the *Per2 -/-* mouse has shown that the loss of this clock gene results in reduced wheel running activity compared to the WT mice but with no other physiological i.e. muscle mass, body mass or muscle contractile differences (Bae et al. 2006; Yang et al. 2009). This observation links nicely with a stress-induced escape response, in that if the ability to run is reduced with the loss of *Per2*, then the protein must have an effect when present. In the study by Bae et al (2006) they looked at the effect of the loss of *Per2* on proteins involved in muscle contraction, metabolic pathways, stress proteins and regulatory proteins. The reduced running ability of the *Per2 -/-* mice was hypothesised to be due to an increased dependence on glycolysis, as key enzymes in the process were increased in the *Per2 -/-* mouse. Triose-phosphase isomerase and phosphopyruvate dehydratase were both increased in the absence of PER2. However, the link between PER2 protein and these enzymes remains undetermined. Furthermore, the enzyme carbonic anhydrase III was reduced in the *Per2 -/-* genotype. This enzyme buffers the cells and blood against acidic conditions, as it converts $CO_2 + H_2O$ to $HCO_3 + H^+$ and vice-versa, and at a reduced level this will result in serious discomfort to the individual, particularly as an increase reliance on glycolysis will result in increased H^+, which will acidify the local environment. These data indicate that PER2 is involved in metabolic processes in the cell, which are enhanced in a stress response, and without PER2 the animal is severely hindered, particularly in terms of behavioural activity. There is a

limited amount of published literature on the range of genes induced by effects of clock protein transcription, but above gives a small insight into the wide range of effects clock genes can have on the organism, in response to various stimuli.

7.4 The circadian inflammatory response

Evidence presented in this thesis (Chapter 5) and in the published literature has shown that the innate immune response is under circadian control, but is it direct (i.e. by CLOCK:BMAL1) or indirect (i.e. influenced by systemic factors)? If it is under direct control it is perhaps important to ask the question, 'what is the advantage of this control?'

Isolated immune cells have been shown to oscillate in terms of their raw numbers in the circulation (Arjona and Sarkar 2005; Haus and Smolensky 1999). Also tissues from the immune system have been shown to oscillate both as tissue explants (using the PER2::luc mouse, Chapter 3) and as mRNA (*mPer2* and *mRev-erbα*) extracted from mice in constant darkness over the 48h (Keller et al. 2009). Furthermore, mediators of the inflammatory response have been shown to oscillate independently and in response to LPS challenge in both humans and mice (Chapter 5 and (Guan et al. 2005; Keller et al. 2009; Petrovsky et al. 1998). It could be, at least in-vivo that the circadian oscillations of the various cells, genes and proteins of the inflammatory system are due to synchronising factors such as Gc or melatonin. Gcs are released in a circadian manner and have been shown to negatively correlate with cytokine production (IFNγ, TNFα and IL-12) i.e. peak Gc expression correlates with minimal cytokine expression (Petrovsky et al. 1998). Other studies, using human volunteers have shown that IL-6 and IL-1β expression do not correlate with Gc release but IFN-γ, IL-8 and TNFα do, so it seems that circulating Gc expression does, affect circulating cytokine expression. An association between Gc and TNFα production has been shown in serum (Parant et al. 1991), where pre-treatment of adrenalectomised mice with Gc stopped TNFα release, in response to LPS. Yet, there is no difference in TNFα or IL-6 circadian expression between the spleens of WT and adrenalectomised mice, removed and then treated with LPS over the circadian day (Keller et al. 2009), and so are therefore devoid of Gc signals. This suggests that although *in-vivo* cytokine production can be influenced by circulating Gcs other factors have an effect or, more likely, that they are direct outputs of the circadian clock, as some cytokines (TNFα and IL-6) remain rhythmic even their absence. Furthermore, Keller et al. (2009) showed through an array study that 8% of the macrophage transcriptome is under circadian control with multiple

levels of the inflammatory response. Genes involved in wound healing, the stress response and phagocytosis all exhibit circadian expression. Also genes, whose protein products act in complexes like cFOS and cJUN (AP-1) and CD-180 and MD-1 (TLR4 inhibitory molecules) were in-phase with each other, suggesting firm circadian control. Taken together, although circulating factors such as Gcs can, and do affect cytokine production and inflammatory cells, cytokines like TNFα and IL-6, as well as other genes involved in the inflammatory response could be under direct circadian control, rather than influenced by systemic factors.

To address the question set out at the beginning, 'what is the advantage of this control?' One hypothesis may be that at different times of the day an inflammatory response may be more harmful (i.e. lethal) than at other times. Doses of TNFα that are high enough to kill mice, are less likely to kill them when administered at the mid to late activity phase (subjective night) (Hrushesky et al. 1994), a time when endogenous levels are low. In the Keller et al. (2009) study, the peak TNFα and IL-6 expression occurred during the mid to late subjective day (prior to the onset of activity), alongside macrophages and monocytes, reaching their maximum number at time that corresponds with a high degree of TNFα induced mortality in mice (Hrushesky et al. 1994). This suggests that if TNFα is induced at a time of peak expression this will readily kill the animal. Evolutionary speaking it will be an advantage to the individuals' survival to be better prepared against bacterial infection at certain times of day than others. For example, it could be a waste of energy to maintain a high level of circulating cytokines/chemokines at times of rest and in relative safety, whereas when hunting or foraging for food (onset of night for the nocturnal rodent) it is very likely that an immune response will be required. Therefore, to better defend this more likely attack, a higher level of circulating cytokines/chemokines would be beneficial to the organism, and hence a circadian oscillation is born. Linking back to the previous point (TNFα and IL-6 being highest prior to the onset of activity) this may be in preparation for moving out of the relative safety of the nest, a time when the animal could be disorientated and susceptible to attack. Later on during the late night, the animals' senses should be more heightened and is overall more alert to the environment, therefore requiring less circulating cytokines. However, very tight regulation would be required so as to not produce too much of a response, resulting in un-necessary death, and so the circadian clock has a controlling role. Furthermore, circadian control of the inflammatory response would be beneficial to allow it to keep in phase with the rest of the organ system. An example

here would be to better prepare the body for any ingested pathogens taken in when eating, and putting the immune response in phase with the metabolic response would allow this to occur, and increase the organisms' survival rate.

The immune system can directly influence the core clock as well as the clock causing the oscillation of cytokine expression, and the relationship is therefore bi-directional. LPS can suppress E-box mediated transcription (*Per2*, *Cry1* and *Rev-erbα*) via TNFα (Cavadini et al. 2007) which is most likely another 'pro-survival' mechanism as the loss of *Per2* has been shown to increase survival after LPS administration (Liu et al. 2006b). However, this is opposite to the effects previously described above where TNFα is less likely to kill when administered during the late subjective night, when PER2 protein is at it highest. Therefore, a lot more work needs to be carried out to further clarify this bi-directional relationship between the inflammatory response, the clock and ultimate survival.

7.5 Caveats and improvements

If I had my time again, and with the benefit of hindsight, I would have approached a number of things differently. Perhaps the first thing to mention is that I would not pursue the C/EBPα story, as this turned out to rather a bust line of research, although there is clear evidence in the literature that C/EBPα affects the clock, in particular *Per2* (Gery et al., 2005) and the work by MacDougald et al. 1994 quite clearly showed that C/EBPα (both transcript and protein) was inhibited after DEX treatment. So although not pursued without good evidence, it was sadly not as fruitful as it could have been. Most probably I would not have worked on the gated inflammatory response either. Although this work produced good results i.e. showing that the gated response of certain cytokines was lost in the absence of *Rev-erbα*, the work contained in Chapter 5 was very time consuming and fairly labour intensive (qPCR and repeating the *in vivo* finding in the PECs). This meant that the time I could commit to my other projects (Chapters 3 and 4) had to be shared, yet on reflection it would have been better to concentrate more of my time on the findings and progression of Chapter 4, rather than pursuing the work in Chapter 5. However, most of the work in Chapter 4 happened towards the end of my research time, so it is with hindsight that I make this statement.

In Chapter 3, with regard to PRC in Fig 3.4, it would have been wise to carry out further treatments between the change from delay to advance (CT10-12), perhaps treating the

slices every 10 to 20min to better define the cross-over point. This is not only important in its own right, but CT12 (the peak of the PER2::luc rhythm) was the treatment time I chose for the rest of the work in that Chapters 3 and 4. Therefore, without knowing more accurately when the delay to advance switch occurred, I could have treated subsequent experiments in the delay phase and then next time in the advance phase, thereby altering the findings. Even though this could have been the case I think that the results in Chapters 3 and 4 were reproducible enough to say that time of the treatments were given in the same phase of the PRC, or that perhaps it does not matter what phase the treatment is administered. In Fig 3.7 although Gibbs et al., 2009 showed co-localisation of PER2 and CLOCK with CCSP, in order to show that the clock proteins were expressed in the Clara cells, there was no co-localisation of GR. Therefore, it would have been wise for me to show GR and MR in the Clara cells, which they appear to be. In the end of Chapter 3 (Fig 3.22-3.25) I showed the development of the flow-through system. Although I do not regret pursuing this line of work, it was very time consuming and did not yield the results I had hoped for, and because of this I think my time would have been more wisely spent on other research i.e. what is contained in the early part Chapter 3 and Chapter 4. The flow-through work needs a PhD all to itself, or someone who can dedicate far more time than I.

One of the major problems (or even hardships) with study in circadian biology is the fact that to categorically show that something is circadian it requires experimental setups which include multiple time points, and possibly over more than 24h. This experimental design then requires someone to collect samples (mice, cells or tissues) over the 24 to 48h and then to analyse the results. This is often too difficult or impractical i.e. the numbers of mice would be too great, and is why two time points are usually used 12h apart (CT0 and CT12). This in itself creates a slight problem with the interpretation of borderline or apparent non-circadian results, such as those seen in Fig 4.8a. In this Figure the differential expression of *mBMAL1* in the *Cav-1* -/- cells was greatly dampened with respect to the WT control. As a result I concluded that the loss of *Cav-1* dampened the *BMAL1* mRNA rhythm. Indeed this could be the case, and that it is mediated through REV-ERB stabilisation (section 4.4.2), yet it could also be true that the two time points I chose are 6h out of phase with the peak and trough, and therefore I measured the rising and falling portions of the *BMAL1* oscillation and measured almost equal quantities. To resolve this issue 4 time points, over 24h, taken every 6h would need to be carried out, then it would be obvious to see if the rhythm is dampened or just out of phase.

This same problem occurred with the *in vivo* LPS challenge in Chapter 5. In Fig 5.2 and 5.4 and table 5.1 and 5.2, mice were only injected at two time points (CT0 and CT12) although these mice were under a previous LD cycle (12h:12h), it again could have been the case where we missed the peak and trough of the rhythm and hit the rising and falling arms. If this happened I could have reported false negative results i.e. cytokines that did not appear gated but would have been found to be gated if further time points were carried out. To that end if the work was done again, and the mice were in plentiful supply, then CT6 and CT18 should be included too. This was not and issue with the PEC studies as a reference oscillation was used to define the treatments at the peak (CT12 and trough CT0 of the PER2::luc rhythm). Furthermore, the mice used for the WT versus *Rev-erb* -/- studies were mixed sex, although for each experiment and equal distribution was used e.g. 3 males and 4 females for each genotype. This mix of sexes could have resulted in the difference between gating seen in the WT animals i.e. TNFa was not gated in the WT males used in Fig 5.2 but was gated in the WT males/females mix in Fig 5.4. Therefore, if carried out again, and with sufficient mice, the work should be carried out with males only and then females. In this case sex differences can be analysed thoroughly. Also a LPS PRC should have been carried out to define the relationship between time and LPS, which expands on Fig 5.6. If this was carried out, parallels and differences could be characterised between LPS and CORT in terms of amplitude and cross-over from delay to advance. However, this would require a lot of time to complete and therefore was not done in this thesis.

7.6 Future directions

The main work in this thesis has been concerned with Gc and the clock, and in the work in Chapter 4 with the immediate re-setting effects. This work is very promising and should be further explored, and to my knowledge no other groups are working on this theme so is, as it stands, novel work:

- Even though *Cav-1* -/- cells had an effect on the induction of *mPer2* and disruption of the lipid raft with methyl cyclodextrin inhibited both the acute amplitude response and phase change, no defined pathway has been described. Therefore, the first experiment would be to grow up some WT-non PER2::luc primary lung fibroblasts and treat with either CORT or the GR agonist 67a over a 4h time course (in line with the rise in luciferase signal Fig 3.18) and Western blot

for the phosphorylated forms of GSK3β, MAPKp42/44, PKB and GR, as in Matthews et al., 2008, and compare the differences. The hypothesis would be that specific phosphorylation events will be apparent in the CORT treated samples but no in the 67a treated samples. This would then provide a mechanism for the differences seen in Fig 3.18.

- The next step is to treat PER2::luc lung slices or PER2::luc primary fibroblasts with specific inhibitors (based on the results of the Western blot) in the presence or absence of either CORT or 67a. The hypothesis would be that specific inhibitors would disrupt the rapid amplitude rise in the PER2::luc signal after CORT, but would not affect the 67a response and give additional weight to the Western blot data.

- It may be of interest to characterise if CAV-1 and PER2 co-localise in the cytoplasm, like that seen with CAV-1 and GR (Matthews et al., 2008). This could easily be done with co-immuno precipitation, and if CAV-1 and PER2 did co-localise this could add to a new hypothesis of how PER2 is regulated i.e. in the presence of CORT PER2 is sequestered to the Caveolae and subsequently stabilised, yet in the presence of the agonist 67a PER2 is not sequestered to the membrane Caveolae.

- Co-immunofluorescence imaging of GR, CAV-1 and PER2 would be useful in specific cell types of the lung e.g. Clara cells with CCSP as well as CAV-1 and PER2 together in the lung. This would give a good indication as to how this interaction formed *in vivo* and what specific cell types it occurred in.

- Perhaps not of immediate interest but a PRC to the GR agonist 67a would shed light on differences in their relative responses. Is the amplitude of advance and delay the same in both cases and does the delay to advance cross-over occur at the same time as CORT i.e. CT10-12.

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Appendix A

Recording Medium

The base medium:

- 1 pot of DMEM powder with 1000 mg/L glucose and L-glutamine, without sodium bicarbonate and phenol red (Sigma, UK).
- 4g glucose, cell culture grade (Sigma, UK).
- 4.7ml of 7.5% sodium bicarbonate solution (Sigma, UK) to give a 0.035% final concentration.
- 10ml of 1M HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Sigma, UK), to give a 10mM final concentration.
- 2.5ml of 10mg/ml streptomycin and 10,000 Units of penicillin to give a final concentration of 25µg/ml streptomycin and 25 Units of penicillin.

The above were dissolved and made up to a final volume of 1 litre, in deionised H_2O, and filter sterilised through a 0.22µm membrane (Fisher, UK). The medium was stored at 4^{o}C and only opened in a sterile environment. Prior to experimental use working aliquots of the medium were made.

The working medium:

- 47.5ml of the base medium was aliquoted in to a sterile tube, wrapped in tin foil to avoid light exposure.
- To that 2.5ml of FBS (Sigma, UK) was added to give a final concentration of 5%. (B27 supplement can be used as an alternative (Gibco, Invitrogen, UK)).
- 50µl of 0.1M Beetle Luciferin K salt (Promega, UK) was added to give a final concentration of 0.1mM.
- The luciferin is light sensitive so prolonged exposure was avoided.
- The working recording medium was be stored at 4^{o}C for no longer than 4 weeks.

Appendix B

Cell Lysis Buffer

In to a final volume of 500µl of deionised H_2O the following was added:

Volume (ml unless stated) or mass (g)	Reagent	Final concentration	Function
5	Triton X -100	1%	Non-ionic detergent to stabilise membrane proteins
15	5M NaCl (sodium chloride)	150mM	To lyse the membrane
5	1M Tris-HCl pH 7.4	1M	Buffer to maintain pH
1	0.5M EDTA pH 8	1mM	Chelates divalent ions (Ca^{2+} and Mg^{2+})
0.19 g	EGTA (ethylene glycol tetraacetic acid	1mM	Chelates specifically Ca^{2+}
2.5	NP-40	0.5%	Isolates membrane complexes

When all reagents were in solution the buffer was stored at RT. Prior to cell lysis a general protease inhibitor cocktail (Roche) was added and 0.1M phenylmethanesulfonylfluoride (PMSF) (Sigma,UK) was added at a 1 in 500 dilution, as a serine protease inhibitor.

Immuno blot buffers. All reagents are from Sigma, UK unless otherwise stated.

Resolving gel - 0.8% bis-acrylamide

11.5ml of deionised H_2O; 6ml of 1.5M Tris pH 8.8; 6.4ml of 30% acrylamide ProtoGel (National diagnostics); 100µl of 10% amino persulphate (APS) and 10µl of TEMED were mixed.

Stacking gel

4ml of deionised H_2O; 1.5ml of 0.5M Tris pH 6.8; 1ml of 30% acrylamide ProtoGel, 100µl APS and 10µl TEMED were mixed.

Tris buffered saline (TBS) and TBS-Tween 20 (TBST)

10 X TBS was made by dissolving the 0.2M Tris and 1.37M) sodium chloride (NaCl) into 1 litre of H_2O. The buffer was reduced to pH 7.6 with hydrogen chloride (HCl) and stored at RT, (all regents Sigma, UK).

To use, the 10 x TBS was diluted to 1 x in deionised H_2O and 0.1% Tween20 (Sigma, UK) was added and mixed and stored at RT.

Running buffer

10 X running buffer was made by dissolving 1.92M glycine and 0.25M Tris into 5 litres of deionised H_2O and to this SDS was added to a final concentration of 0.1% and stored at RT.

Transfer buffer

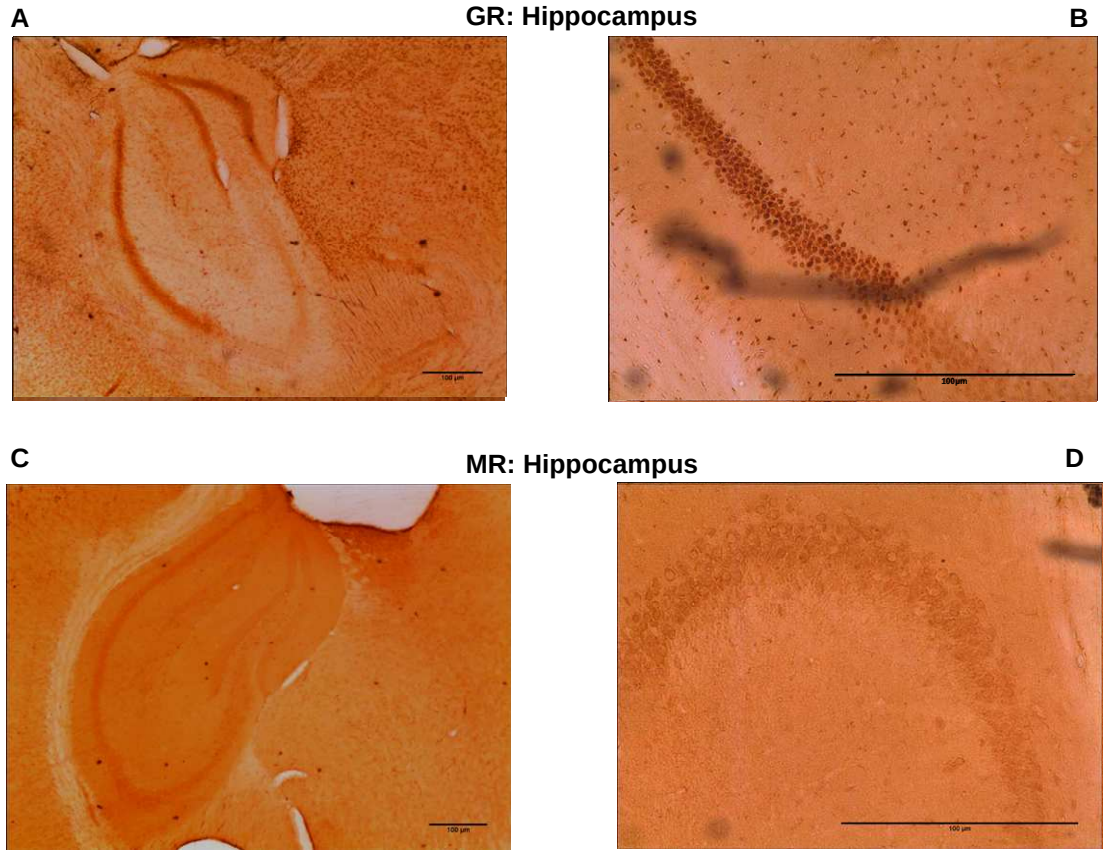
200ml 100% methanol was added to 1 X running buffer and made to 1 litre with deionised H_2O and stored at 4^oC.

Loading Dye

6 x loading dye was prepared using 6% (w/v) SDS, 45mM Tris-HCl pH 6.8, 15% (v/v) β-mercaptoethanol and 60% (v/v) glycerol. This was mixed and stored at -20^oC until used.

Appendix C

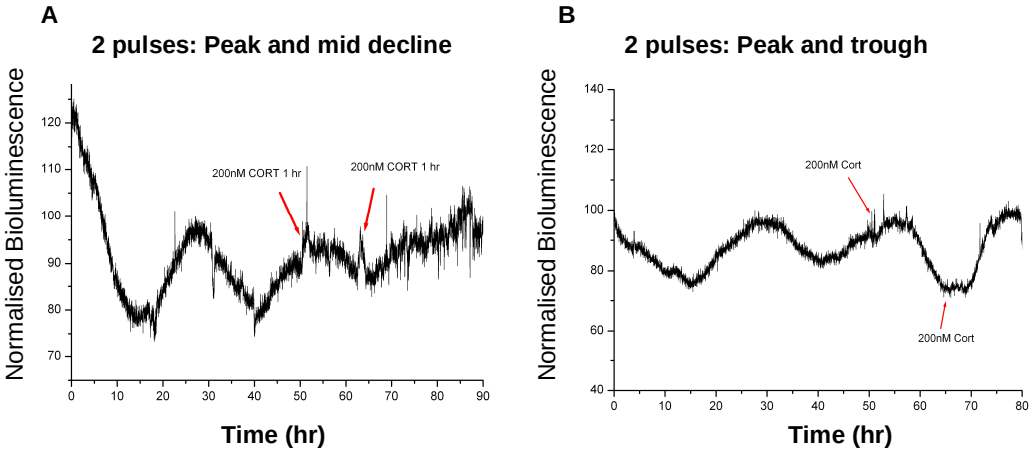
Hippocampal expression of GR/MR



Appendix C: The mouse hippocampus was stained with an anti-GR (A and B) and an anti-MR (C and D) antibody at 1 in 2500 and 1 in 250 dilution for GR and MR respectively. The brains were fixed in Bouin's solution and imaged using a x 10 magnification (A and C) and x 40 magnification (B and D) of the same sample. Primary negative samples are not shown due to the image being too light to define relevant nuclei. Scale bar is equivalent to 100µm.

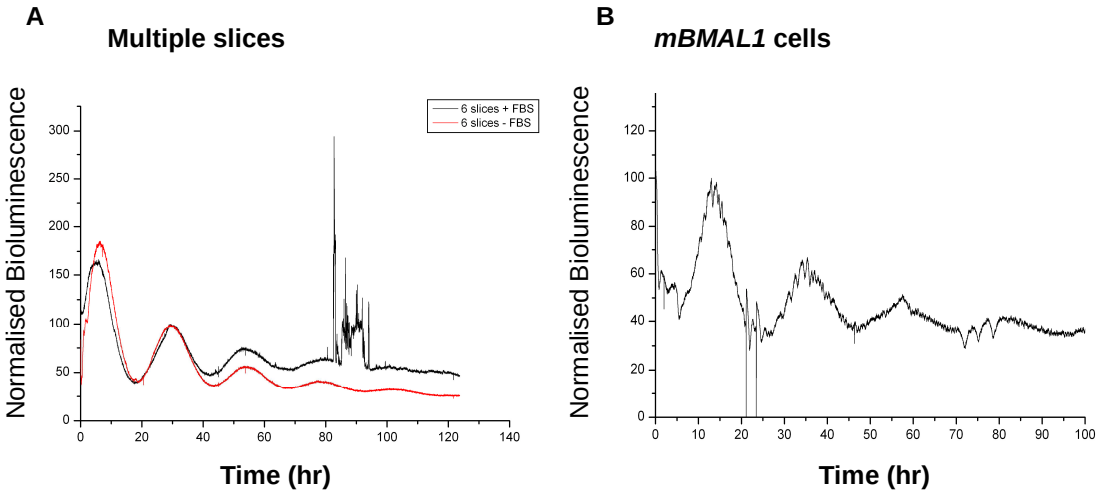
Appendix D

Flow-through culture: Multiple pulses



Appendix D1: Multiple pulses on the same sample: Two 60 min pulses with 200nM CORT given at the peak (CT12) and mid decline (CT18, A) or trough (CT0, B) shown by the arrows, using the PER2::luc lung slice, under a 6.8ml/h constant flow rate. Experiments are single slices and n=1.

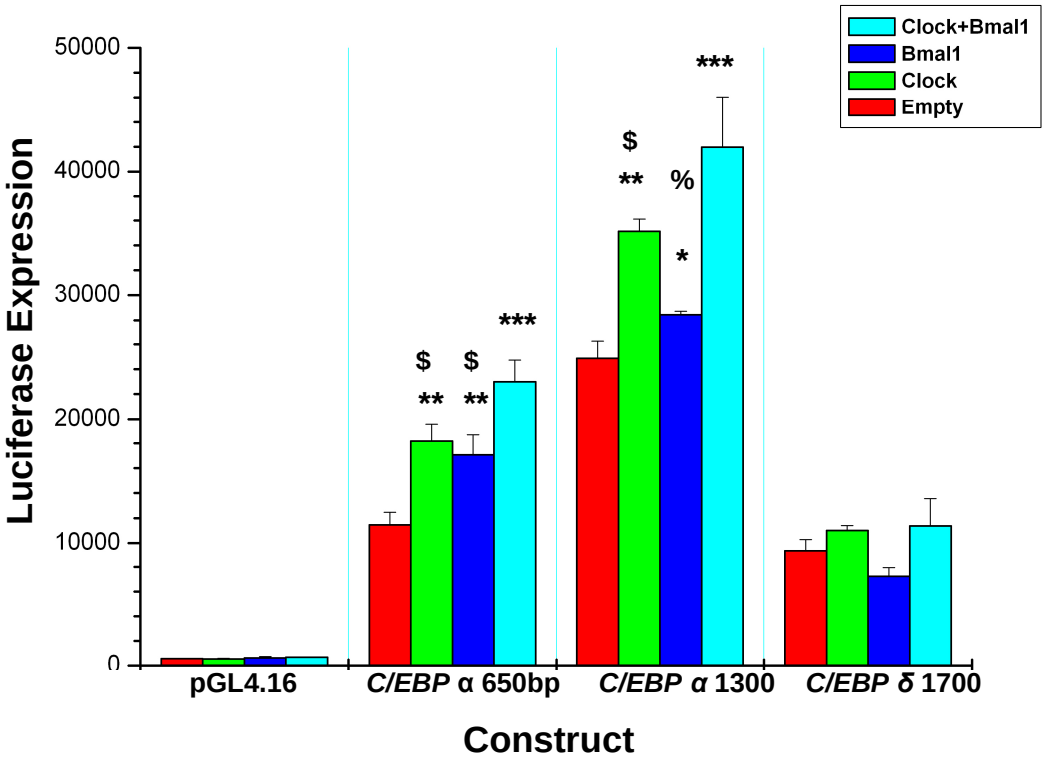
Flow-through culture: Multiple slices and cells



Appendix D2: Multiple lung slices and *BMAL1* reporter cells: Multiple PER2::luc lung slices were assayed at the same time (A) or m*BMAL1::luc* reporter cell line (B), under flow-through conditions, using a 6.8ml/h flow rate. The multiple slices were assayed with (black) and without (red) serum.

Appendix E

CLOCK/BMAL E-box induction of C/EBP alpha and delta constructs



Appendix E: Clock and Bmal1 induced expression of C/EBP alpha and delta constructs: C/EBP alpha 650bp, 1300bp and C/EBP delta 1700bp luciferase constructs were induced with empty vector (red), *mclock* (green), *mBmal1* (dark blue) or *mClock* and *mBmal1* (light blue) for 24h before analysis. Error bars are SEM of n=3. Statistical analysis was by One-way ANOVA (P<0.0001) with a Bonferroni post-hoc analysis, * P< 0.05, **P< 0.01, ***P<0.001 versus empty vector; $ P<0.05 versus Clock + Bmal1; % P<0.01 versus Clock + Bmal1. Work carried out by Dr. Qing-Jun Meng.

Appendix G

CCL5/RANTES - 3000bp 5’ to the ATG

Read as 5’ to 3’

cacacaaatgaaaataagtaaataaatttgtaaaagatactatgctcacagagttaagagacaatgctgtaatgtcaacaccacagaga
atgaggaagtaaatttaaggtcaactgggtttcagtatgagcaacagaaaagacaaaagacaaggccagtaaaaggtggttcacaatta
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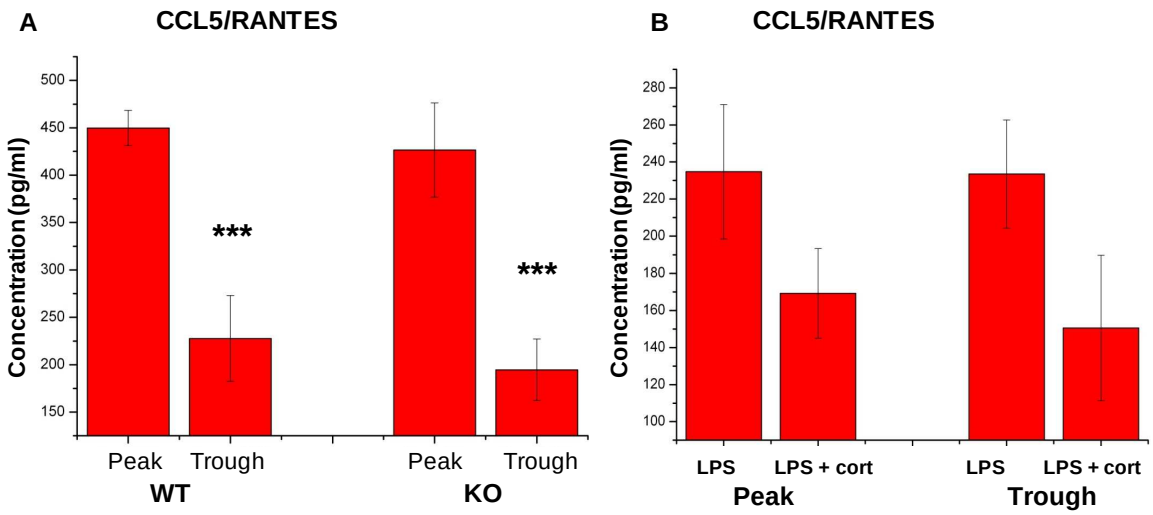
gagggagtcatcctggactggagggcagttagaggcagagtcatacttccaagggtgatttcagttttcttttccattttgtgttttcattttatgac
agcaacaagtgtttggtgtcttttgtggaaactccccaagtcctggggctgggggggggcacttcctctgctaccctggctccctataaaagg
tctgcctgagctgcagagcaccc**……start of transcription**

- 10 non-canonical E-box motifs (CACATG) that fit the generic description of the E-box CANNTG.

Appendix H

CCL5/RANTES expression in murine PECs

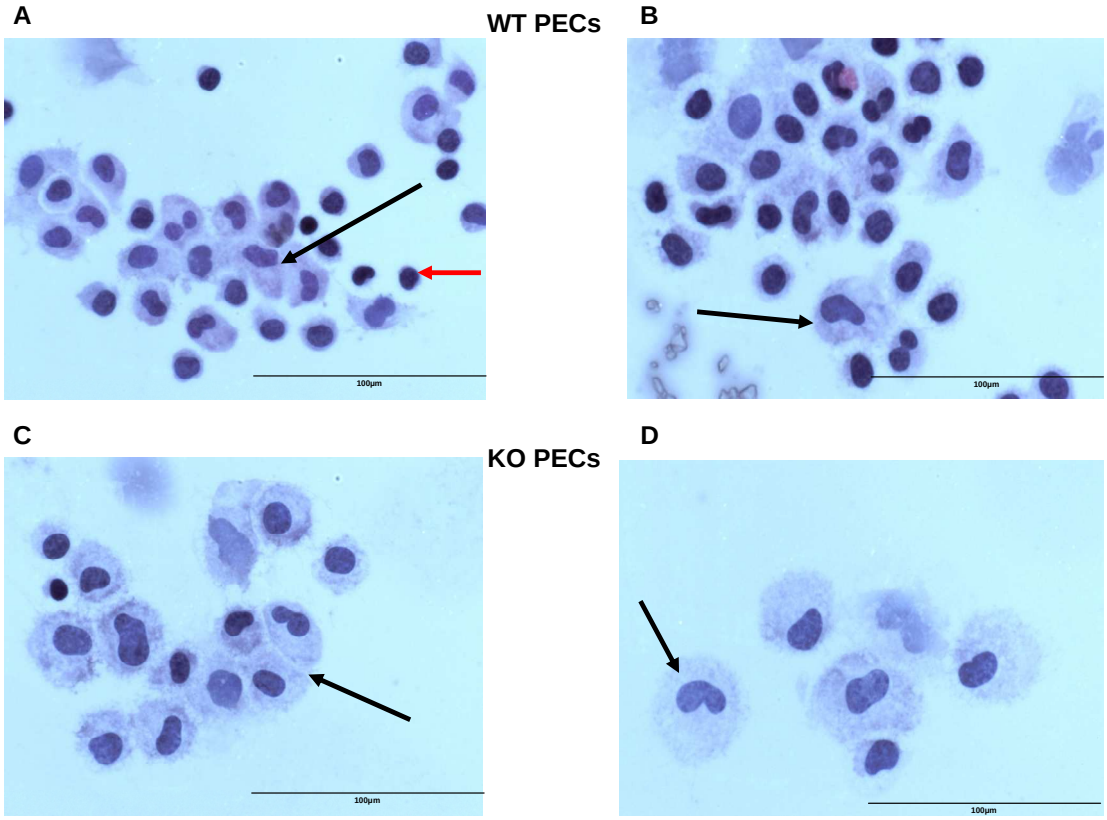
Effect of a timed LPS challenge on inflammatory mediator release in isolated mouse PECs



Appendix F: Timed LPS challenge on inflammatory mediator release in isolated mouse PECs: PECs from WT or *Rev-erb alpha* knock out mice were treated with 100ng/ml LPS for 4h prior to the quantification of CCL5/RANTES release (A) or WT PECs were treated with 100ng/ml LPS with or without 100nM cort prior to CCL5/RANTES quantification (B). Error bars are standard deviation from 3 internal replicates of pooled PECS from 3 mixed sex mice (A) or 5 male mice (B), (overall n=1). Statistical analysis was by Two-way ANOVA (in A: $P<0.0001$ for time of treatment and $P=0.24$ for genotype interaction; in B: $P=0.026$ for time and treatment interaction) with a Bonferroni post-hoc analysis, *** $P<0.001$.

Appendix I

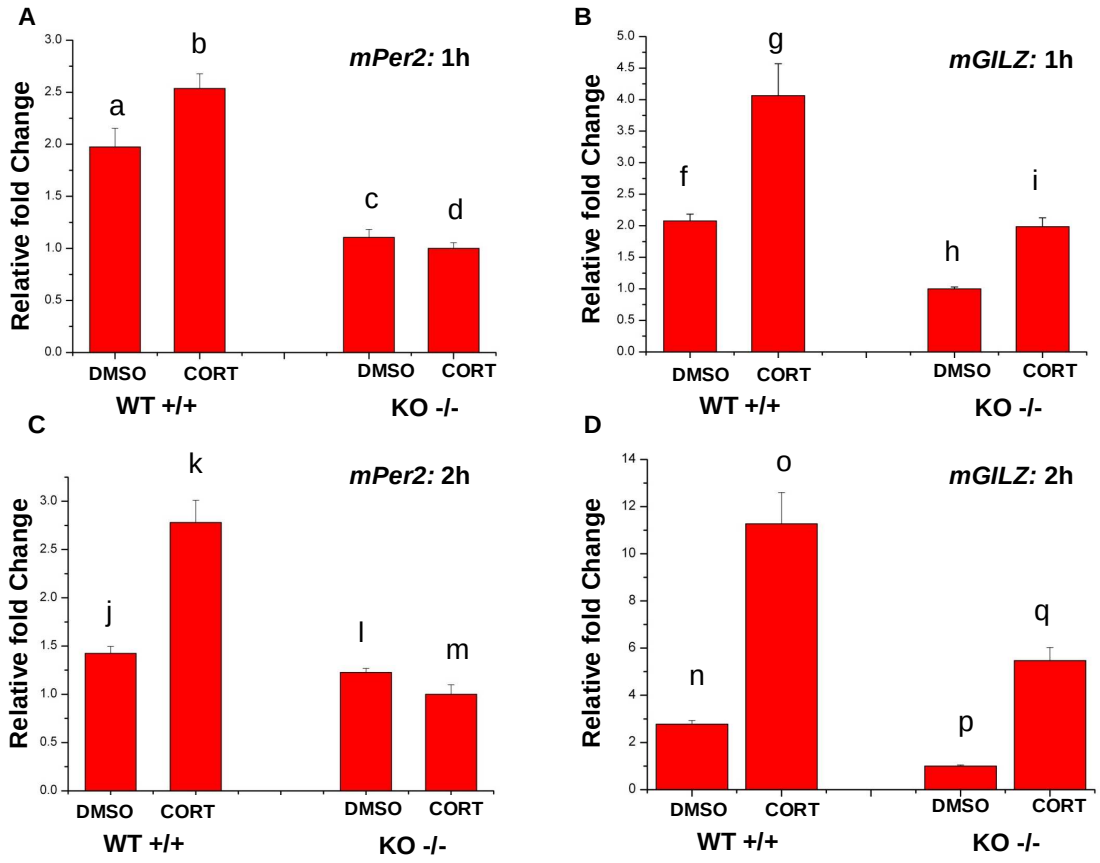
Composition of the peritoneal exudate used for the cellular LPS studies



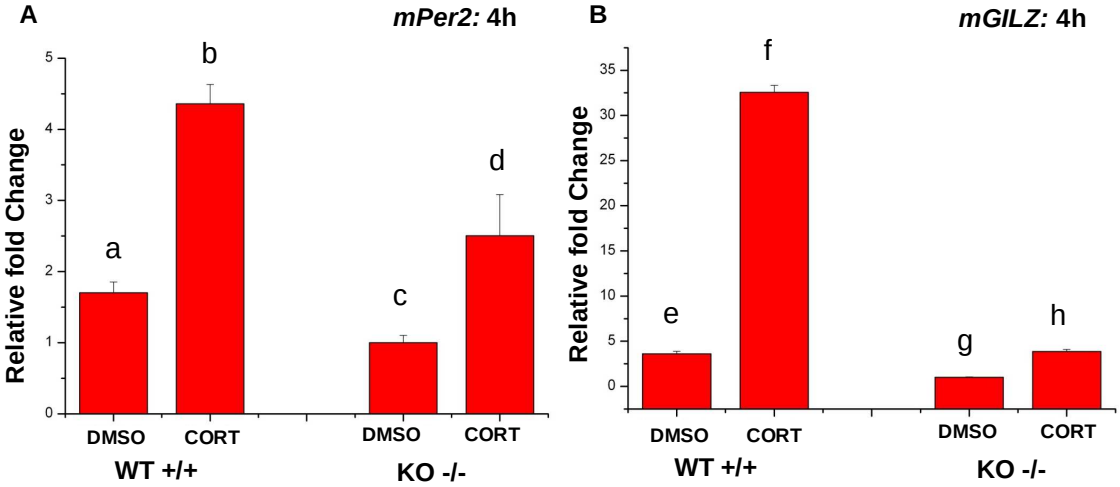
Appendix I: WT and *Rev-erb alpha* knock out PECs: Peritoneal cells were isolated (section 2.2.6) and cultured from WT (A and B) and *Rev-erb alpha* knock out (C and D) mice and prepared for staining (section 2.2.6.3), representative cells are shown above. The arrows refer to the resident macrophages, evident due to their large cytoplasm surrounding the nucleus. The red arrow points to the other obvious cell type the T-lymphocyte or natural killer cell, noted for its large nucleus. Scale bars is equal to 100µm.

Appendix J

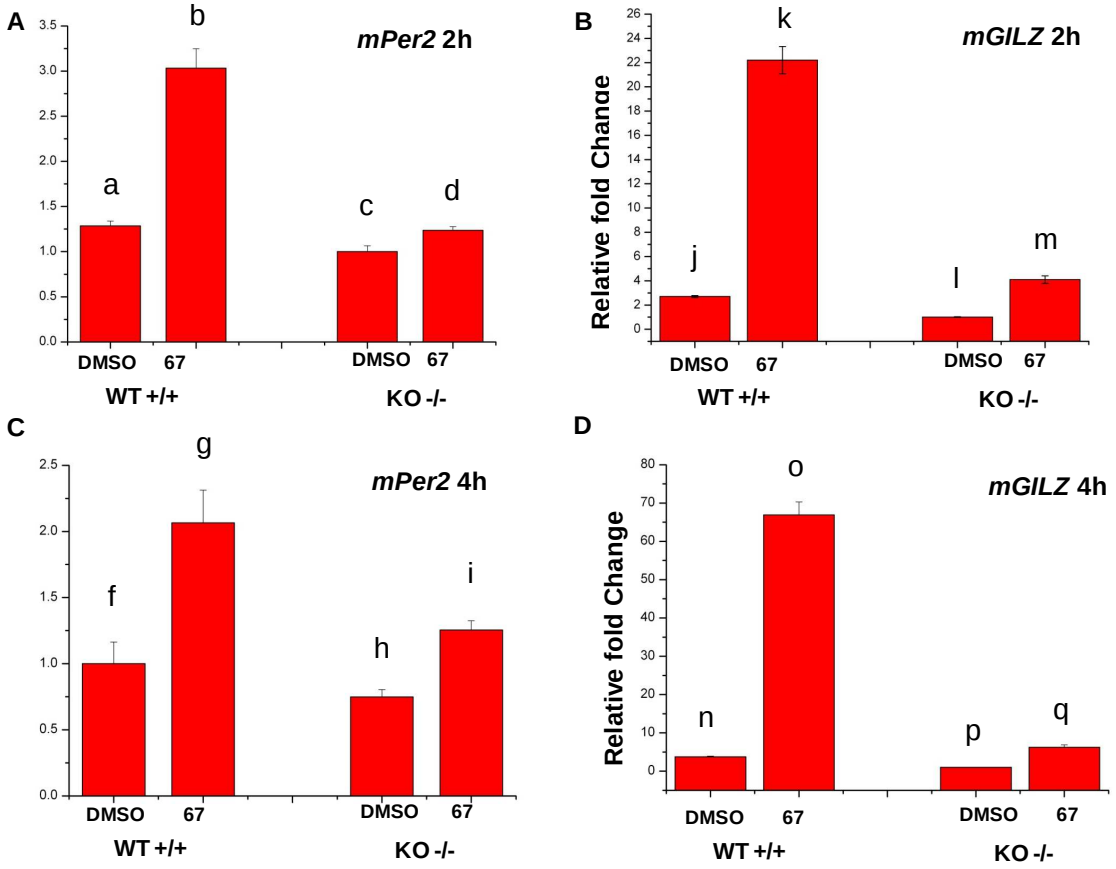
***mPer2* and *mGILZ* expression after CORT or GR agonist administration in WT and *Cav-1* -/- cells**



Appendix J1: *Per2* and *GILZ* transcript expression in WT and *Cav-1* knock out cells after 1h and 2h CORT exposure: WT or *Cav-1* -/- cells were exposed to CORT for 1h (A and B) or 2h (C and D) prior to RNA extraction. *Per2* (A and C) or *GILZ* (B and D) transcripts were assayed from the same RNA sample. Expression is given as fold change relative to the lowest value. Error bars are SEM of n=3. Statistical analysis was by Two-way ANOVA (in A: P=0.1 for treatment and P=0.027 for genotype interaction; in B: P=0.0006 for treatment and P=0.0004 for genotype interaction; in C: P<0.0027 for both treatment and genotype interaction; in D: P<0.0008 for both treatment and genotype interaction) with a Bonferroni post-hoc analysis. With regard to A: (a) versus (b) P<0.05; (a) versus (c) and (b) versus (d) P<0.01; (c) versus (d) n.s. With regard to B: (f) versus (g) P< 0.01; (f) versus (h) P< 0.05; (g) versus (I) P< 0.001; (h) versus (I) n.s. With regard to C: (j) versus (k) and (k) versus (m) P<0.001; (k) versus (m) and (j) versus (l) n.s. With regard to D: (n) versus (o) P<0.001; (p) versus (q) P<0.01; (o) versus (q) P< 0.001; (n) versus (p) n.s.



Appendix J2: *Per2* and *GILZ* transcript expression in WT and *Cav-1* -/- cells after 4h cort exposure: WT or *Cav-1* -/- cells were exposed to cort for 4h prior to RNA extraction. *Per2* (A) or *GILZ* (B) transcripts were assayed from the same RNA sample. Expression is given as fold change relative to the lowest value. Error bars are SEM of n=3. Statistical analysis was by Two-way ANOVA (in A and B $P<0.005$ for both treatment and genotype interaction) with a Bonferroni post-hoc analysis With regard to A: (a) versus (b) and (c) versus (d) $P< 0.05$; (b) versus (d) $P< 0.01$, (a) versus (c) n.s. With regard to B: (e) versus (f) and (f) versus (h) $P<0.001$, (g) versus (h) and (e) versus (g) $P< 0.01$;



Appendix J3: *Per2* and *GILZ* transcript expression in WT and *Cav-1* -/- cells after GR agonist (67a) exposure: WT or *Cav-1* -/- cells were exposed to 67a for 2h or 4h prior to RNA extraction. *Per2* 2h (A), *Per2* 4h (B), *GILZ* 2h (C) or *GILZ* 4h (D) transcripts were assayed from the same RNA sample (with respect to time). Expression is given as fold change relative to the lowest value. Error bars are SEM of n=3. Statistical analysis was by Two-way ANOVA (in A to D $P<0.001$ for treatment and genotype interaction) with a Bonferroni post-hoc analysis. With regard to A: (a) versus (b) and (b) versus (d) $P<0.001$, (a) versus (c) and (c) versus (d) n.s. With regard to B: (j) versus (k) and (k) versus (m) $P< 0.001$, (l) versus (m) $P< 0.05$, (j) versus (l) n.s. With regard to C: (f) versus (g) $P< 0.01$, (g) versus (i) $P< 0.05$, (h) versus (i) and (f) versus (h) n.s. With regard to D: (n) versus (o) and (o) versus (q) $P< 0.001$, (p) versus (q) and (n) versus (p) n.s.