THE IMPACT OF CIRCADIAN ENTRAINMENT AND MELATONIN ON KIDNEY DAMAGE ASSOCIATED WITH ISCHAEMIA-REPERFUSION INJURY

DANNY DAREN DA'VAL

A thesis submitted in partial fulfilment of the requirements of the University of Kent and the University of Greenwich for the Degree of Master of Philosophy

March 2025

Declaration of Originality

I certify that this work has not been accepted in substance for any degree and is not concurrently being submitted for any degree other than that of Master of Philosophy being studied at the Universities of Greenwich and Kent. I also declare that this work is the result of my own investigations except where otherwise identified by references and that I have not plagiarised the work of others.

Danny Da'Val,

March 2025

Word Count: 52789

Acknowledgements

Firstly, I would like to express my immense gratitude to Dr Gurprit Lall and Dr Claire Peppiatt-Wildman for their invaluable patience, guidance and efforts to aid me during my study. Thank you for giving me the opportunity to work on this project, providing valuable feedback, and challenging me to grow as a scientist.

I would like to thank the Medway School of Pharmacy, and its staff, for providing me with this opportunity, and assistance I required to undertake this study. I'd like to mention in particular; Katrin Jones and Fani Papagiannouli, who provided kindness and encouragement, that I don't want to go unnoticed. I would also like to thank my fellow peers and colleagues, Rebecca and Mafalda, from whom I learned so much. Your teaching, camaraderie and company through our collaborative work within the lab were both insightful and a joy.

My dearest thanks go to my family, to my parents, and to my sisters, Lucy and Hannah, who listened, encouraged and always backed me throughout this project. Their belief in me has kept my spirits and motivation high during this process.

A special shoutout needs to go to my friend Tyler Wooldridge; for their editing help, late-night feedback sessions, and moral support. Finally, the biggest thank you to Maddy, for your belief, encouragement and faithful support. Your endless provision of care, humour and counsel, during even the most difficult of times

Abstract

Circadian rhythms regulate cellular, behavioural and physiological processes through central and peripheral clocks over a 24-hour period, with the suprachiasmatic nucleus (SCN) acting as the main enforcer of synchronising peripheral oscillators. Increasing evidence links circadian signalling to immune responses and susceptibility to pathological conditions such as ischaemia–reperfusion (IR) injury, an inevitable condition in organ transplantation and major cause of graft dysfunction. The kidneys are particularly sensitive to ischaemic injury, as renal microvasculature is vulnerable to hypoxia. Renal microvasculature, particularly pericytes that regulate capillary blood flow stability and immune cell infiltration, may represent a key site where circadian messengers influence IR onset and severity.

This study aimed to investigate (i) whether circadian messengers; GABA, arginine vasopressin, corticosterone and melatonin directly modulate renal pericyte activity, and (ii) whether GABA can reset circadian responses in the kidney live slice model.

Live kidney slices from C57BL/6J mice were exposed to individual circadian mediators (GABA 50 µM, arginine vasopressin 300 nM, corticosterone 50 µM and melatonin 50 µM) over a period of 500s, then observed to a total 1200s and 1800s and real-time vascular responses of vasa recta pericytes were observed and assessed under microscope. To assess GABA resetting potential and if it impacts the studies on pericyte regulation of medullary blood flow in the kidney slice model, slices were pre-treated with GABA (50 µM) for 30 minutes prior to corticosterone or melatonin exposure.

All tested circadian messengers induced pericyte-mediated constriction, supporting their role in circadian regulation of renal microvasculature and a possible role in IR injury. Corticosterone evoked robust constriction, consistent with glucocorticoid-mediated stress responses, and could play a role in irreversible constriction observed in IR injury and acute kidney injury. Melatonin also constricted pericytes, potentially contributing to nocturnal suppression of micturition activity in mice. GABA induced significant constriction at low concentrations, though effects were inconsistent at 50 µM. Importantly, GABA pre-treatment did not alter pericyte response to corticosterone or melatonin, suggesting there is a capacity to reset peripheral renal clocks in this model.

In conclusion, renal pericytes respond directly to circadian messengers, highlighting a mechanism by which circadian signals may influence renal vascular tone during IR injury. Corticosterone and melatonin emerge as key regulators, while the role of GABA in clock resetting requires further investigation. These findings support the potential for circadian-based therapeutic strategies, particularly targeting melatonin, to mitigate and possibly ameliorate renal IR injury.

Table of Contents

List of abbreviations

5-H-Trp	5-hydroxy-tryptophan
5-HT1A	Serotonin 1A receptors
8-OHdG	8-Hydroxy-2'-deoxyguanosine
11β-HSD	11β- Hydroxysteroid Dehydrogenases
αSMA	α-Smooth muscle actin
AAAD	Aromatic L-amino acid decarboxylase
AANAT	Arylalkylamine-N-acetyltransferase
AC	Adenylyl cyclase
ACE	Angiotensin-converting enzyme
ACTH	Adrenocorticotropic hormone
ADCC	Aromatic amino acid decarboxylase cytotoxicity
ADP	Adenosine diphophate
AFMK	N1-acetyl-N2-formyl-5-metoxykynuramine
AKI	Acute kidney injury
AKT	Protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AP-1	Activator protein-1
APCs	Antigen presenting cells
ARC	Arcuate nucleus
ARE	Antioxidant responsive element
ASMT	Acetylserotonin O-methyltransferase
ATP	Adenosine triphophate
AUC	Area under the curve

AVP	Arginine Vasopressin
BAX	Bcl-2-associated X protein
BBB	Blood brain barrier
BCL2	B-cell lymphoma 2
BDZ	Benzodiazepines
BK_{Ca}	Large conductance, calcium-activated potassium channel
BMAL1	Brain and muscle arnt-like protein-1
BP	Blood pressure
Ca^{2+}	Calcium ions
CaCl2	Calcium chloride
Cl_{Ca}	Calcium-activated chloride channel
CaM;	Calmodulin
cAMP	Cyclic adenosine monophosphate
CalRet;	Calreticulin
CCD	Charged coupled device
CCG	Clock-controlled genes
CCL	Chemokine ligands
CFU-GM	Progenitor cells for granulocytes and macrophages
c-fos	Forskolin
cGMP	Cyclic guanosine monophosphate
CKD	Chronic kidney disease
CLOCK	Circadian locomotor output cycles kaput
CKIε	Casein kinase I-epsilon
Cl^-	Chloride ions
CNS	Central nervous system
CO_2	Carbon dioxide

CP	Ceruloplasmin
CREB	cAMP-response element binding protein
CRF	Corticotrophin-releasing factor
CRP	C-reactive protein
CRY	Cryptochrome like protein
CSF-1	Colony stimulating factor 1
CT	Circadian time
CCL/CXCL	Chemokine ligand
DAMPs	Damage-associated molecular patterns
DBP	D-box binding PAR bZIP transcription factor
DIC	Differential interference contrast
DVR	Descending vasa recta
EC	Endothelial cell
ECM	Extracellular matrix
ENaC	Epithelial sodium channel
eNOS	Endothelial nitric oxide synthase
ePC	Ensheathing pericytes
EPO	Erythropoietin
EPSP	Excitatory postsynaptic potential
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ET	Endothelin
Fos	Forskolin proto-oncogene
Gi/o	G protein inhibitory (i) and other (o) alpha subunit
$G_{q/11}$	G alpha q protein 11
G_s	G protein stimulatory alpha subunit

GABA	Gamma aminobutyric acid
GAD	Glutamic acid decarboxylase
GAT	GABA Transporter
GFR	Glomerular filtration rate
GIRK	G protein-gated inwardly rectifying potassium
GIT	Gastrointestinal tract
GJ	Gap junction
GLU	Glutamic acid
GLUT	Glucose transporters
GPCR	G-protein coupled receptor
GR	Glucocorticoid receptors
GSH	Glutathione
HCl	Hydrogen chloride
HIOMT	Hydroxyindole-Omethyltransferase
HMGB1	High mobility group box 1
HO-1	Hemeoxygenase-1
HPA	Hypothalamic-pituitary-adrenal
HSP	Heat shock proteins
ICAM	Intercellular adhesion molecule
IEG	Immediate early genes
IFNγ	Interferon gamma
IGF-1	Insulin-like growth factor 1
IKK	IκB kinase
IL-	Interleukin
iNOS	Inducible nitric oxide synthase
ipRGCs	Intrinsically photosensitive retinal ganglion cells

IPSP	Inhibitory postsynaptic potential
IRI	Ischaemia reperfusion Injury
JAK	Janus Kinase
JNK	C-Jun N-terminal kinase
K+	Potassium ions
K-ATP	Voltage sensitive potassium channels
K_{IR}	Inwardly rectifying potassium channel
K_V	Voltage-gated potassium channel
KCL	Potassium chloride
KCC2	Potassium-chloride cotransporter 2
Keap1	Kelch like ECK associated protein 1
Kim-1	Kidney injury molecule 1
LAN	Late-at-night
LD	Light-dark
LR	Late Response genes
M-CSF	Antibody-dependent cellular monocyte colony stimulating factor
MAP	Mean arterial pressure
Mas	Mitochondrial assembly receptor
MBF	Medullary blood flow
MCP	Monocyte chemoattractant protein
MDA	Malondialdehyde
Mel1c	Non-mammalian G protein-coupled melatonin receptor
MgSO4	Magnesium sulfate
MHC	Major histocompatibility complex
MIOS	Melatonin-induced opioid system
MLC,	Myosin light chain

MLCK	Myosin light chain Kinase
MR	Mineralocorticoid receptors
mRNA	Messenger RNA
MT	G protein-coupled melatonin receptors
mtPTP;	Mitochondrial permeability transition pore
mTOR	Mammalian target or rapamycin
N	Neutrophils
Na	Sodium
Na acetate	Sodium acetate
NaCl	Sodium chloride
NaHCO3	Sodium bicarbonate
NaH2PO4	Monosodium phosphate
Na2HPO4	Disodium hydrogen phosphate
NaOH	Sodium hydroxide
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate oxidase
Na pyruvate	Sodium pyruvate
NAS	N-acetyl-serotonin
ND	Non-diseased
NETs	Neutrophil extracellular traps
NFIL3	Nuclear factor, interleukin 3
NF-kB	Nuclear factor-kappa B
NG2	Neural/glial antigen 2
NHE3	Sodium hydrogen antiporter
NK cells	Natural killer cells
NKCC1	Sodium-potassium-chloride cotransporter
NO	Nitric Oxide

NOS	Nitric oxide synthase
Notch1	Neurogenic locus notch homolog protein 1
NPY	Neuropeptide Y
NQO1	NAD(P)H quinone oxidoreductase 1
NQO2	NAD(P)H dehydrogenase, quinone 2
Nr3c2	Nuclear receptor subfamily 3 group c member 2
NRF2	Nuclear factor erythroid 2-related factor 2
NSC,	Non-specific cation channels
O2	Oxygen
O2-	Super oxide anion
ONOO	Peroxynitrite
OT	Oxytocin
PAR	Proteinase-activated receptor 1
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase chain reaction
PD-L1/PD-L2	Programmed death-ligand 1/2
PEPT	Peptide transporter
PER	Period
PFC	Prefrontal cortex
PDGF	Platelet-derived growth factor
PDGF-BB	Platelet Derived Growth Factor BB
PDGFRβ	PDGF receptor β
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKG	Protein kinase G

PR	Pineal recess
PRRs	Pattern recognition receptors
PSS	Physiological saline solution
PVN	Paraventricular nucleus
R	Ligand-binding receptor
RA	Rheumatoid Arthritis
RAS	Rat Sarcoma signalling pathway
RAAS	Renin-angiotensin-aldosterone system
RBF	Renal blood flow
Ren1	Renin 1
REV-ERBα	Nuclear receptor subfamily 1 group D member 1
REV-ERBβ	Nuclear receptor subfamily 1 group D member 2
RhoK	Rho kinase
RISK	Reperfusion injury salvage kinase
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROI	Reactive oxygen intermediate
RORα	Retinoid-related orphan receptor alpha
RORβ	Retinoid-related orphan receptor beta
RORE	Retinoic Orphan Receptor Elements
ROS	Reactive oxygen species
RSNA	Renal sympathetic nerve activity
SA	Serum albumin
SAFE	Survivor activating factor enhancement
SCF	Stem cell factor
SCN	Suprachiasmatic nucleus

Ser	Serotonin
sGC,	Soluble guanylyl cyclase
SIRT1	Silent information regulator 2 homolog 1
SK_{Ca}	Small conductance, calcium-activated potassium channel
SOD	Super oxide dismutase
sPC	Stellate pericytes
STAT	Signal Transducers and Activators of Transcription
T	T cells
T1D	Type 1 diabetes
TF	Tissue factor
TGF	Tubular glomerular feedback (
TGF-β	Transforming growth factor-beta
Tie2	TEK receptor tyrosine kinase
TLR	Toll like receptors
TNFα	Tumour necrosis factor alpha
TNF-β	Tumour necrosis factor beta
TPH	Tryptophan hydroxylase
tsPC	Thin-stranded pericytes
TTL	Transcriptional–translational feedback loop
TPH	Tryptophan Hydroxylase
UT	Urea transporter
V1-3R	Vasopressin 1-3 receptors
VDR3:	Vitamin D receptor 3
VEGF	Vascular endothelial growth factor
VGAT	GABA vesicular transporters
VIP	Vasoactive intestinal peptide

VOCC	Voltage-operated calcium channel
VSMC	Vascular smooth muscle cell
ZT	Zeitgeber Time

List of Figures

List of Tables

Chapter 1

Introduction

Chapter 1: Introduction

1.1 The Circadian clock

1.1.1 Introduction to the circadian clock

Most organisms have evolved to have endogenous time-keeping mechanisms adapt to the day-night cycle. In mammals; the light-dark cycles synchronize both behavioural and metabolic processes in response to environmental cues. This is achieved by the circadian clock, which internally generates behavioural and physiological rhythms to anticipate with daily environmental changes. The circadian cycle entails the rhythmic expression of genes over a 24-hour period. Societal changes like the increased night shift work plus other factors cause a demand of people to de-synchronise from the diurnal cycle. Recent research into the circadian clock has revealed that impaired synchronisation is associated with many diseases. This consequentially, increase in the demand to understand the circadian clock.

Mammalian circadian rhythms are regulated by the Suprachiasmatic nucleus, located in the anterior hypothalamus (Lee *et al.*, 2007). The SCN, known as the master pacemaker, acts as the main enforcer of the circadian clock, responsible for coordinating independent peripheral oscillators so the rhythms are organised to a cellular scale. The SCN receives direct input via photon capture by specialised photoreceptors; intrinsically photosensitive retinal ganglion cells (ipRGCs). These ipRGCs convey the information to the SCN for visual processing and to induce non-visual responses, thereby synchronising the SCN pacemaker (Ginty *et al.*, 1993; Curtis *et al.*, 2014; Prayag *et al.*, 2019). The most impactful environmental cue to influence the circadian clock is the light and dark cycle (Crepeau *et al.*, 2006). Other environmental conditions that influence the circadian rhythms are temperature, exercise, sound and food availability. These cues are known as zeitgebers, which occur naturally in a rhythm, and are therefore perfect to regulate the circadian clock. Temperature as a zeitgeber is particularly interesting, as it can alter the phase of the circadian rhythms by moving the cycle to earlier or later stages but still maintain the period length of the cycle (Vitaterna, Takahashi and Turek, 2001). Stages of the circadian cycle are separated in terms of zeitgeber time and circadian time. Zeitgeber time (ZT), relates to the time in relation to a Zeitgeber, typically the 12:12 light dark cycle, with ZT0 and ZT12 indicating when lights are on/off respectively. Circadian time refers to stages of endogenous free-running period of the intrinsic rhythm generated by the organism, independent of environmental cues (Karatsoreos and Silver, 2017). The free-running period typically occurs when an organism is isolated from these cues such as in constant darkness/light. In free-running conditions, onset of activity depends on the animals, with day-active organisms starting at circadian time zero (CT0) and night-active organisms is CT12. The SCN consists of

multiple neurons resonating together to produce a rhythmic signal that emanates to peripheral tissues via molecular and hormonal pathways. This internal rhythm is enforced by molecular oscillations via a conserved transcriptional-translational autoregulatory loop to induce oscillatory expression of 'clock genes' within a cellular level.

1.1.2 The Molecular clock

The current model of the mammalian molecular clock suggests that the central mechanism is comprised of a set of clock genes entwined with a delayed interlocked transcriptional–translational feedback loop (TTFL) with several auxiliary mechanisms to ensure the loop remains robust and stable(Zhang and Kay, 2010, cited in Barclay, Tsang and Oster, 2012). It has become apparent that more than a third of the mammalian genome is influenced by clock genes (Curtis *et al.*, 2014). The rhythms generated by the central circadian clock are enforced by an assortment of multiple genes and proteins. Figure 1 illustrates the central and auxiliary loops and how they interact (Curtis *et al.*, 2014). The integral instigators of the central loop of the TTFL are the molecular components CLOCK and BMAL1, proteins that bind together to form a helix-loop-helix heterodimer (Curtis *et al.*, 2014). This heterodimer binds to the E box area of the promoter region to upregulate the expression of clock-controlled genes. Among the genes promoted are the circadian proteins Cryptochrome-like proteins CRY1-2 and Period PER1-3. These two proteins translocate to the nucleus and interact together to downregulate CLOCK and BMAL1, to repress the positive loop of the circadian oscillation. PER and CRY limit their own expression and as they are gradually degraded, the repression of BMAL1 and CLOCK is relieved, and the cycle starts again (Curtis *et al.*, 2014).

The second loop involves the oscillation of nuclear receptors RAR related Orphan Receptors (RORα+β) and REV-ERBα/β (Curtis *et al.*, 2014). After BMAL1-CLOCK binding to the E-box promoter region is upregulated, these nuclear receptors translocate into the nucleus and bind to the promoter region of *Bmal1*. RORs and REV-ERBs induce and repress the expression of BMAL1 respectively, providing an auxiliary regulation of the central loop. The tertiary loop consists of the D-box binding protein, a transcriptional activator regulated by BMAL1-CLOCK and the repressor nuclear factor interleukin 3 (NFIL3), which is regulated by RORE (Curtis *et al.*, 2014). Both factors synergistically regulate D-box gene expression, including the Per gene. In addition, post translational modifications such as kinase and phosphatase activity, histone modifications and the epigenetic code also provide another layer of regulation to circadian clock (Curtis *et al.*, 2014).

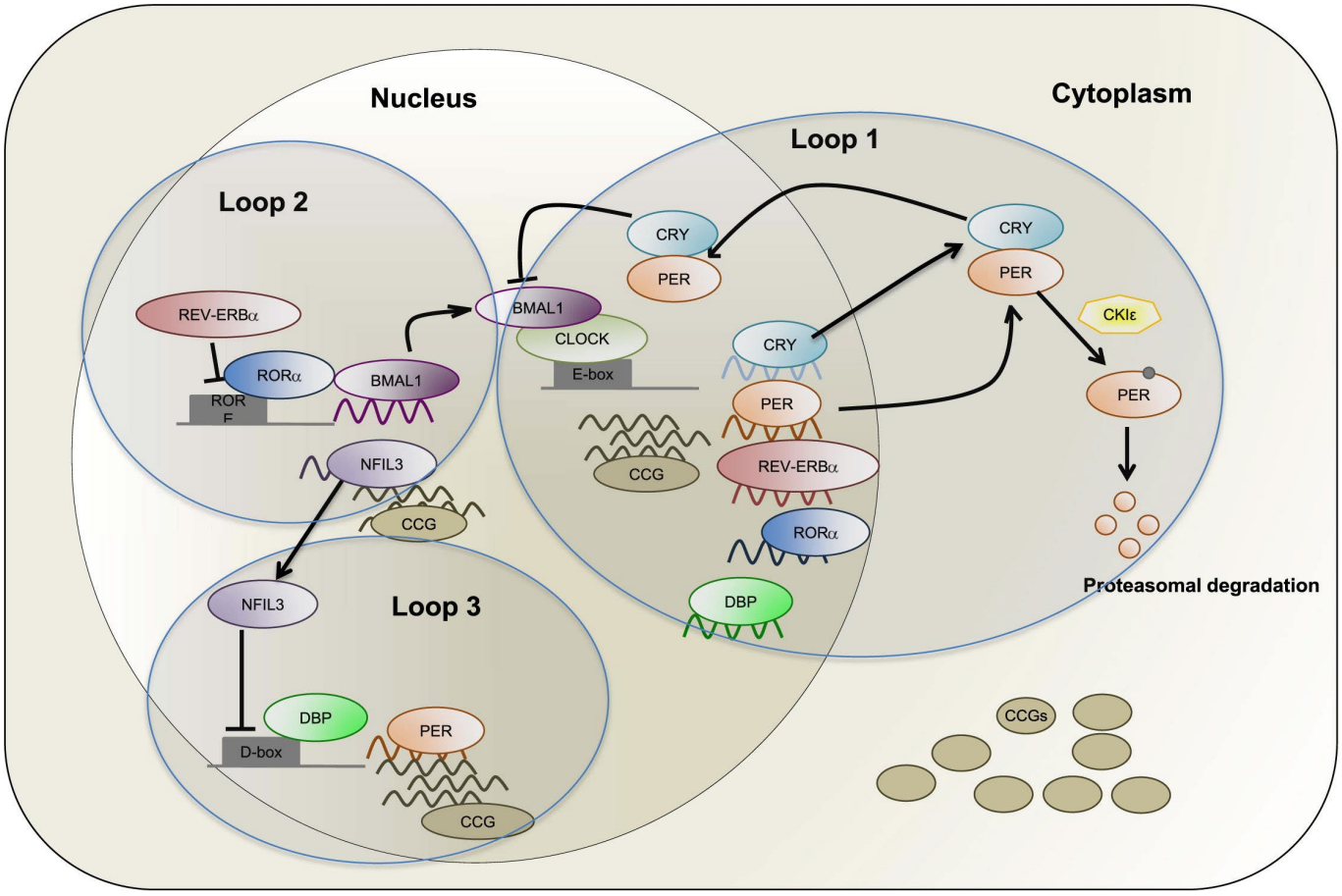

Figure 1 - The Molecular Clock model taken from Curtis *et al* (Curtis *et al.*, 2014).

This model of the molecular clock by Curtis *et al.* illustrates the Interlocking Feedback Loops (Curtis *et al.*, 2014). In Loop 1, BMAL1 and CLOCK bind to E-box elements within genes of repressor proteins such as PER, CRY, REV-ERBα, RORα and DBP. Over time, PER and CRY accumulate, translocate to the nucleus and repress these repressor proteins' expression. Posttranslational modifications like the phosphorylation of PER by CKIε also regulate repressor proteins as this marks proteins for degradation. Loop 2 is governed by REV-ERBα and RORα that bind to RORE promoter elements, which act upon *Bmal1* and *Nfil3*. Loop 3 is an alternate regulation by NFIL3 and DBP on D box promoter elements. Transcription factors within each loop also regulate genes referred to as clock-controlled genes (CCGs). These genes have circadian expression but don't feedback to alter the molecular clock. If some CCGs are themselves transcription factors, the targeted genes will also have a circadian expression. Therefore, core clock components and CCGs regulate transcription of many cellular components independent of what their function within the molecular clock.

The immediate early gene (IEG) has been revealed to be tightly correlated with entrainment of the SCN regulated rhythms (Ginty *et al.*, 1993). Multiple studies link this entrainment with the phosphorylated form of cAMP response element binding protein (CREB) in response to light (Ginty *et al.*, 1993; Lee *et al.*, 2010; Wheaton *et al.*, 2018). Upon exposure of light, the transcriptional regulatory site, Ser133 is phosphorylated on CREB, inducing transcription. Phosphorylation occurred after light induced IEG expression and induced a phase shift in the circadian rhythms. In addition, phosphorylated CREB effects the expression of several genes that influence the functional properties of the SCN (Lee *et al.*, 2010;

Wheaton *et al.*, 2018). This implicates CREB in neuronal signalling in the hypothalamus and suggests that the light-regulated molecular responses of the circadian clock are partially coordinated by CREB phosphorylation.

The Forskolin (*c-fos*) proto-oncogene in SCN cells has also been identified to coordinate photic entrainment (Ginty *et al.*, 1993). The Fos protein is a transcription factor that regulates the expression of late response (LR) genes that contain AP-1 binding sites within the regulatory region. The selective expression of LR genes may determine long term cellular responses. Light exposure induces *c-fos* by CREB signalling, but only when the behavioural rhythms undergo a phase shift, with both processes having similar photic illumination thresholds (Wheaton *et al.*, 2018). CREB mediates *c-fos* expression in response to intracellular increase of cAMP or Ca^{2+}. This triggers the phosphorylation of Ser^{133}, essential for binding site conformational change to allow transcription. There is an established link between the circadian phase dependence on light-induced phosphorylation of CREB and light-induced transcription of *c-fos* (Ginty *et al.*, 1993). In turn, CREB participates in the control of *c-fos* transcription in SCN cells (Wheaton *et al.*, 2018). Regulation of gene expression in the SCN by CREB may be important for entraining the pacemaker that regulates behavioural and hormonal rhythms. In addition, the components of the light sensitive molecular responses in the SCN may act upstream of CREB phosphorylation (Ginty *et al.*, 1993).

1.1.3 Circadian Entrainment, Central-Peripheral clock communication

One core principle of the circadian clock is that the rhythm of the internal clock is self-sustained, meaning all diurnal rhythms can persist without the external environment; a concept known as “free running” cycle (Vitaterna, Takahashi and Turek, 2001). This property has been identified in cell cultures and explanted tissues, proving the internal clock will attempt to persist even without the rest of the circadian clock (Husse, Eichele and Oster, 2015). The free running clock in humans has a rhythmic cycle approximately 24.35 hours so without alignment to the 24-hour light-dark cycle, the internal clock will desynchronise ((Vitaterna, Takahashi and Turek, 2001; Crepeau *et al.*, 2006). This means the internal time-keeping system needs to be accelerated daily to align with the day-night cycle, which as described by Vitaterna *et al.,* “results in greater precision in controlling the timing of the expressed rhythms” as the system is constantly adjusted and therefore tightly regulated (Vitaterna, Takahashi and Turek, 2001). This process of synchronising to external zeitgebers is known as entrainment. In terms of the light-dark cycle, the circadian clock is entrained by light exposure. Light intensity also affects the entrainment of the clock as stated in Aschoff’s first rule; which states “the free-running circadian period that is observed in complete darkness, will shorten for diurnal animals but lengthen for nocturnal animals when they are exposed to

constant light" (Aschoff, 1979; Goltsev et al., 2022). The human circadian system has been found to be the most sensitive to short wavelength (Blue) light between 460 and 480nm as intrinsically photoreceptive retinal ganglion cells, which are highly sensitive to blue light, are the cells involved in circadian transduction (Crepeau *et al.*, 2006; Wahl *et al.*, 2019). Blue light exposure during the day is important in suppressing melatonin production and shifts its rhythm, which can induce a chronic sleep debt and therefore reduces alertness and cognitive performance if blue light is prolonged to just before bedtime (Crepeau *et al.*, 2006; Wahl *et al.*, 2019). In terms of circadian clock entrainment, morning exposure to light induces a phase advance of the circadian clock and delays it if exposed in the evening. This advancement/delay of the clock affects other rhythms induced by the SCN as shown by an example discussed by Crepeau *et al.,* which stated that after east-west travel, the delay shift in the clock induces a delay in temperature and melatonin rhythms (Crepeau *et al.*, 2006).

The most accepted theory to which the circadian clock is organised is the Hierarchal model or the "orchestra model" (De Assis and Oster, 2021). The model considers the SCN as the conductor, providing information to coordinate the musicians (peripheral clocks). This model was generated after analysis of SCN lesion experiments, which found that lesions disrupted both SCN neural connections but also its neighbouring connections (De Assis and Oster, 2021). The SCN has been demonstrated the most critical in zeitgeber-free environments, as peripheral clocks are coordinated by the light-dark cycle in absence of a functional SCN. It is also essential under conditions where zeitgebers can conflict, such as with jet lag. A vast body of literature supports the Hierarchal model of circadian entrainment, principally the information of the SCN receiving input from the light zeitgeber and subsequently synchronising the peripheral clocks, suggesting a strict "top-down" system (Husse, Eichele and Oster, 2015). However, despite support for the hierarchal model, there has been increasing evidence that suggests that this model is not the full story. Light exposure has been found to acutely activate clock gene expression and glucocorticoid release via sympathetic innervations, independent to the photic response by the SCN (Husse, Eichele and Oster, 2015). Stokkan *et al* found that some peripheral organs, in this case the liver, can experience a rhythm shifted by restricted feeding despite the light-dark cycle phase remaining constant (Stokkan *et al.*, 2001). This information provokes the notion that the central-peripheral communication may not be as simple as a hierarchal system but more complex. This information led to the suggestion that other cyclic environmental inputs such as temperature, social cues or access to food can also act as entraining agents but influence of these signals onto the circadian clock and the SCN are considered weaker signals to enforce behaviour (Husse, Eichele and Oster, 2015). More notably, the circadian oscillators involved with restricted feeding were described to be distinct from those involved

with light entrainment (Stokkan *et al.*, 2001). This led to the concept of the Federated model, where different zeitgebers are able to act independently upon different peripheral clocks (Figure 2) (Husse, Eichele and Oster, 2015). The federated model of the circadian organisation allows a more tailored response, where peripheral clocks attune to the more relevant zeitgebers. Another advantage of the federated model is the circadian timekeeping has increased noise resistance to circadian timekeeping, as parallel and independent synchronisation pathways would enforce the same signal, increase the signal's stability and prevent unwanted shifts in a noisy zeitgeber environment (Husse, Eichele and Oster, 2015). Overall, the organisation of the mammalian circadian clock was initially seen to be Hierarchal and follows this model in the absence of a periodic zeitgeber. However, in presence of Zeitgebers the organisation resembles a more federated model, which could hint the circadian clock has a more complex multi-model system. This organisation could exist to provide a compromise to the circadian clock, to allow fine-tuned adaption to the complex environment yet still maintain stability under conditions of less or conflicting Zeitgeber signals.

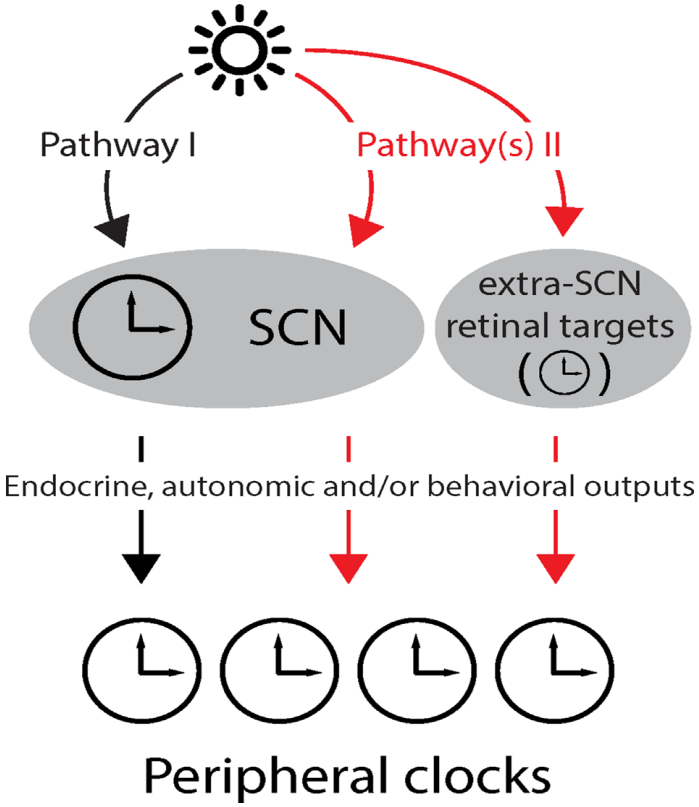

Figure 2 - The proposed system for Circadian Entrainment, taken from Husse *et al*. (Husse, Eichele and Oster, 2015).

Circadian entrainment is considered to synchronise peripheral clocks to the LD cycle by parallel pathways. Light is considered the most important zeitgeber for circadian clock and can entrain peripheral clocks via multiple routes. In pathway 1, light is transmitted from the retina to the SCN through the retino-hypothalamic tract. The SCN clock then transmits information to peripheral clocks along neuronal/endocrine pathways. The 2nd pathway theorises that photic information can bypass the SCN to reset peripheral clocks.

The SCN conveys time-of-day information to the rest of the brain and body via Sympathetic and parasympathetic stimuli, humoral pathways, oscillations in temperature and fast-feeding systems (De Assis and Oster, 2021). The SCN receives input from the hypothalamus and extra hypothalamic regions, pathways critical for regulating SCN physiology (De Assis and Oster, 2021). In response, the SCN produces a combination of efferent projections and diffusible signals terminating at various regions in the brain; including the preoptic area, paraventricular nucleus (PVN) and Arcuate nucleus (ARC) (Curtis *et al.*, 2014; De Assis and Oster, 2021). These projections are utilised as an entrainment mechanism between the central and peripheral clocks. The first pathway of the SCN is the autonomic pathway. Photic stimulation of the retina and electric stimulation of the optic nerve activates SCN neurons which enforces neuronal firing and the rhythmic expression of *Clock* genes (Brown and Piggins, 2007). This method does not work for every peripheral clock however, as shown in the liver, where *Clock* gene expression remains unaltered after autonomic denervation, revealing that it must be influenced by another pathway. An additional pathway for the SCN is via astrocyte/glial cell activity. The activity of astrocytes occurs within the circadian night which is in antiphase to SCN neurons (Hastings, Maywood and Brancaccio, 2019). Astrocytes are important in controlling cerebral vasculature and synapse delineation. In terms of SCN signalling, SCN astrocytes were found to have the capacity to drive and direct behavioural rhythms by acting on the SCN when it conflicts with the neuronal TTFL (Hastings, Maywood and Brancaccio, 2019). This is achieved by releasing gliotransmitters, neuroactive compounds such as glutamate, which perform region specific roles across the brain (Hastings, Maywood and Brancaccio, 2019). Another pathway is the humoral pathway, primarily comprised of the hypothalamic-pituitary-adrenal (HPA) axis. Glucocorticoids and Catecholamines (adrenaline/Noradrenaline) are primary contenders of this pathway, rhythmically released by the adrenal cortex during the active phase of the circadian clock (Curtis *et al.*, 2014; Druzd, De Juan and Scheiermann, 2014; De Assis and Oster, 2021). These hormones are induced by corticotropin releasing hormone secretion via the PVN, which controls the rhythmic release of adrenocorticotropic hormone (ACTH) in the pituitary to act downstream upon the adrenal cortex. Notably, the Glucocorticoid influence on the circadian clock is achieved by Glucocorticoid response elements whose activation induces gene transcription and translation of circadian components (De Assis and Oster, 2021). Another synchroniser messenger is melatonin, which is secreted exclusively at night in diurnal and nocturnal species. During darkness, Glutamatergic signalling to the PVN induces noradrenaline release towards the pineal gland and subsequently melatonin synthesis (De Assis and Oster, 2021). Light exposure, whether during the day or light-at-night (LAN), causes the SCN to send inhibitory signals to the PVN, therefore inhibiting melatonin synthesis. Food is an important zeitgeber for metabolic tissues, synchronising local

clocks and inducing few effects upon the SCN, affecting locomotor activity and melatonin synthesis (Cipolla-Neto and Do Amaral, 2018, cited in De Assis and Oster, 2021). The SCN's final pathway is by regulating the internal temperature rhythms by the preoptic nucleus. Diurnal variations in temperature have been identified between the active and resting phases which play an important role in peripheral clock synchronisation. However, how the temperature is regulated currently remains elusive (De Assis and Oster, 2021).

Central-peripheral communication is not exclusive to central transmission to peripheral clocks; Peripheral clocks have been shown to send feedback mechanisms to enforce the rhythm of the central clock. Melatonin acts as one of these feedback messengers, acting upon melatonin receptors found in the SCN, altering gene expression within the SCN and even directly reset the SCN in rats (De Assis and Oster, 2021). Leptin, the satiety hormone, is another feedback messenger of the SCN, altering the phase shifting effects of LAN exposure as found in female mice (De Assis and Oster, 2021). A final one of note is Ghrelin, a metabolic hormone coordinated directly by the SCN, is synthesised to anticipate feeding times by acting on the ARC. *In vivo* experiments have identified that ghrelin and its analogues induce phase advances in locomotor activity in food deprived mice subjected to constant darkness conditions (De Assis and Oster, 2021).

1.1.4 Circadian messengers

Central peripheral communication is coordinated using circadian messengers. For this study, our work will focus on GABA, arginine vasopressin, corticosterone and melatonin.

1.1.4.1 Gamma aminobutyric acid

Gamma aminobutyric acid (GABA) modulates inhibition through the mammalian CNS by acting on GABAA, GABAB and GABAC receptors (Sarang et al., 2001; Takano et al., 2014). GABAA and GABAC receptors are ligand-gated Chloride channels, consisting of a pentamer of α, β, γ, δ, ε, π, and θ subunits, (Takano et al., 2014). GABAA receptors are subdivided into GABAA PHASIC and GABAA TONIC receptors, which have synaptic and extra synaptic functions respectively (Figure 3) (Albers et al., 2017). PHASIC receptors respond to GABA release in the synapse at concentrations greater than 1 mM, producing inhibitory post synaptic potentials which peak and decay in milliseconds. TONIC receptors mediate currents outside the synapse; inducing stronger excitation and inhibition in response to concentrations between 0.5 and 1 μM. Activated chloride channels cause an influx of Cl^- ions into the postsynaptic neuron, causing

hyperpolarisation (Paulus and Rothwell, 2016). GABAB receptors are metabotropic G-protein receptors coupled with the Gi/o α subunit on presynaptic and postsynaptic terminals. Presynaptic GABAB receptors induce inhibition by suppressing voltage-sensitive Ca^{2+} channels/ K^{+} channels, reducing transmitter release. On postsynaptic terminals, the Gi/o α subunit binds to the GIRK channel which coordinates potassium efflux into the synaptic cleft (Takano *et al.*, 2014; Paulus and Rothwell, 2016). Both responses hyperpolarize the membrane and increases membrane conductance.

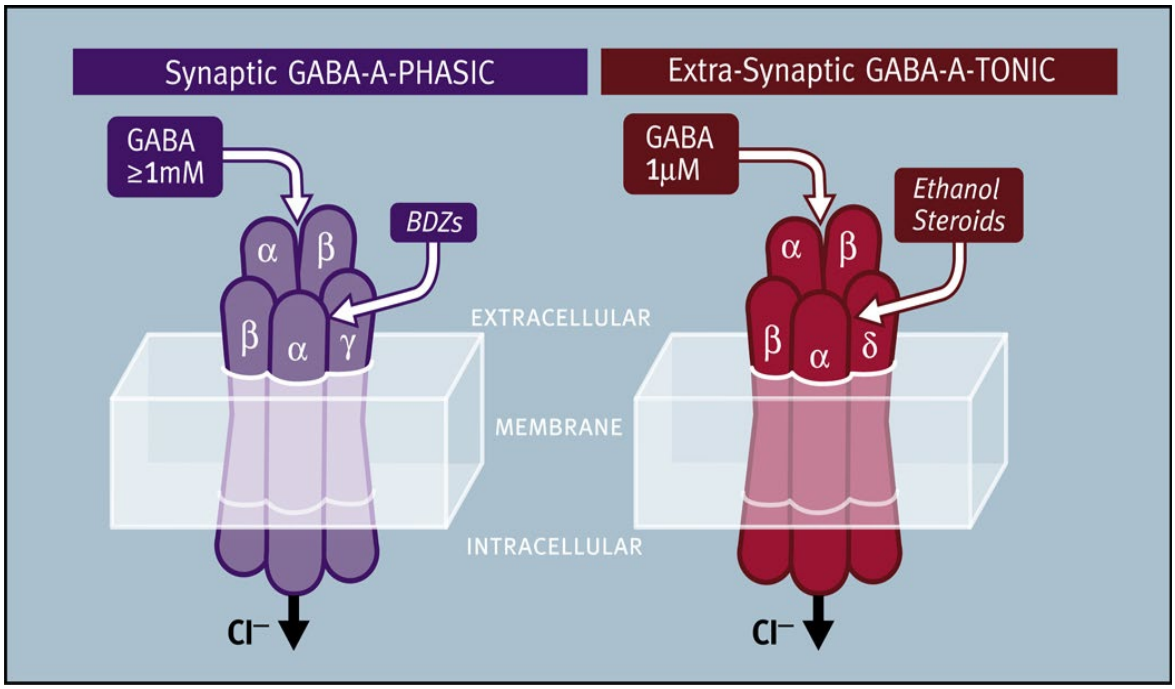

Figure 3 - The properties of GABAA-PHASIC and GABAA-TONIC receptors taken from Albers *et al.*, (Albers et al., 2017).

$GABA_A$-PHASIC receptors are characterized by having the γ subunit and respond to GABA concentrations of >1 mM. After binding to GABA, these receptors produce inhibitory postsynaptic currents that desensitize rapidly. Extra-synaptic $GABA_A$-TONIC receptors are known for the possession of the δ subunit. These receptors respond to lower concentrations of GABA (0.5–1.0 μM) and are activated after long term exposure. For both types of receptors, the pocket formed between the type-specific subunit and α subunit allows for benzodiazepines and ethanol/steroids to bind to $GABA_A$-PHASIC and $GABA_A$-TONIC receptors respectively.

During early stages development of the CNS, GABA acts in an excitatory role (Ben-Ari *et al.*, 2007; Li and Xu, 2008). Unlike in adult CNS, GABA perfusion induces a rise in intracellular Ca^{2+} levels within the postsynaptic neuron, inducing depolarisation. It is theorised that the Cl^- ion gradient is involved in GABAA receptor's excitatory role as this gradient is inversed in prenatal and early post-natal stages, with higher concentrations inside the cell than the synaptic cleft. Therefore, GABAA receptors transport Cl^- ions out of the cell, causing depolarisation. As the CNS develops the intracellular Cl^- concentration declines below

the extracellular concentration, which causes GABAA tonic receptors to become inhibitory. A need for this switch may be due to the theory that GABA neurons are generated before Glutamate receptors, therefore act as an excitatory neuron during development until Glutamate receptors form (Ben-Ari *et al.*, 2007). The function of GABAA receptors as an excitatory or inhibitory neuron is due to the cation chloride transporter expressed (NKCC1 or KCC2) (Li and Xu, 2008). GABAA receptors induce depolarisation in the SCN and are essential in neuron proliferation, migration and differentiation (Spitzer, 2010; Albers *et al.*, 2017).

1.1.4.2 GABA as a signalling molecule in the SCN

Virtually all neurons within the SCN communicate via GABAergic signalling (Albers *et al.*, 2017; Ono *et al.*, 2018). Studies speculate GABA and neuropeptide presence within neurons modulated by the circadian cycle, with co-expression of GABA with vasoactive intestinal peptides (VIP) in the ventral core and arginine vasopressin (AVP) in the dorsal shell (Albers et al., 2017). GABAA TONIC receptors and GABAA PHASIC receptors are found to be in greater abundance during the night and day respectively, revealing the change in GABAA at different circadian phases (Albers et al., 2017). Recent studies in rats analysed GABAA tonic activity in the SCN through spontaneous postsynaptic currents and demonstrated diurnal rhythm, with a peak at ZT7-8 and nadir at ZT19-20, the middle of day and night respectively (Moldavan, Cravetchi and Allen, 2021). The GABAA tonic current increased with postsynaptic current frequency, revealing rats' nocturnal behaviour induce these rhythms by GABAA tonic receptors. Inhibitory synaptic transmission also showed a daily rhythm, as mice under the 12hr light: dark cycle showed a peak in the dorsal SCN at ZT 11-12, and significant elevation at ZT13-15. This revealed rhythm in the dorsal region, was consistently higher than the ventral SCN (Itri *et al.*, 2004). GABA excitatory neuron activity exhibits a peak at ZT 17-20 and Nadirs at ZT5-8, inversely proportional to the GABAA postsynaptic current (Choi *et al.*, 2008). NKCC1 expression in the dorsal SCN coincides with this excitatory activity, meaning NKCC1 expression is responsible for GABA excitatory signalling. GABAB receptors also seem to be more predominant within the dorsal SCN, producing inhibitory signals in SCN neurons (Albers et al., 2017). Blocking Gi/o activity (component specific for GABAB receptors) induces three changes in SCN rhythmicity; decreasing the proportion of rhythmic neurons in the SCN by approximately 40%, decreasing rhythmic signal amplitude, and desynchronization for the remaining neurons (Aton *et al.*, 2006). Gribkoff *et al* identified the GABAB agonists have a greater potency to inhibit SCN activity at night than the day, suggesting a rhythmic expression (Gribkoff, Pieschl and Dudek, 2003). Most data suggest that GABAB receptors predominantly responsible for presynaptic inhibition although there is a small number found on postsynaptic terminals (Albers et al., 2017). Figure 4 shows an overview of GABA presynaptic and post synaptic signalling.

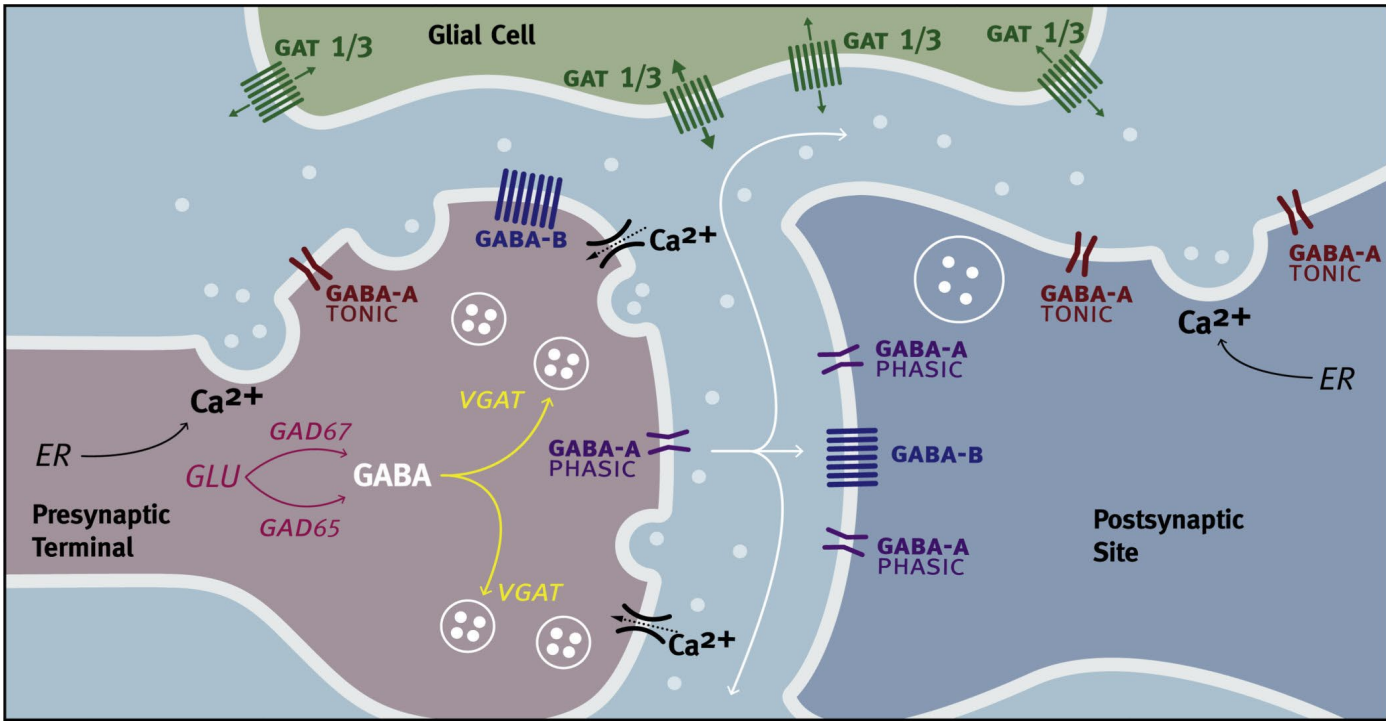

Figure 4 - GABA signalling in presynaptic and postsynaptic neurons, taken from Albers *et al*, (Albers *et al.*, 2017).

Glutamic acid (GLU) catalysed into GABA in presynaptic neurons by glutamic acid decarboxylase (GAD). GAD67 and GAD65 synthesizes GABA in TONIC and PHASIC receptors respectively. GABA vesicular transporters (VGATs) transport GABA into synaptic vesicles (yellow). GABA is released into the extracellular space which increases intracellular calcium (Ca^{2+}). Ca^{2+} influx can occur through voltage-gated ion channels as the result of action potentials or by intracellular Ca^{2+} release from the endoplasmic reticulum (ER). GABAA-PHASIC (purple), GABAA-TONIC (dark red) and GABAB (blue) receptors are located on presynaptic and postsynaptic terminals. GABAA-PHASIC receptors are typically found at synaptic regions while GABAA-TONIC receptors are at extra-synaptic sites. GABAB receptors are found in both synaptic and extra-synaptic regions. GABA transporters (GATs) can remove GABA from the extracellular space or release GABA into it. GAT1 and GAT3 (Green) are found in the SCN on astrocytic processes extra-synaptic regions.

Despite GABA's ubiquitous expression and its receptors in SCN neurons, the function of GABA within the SCN is controversial (Aton *et al.*, 2006). One possible role is GABA aids in the coupling of circadian clock cells. The circadian pacemaker is a complex of circadian clock neurons that need to be coordinated to produce coherent outgoing signals. GABA is theorised to coordinate clock neuron coupling by GABAA TONIC receptors, as part of extra-synaptic activity. Evidence GABA contributes to coupling cellular clocks in the SCN was found studying GABA and GABA agonists on circadian firing of individual SCN neurons in cell culture (Liu and Reppert, 2000). Acute application of Muscimol (GABAAR agonist) and baclofen (GABABR agonist) induces inhibition of neuronal firing when applied at various phases of the circadian cycle (Albers et al., 2017). Long term application caused phase delays for GABA and muscinol but not baclofen between circadian times (CT) 8 and 14, therefore indicating phase shifting by GABA is coordinated by GABAA receptors (Aton *et al.,* 2006). Aton *et al* inhibited endogenous GABA activity in the

SCN to discover if clock neurons would desynchronise but found they had increased synchronicity (Aton *et al.,* 2006). This implies that GABA can induce phase shifts in the SCN cultures, but it may not be necessary for their synchronisation (Albers et al., 2017).

The ventral core and dorsal shell can function independently as oscillators but also require coupling (Albers et al., 2017). It was noted GABAA receptors produce primarily inhibitory effects on the ventral SCN and primarily excitatory effects in the dorsal SCN (Albus et al., 2005). Albus *et al* examined GABA's role in resynchronising ventral and dorsal oscillators and found the dorsal SCN took longer to reset than the ventral SCN, and phase resetting is prevented by bicuculline, a GABAA antagonist (Albus et al., 2005). VIP acts together with GABAA signalling to promote resynchronisation when the ventral and dorsal SCN are out of phase (Albers et al., 2017). However, when in phase, VIP opposes GABAA signalling, therefore opposes resynchronisation. This could be due to the changing of GABAA TONIC signalling between excitatory and inhibitory with the NKCC1 and KCC2 chloride channels throughout the diurnal cycle. Additionally, mice had a higher NKCC1:KCC2 ratio (Excitatory: inhibitory ratio) in the dorsal region compared to the ventral region so a difference in these receptors in the ventral and dorsal SCN could account for the coupling changes (Myung *et al.*, 2015). Gabazine, a GABAA antagonist, disrupts phase and period changes due to long photoperiod entrainment. Together this suggests GABAA induced excitation "uncouples" the ventral and dorsal SCN by pushing the phase of the oscillators apart (Albers et al., 2017).

GABA activity is linked to entraining the circadian pacemaker. Short- and long-term activation of SCN GABA receptors mediates the effects of photic and non-photic stimuli on the circadian pacemaker (Albers et al., 2017). With non-photic manipulation of the SCN, systemic injection of GABAA agonist benzodiazepines (BDZs) causes phase shifts like NPY into the SCN. Short acting BDZs reduces locomotor activity and long acting BDZs increase locomotor activity respectively. It was identified that some neurons of the geniculo-hypothalamic tract (GHT) produce GABA and NPY, alluding to NPY and BDZs having similar phase shifting effects in the SCN (Albers et al., 2017). BDZs act upon GABAA PHASIC receptors, which appear more in the subjective day, and produce opposite effects in diurnal species than in nocturnal animals. GABAA receptor activation during the day alters *Per1* and *Per2* expression, producing phase advances in nocturnal animals via *Per1* expression, yet phase delays via *Per2* suppression in diurnal species (Albers et al., 2017).

GABA entrainment by photic stimuli is more complex, as GABAA receptor activation produces different effects depending upon the route of activation. GABAA modulates phase shifts by light entrainment during the dark phase of the LD cycle, with agonists accelerating re-entrainment and antagonists blocking phase delays respectively (Albers et al., 2017). GABAA activity is implicated to alter NPY activity, which inhibits light induced phase shifts but not its ability to reduce light induced phase delays (Lall and Biello, 2002, 2003). Phase advancing effects of NPY are still mediated by GABAA receptors though. Combined evidence supports that GABAA activation induces phase delays for the circadian pacemaker (Albers et al., 2017). Sustained ventral SCN activation is suspected to induce activation of the dorsal SCN. The excitation: inhibition ratio of GABAA TONIC receptors in the SCN is considered to contribute to circadian entrainment by modulating the coupling of ventral and dorsal SCN. Acute activation of GABAB receptors effects light phase shifts when administered systemically and within the SCN. Systemic administration prior to light exposure inhibits phase delays and advances as well as *Fos* and *Per1* induction in the SCN (Albers et al., 2017). GABAB activity blocks light phases completely if administered just after the light pulse. GABAB activity also modulated Fos protein activity like light induced phase shifts. Presynaptic GABABR activity inhibits EPSPs that situate from the optic nerve in both daytime and nighttime, and reduces glutamate and serotonin release in the SCN, showing GABAB receptors are the primary site to entrain the SCN via photic stimuli. The SCN regulates the daily rhythm of plasma melatonin via both excitatory Glutamatergic and inhibitory GABAergic signalling to the PVN (Albers et al., 2017). GABAergic signalling was suggested to inhibit melatonin expression and synthesis during the day whereas Glutamatergic signalling promotes melatonin synthesis and release at night (Perreau-Lenz *et al.*, 2005) . GABAergic signals from the SCN mediate PVN neuron activity that controls parasympathetic input, including the rhythmic release of oxytocin and AVP (Albers et al., 2017). GABA also uses hormone release to alter output signals such as GABAA neuron activation in SCN slices coordinates AVP release.

1.1.4.3 Arginine Vasopressin

Vasopressin is a neuropeptide hormone, synthesized in the hypothalamus, and released via the posterior pituitary gland into the circulation (Hyodo, 2016). Vasopressin is secreted in response to increased plasma osmolality, to promote water reabsorption and increase arterial blood pressure (Garrahy and Thompson, 2019). Thirst is also induced by osmoreceptors located near those that control vasopressin release suggesting that both are activated together in the anterior hypothalamus. Vasopressin also responds to blood volume, inducing rapid release of vasopressin during hypovolemia. Response to hypovolemia and hypotension is the most important non-osmotic stimuli to prevent blood pressure related shock. To

prevent this, a blood pressure decrease induces the unloading of baroreceptors, then a massive increase in plasma vasopressin from the posterior pituitary that exceeds physiological levels during dehydration.

Vasopressin responses are mediated by action-specific vasopressin receptors on the cell surface (Garrahy and Thompson, 2019). Vasopressin receptors are GPCRs with 3 subclasses; V1, V2 and V3 receptors, expressed in different areas of the body. V1 receptors are predominantly expressed in vascular smooth muscle. When activated, V1 receptors induce the phosphatidyl-inositol intracellular cascade which generates intracellular calcium increase. This causes vasoconstriction when the concentration of arginine vasopressin exceeds physiological levels which could act as a safety mechanism in response to shock. V1 receptors are also found in platelets, hepatocytes and the myometrium, inducing thrombosis, glycogenolysis and uterine constriction respectively. V2 receptors are similarly expressed in vascular smooth muscle to V1 and on the basolateral surface of the collecting tubules. V2 receptors activate adenyl cyclase, cAMP and protein kinase A, mediating vasoconstriction and expression of water channels in the renal collecting ducts (Garrahy and Thompson, 2019). V2 receptors were also found to cause vasoconstriction in vascular endothelium but in the forearms, can induce vasodilation instead, mediated by nitric oxide (Tagawa *et al.*, 1993; Garrahy and Thompson, 2019). V3 receptors have limited expression, currently being found only in the anterior pituitary gland. Characteristically, V3 receptors bear remarkable resemblance to V1 receptors, inducing phosphatidyl-inositol signalling, and calcium influx. V3 receptors differ because they activate several signalling pathways in addition to $G_{q/11}$. $G_{q/11}$ G-protein induces ACTH secretion from the anterior pituitary cells via protein kinase C in a dose dependent manner. G_s, G_i, and $G_{q/11}$ on the other hand, are all implicated in the induction and maintenance of ACTH secreting tumour cells (Holmes, Landry and Granton, 2003).

1.1.4.4 Arginine vasopressin as a signalling molecule in the SCN

Arginine vasopressin is a major messenger for the circadian system in the SCN. AVP is in the dorsomedial subdivision of the SCN and exhibits a circadian rhythm, displaying a peak at ZT9 and its lowest point at ZT21 (Yambe et al., 2002). Interestingly, the rhythm is maintained under constant dark conditions *in vivo* and *in vitro*, revealing that AVP rhythm is controlled by an endogenous mechanism rather than external input (Yambe et al., 2002). The expression of AVP and release acts on the hypothalamic-pituitary-adrenal axis and gonadotropin releasing hormone (Yambe et al., 2002). It is documented that less urine is produced and excreted during the night than during the day, partially due to the nocturnal secretion of antidiuretic hormone. Plasma AVP in humans increased progressively during the night, with peaks between 2400 and 0400h (George *et al.*, 1975) . Plasma AVP is influenced by; plasma osmolality, blood

volume and arterial pressure, all which induce a rise in plasma AVP (George *et al.*, 1975; Garrahy and Thompson, 2019).

AVP signalling is located within the dorsal shell and identified to couple SCN neurons by V1 signalling in the SCN (Yamaguchi, 2018). This coupling persists during constant darkness, as the coherent signal generated and distributed by the SCN is robust. An abrupt change in the LD cycle desynchronises the internal clock from the environment. The neuronal circuit that mediates V1 receptor signalling plays a crucial role in the circadian clock tolerance to jet lag (Yamaguchi, 2018). V1a/V1b receptor KO mice exhibited almost instantaneous re-entrainment of *Clock* expression, body temperature and locomotor activity. This immediate adaptation has been observed in mice that lack the *Clock* gene but still exhibit a functional circadian clock (Yamaguchi, 2018). AVP has also been speculated to be one mediator of the circadian period length (Mieda, Okamoto and Sakurai, 2016). This was found by casein kinase 1δ deletion in SCN AVP neurons, which increased the period length of circadian behaviour. This hints the necessity of AVP neurons within the SCN to produce stable expression of the circadian period by enhancing SCN neuron coupling.

1.1.4.5 Corticosterone

Corticosterone is a glucocorticoid produced in the zona fasciculata of the adrenal cortex in many animals (Katsu and Iguchi, 2015). In rats, mice and other animals, corticosterone is the principal glucocorticoid, whereas in humans, the main glucocorticoid is cortisol. Whether cortisol or corticosterone is the primary glucocorticoid is determined by the expression of 17α-hydroxylase, the enzyme that produces cortisol in the zona fasciculata which is expressed in humans but not rodents (Gomez-Sanchez and Gomez-Sanchez, 2014). Physiological functions have been shown to differ between cortisol and corticosterone. Both steroids play a role in the functional zonation of the adrenal gland but act differently on adrenal steroidogenesis (Katsu and Iguchi, 2015). Cortisol inhibits 18-hydroxydeoxy-corticosterone and aldosterone production in adrenal tissue culture, whereas corticosterone inhibits cortisol production but stimulates androgen production (Katsu and Iguchi, 2015).

Glucocorticoids like cortisol and corticosterone act via either mineralocorticoid receptors (MR) or Glucocorticoid receptors (GR) (Gomez-Sanchez and Gomez-Sanchez, 2014). In most tissues, glucocorticoids typically activate MRs at basal levels and GRs at stress levels. This suggests glucocorticoids have a possible role in homeostatic regulation in addition to being a stress response molecule (Gomez-Sanchez and Gomez-Sanchez, 2014). In general, corticosterone acts through GR, for example to mediate

blood pressure in the hindbrain (Katsu and Iguchi, 2016). Mineralocorticoids like aldosterone, bind exclusively to MR and MRs have equal binding affinity for aldosterone, cortisol and corticosterone, suggesting glucocorticoids mediate aldosterone activity through competitive inhibition (Gomez-Sanchez and Gomez-Sanchez, 2014). Despite sharing MRs, cortisol and corticosterone has diverse purposes to aldosterone and is regulated differently. Aldosterone, a constituent of the RAAS system, is crucial for haemodynamic homeostasis, whereas glucocorticoids regulate stress response and dampen inflammation. Glucocorticoids are affected by 11β-Hydroxysteroid dehydrogenase (11β-HSD) that alters glucocorticoid structure to their active or inactive form. There are two isozymes; 11β-HSD1 that converts the inactive cortisone and 11-dehydrocorticosterone into cortisol and corticosterone respectively, and 11β-HSD2 that converts these glucocorticoids back into their inactive form (Figure 5) (Gomez-Sanchez and Gomez-Sanchez, 2014). Cortisone is implicated in MR regulation, as it was observed to inhibit MR activity by aldosterone *in vitro*. Corticosterone is utilised as an intermediate to produce aldosterone in the zona glomerulosa. Corticosterone also initiates transcription of effector proteins by binding to DNA. Aldosterone and cortisol/corticosterone are also speculated to mediate rapid non-genomic effects independent of MR or GR, likely by GPCRs or activating cell signalling pathways (Figure 6) (Gomez-Sanchez and Gomez-Sanchez, 2014).

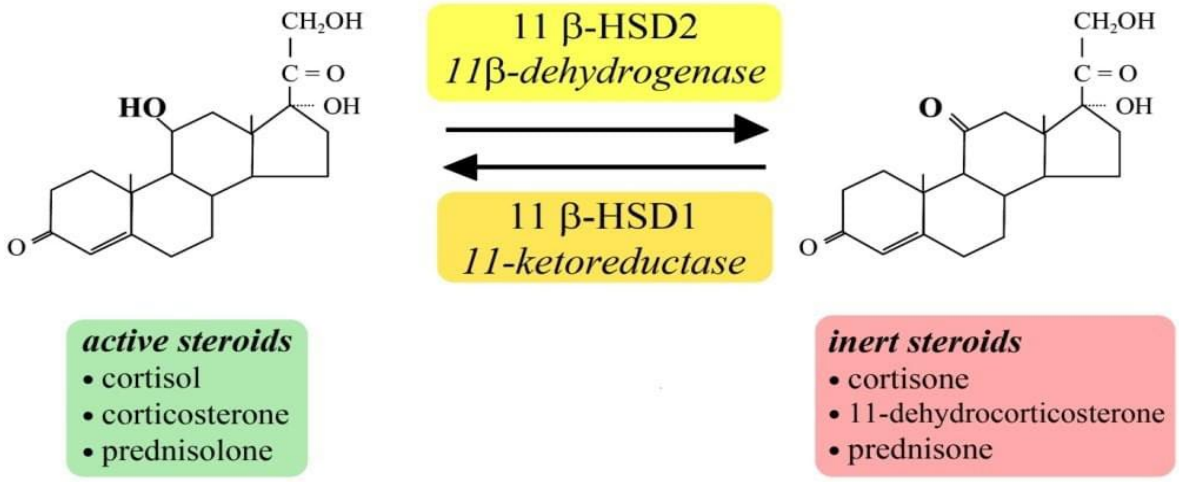

Figure 5 - The conversion of Cortisol and Corticosterone into Cortisone and 11-dehydrocorticosterone, taken from Creative-Biostructure, (Custom MemProTM 11-beta Hydroxysteroid Dehydrogenases (HSD-11β) - Creative Biostructure, 2020)

11β-HSD enzyme is a key regulator of Cortisol or Corticosterone activity. This enzyme is subdivided into 2 isoforms; 11β-HSD1, 11-ketoreductase and 11β-HSD2, 11β-dehydrogenase. 11β-HSD1 converts cortisone and 11-dehydrocorticosterone into cortisol and corticosterone. 11β-HSD2 converts these active steroids into cortisone and 11- dehydrocorticosterone. Of note, 11β-HSD1 is NADPH dependent and 11β-HSD2 is NAD^+ dependent to function.

MR activation is known to modulate ion and fluid transport, crucial for osmotic and hemodynamic homeostasis, as well as effecting membrane excitability in neurons and muscle cells. GR are essential for energy homeostasis and dampen inflammatory responses as part of the stress response (Gomez-Sanchez and Gomez-Sanchez, 2014). MR also effects stress and is considered the early response whereas GR are activated as a later response, even suggesting GR dampens MR functions, possibly as a stress reliever. This is essential as prolonged MR activation causes a rise in reactive oxygen species, inflammation and progression/onset of cardiovascular and renal disease (Gomez-Sanchez and Gomez-Sanchez, 2014).

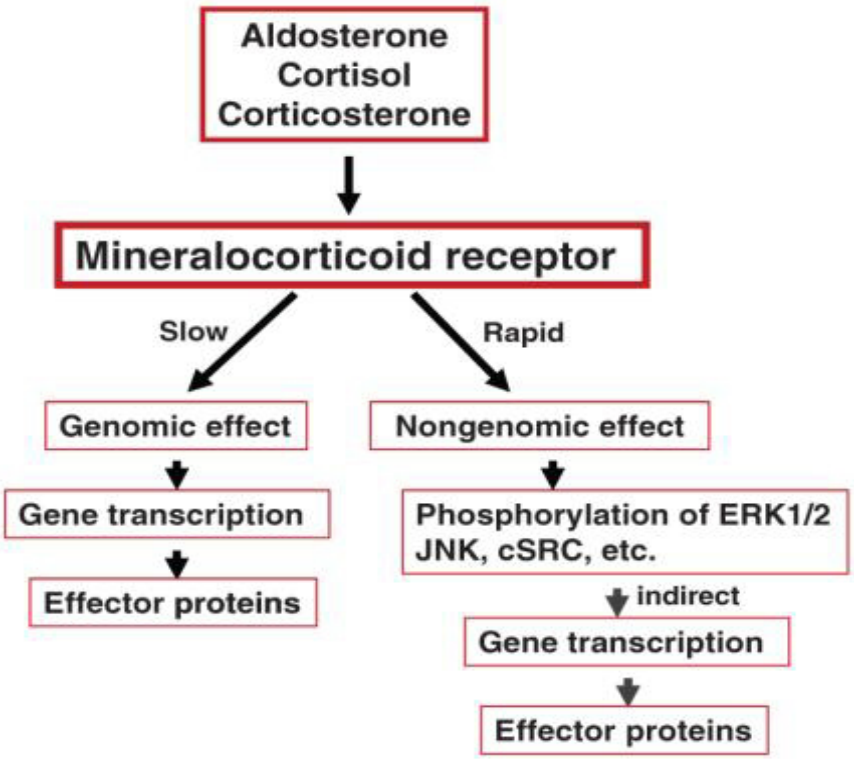

Figure 6 - Action of Corticosterone and similar steroid hormones, taken from Gomez-Sanchez and Gomez-Sanchez, (Gomez-Sanchez and Gomez-Sanchez, 2014)**.**

Aldosterone, Cortisol, and Corticosterone bind with similar affinities to the mineralocorticoid receptors. Binding can initiate both slow transcriptional effects and nongenomic effects that occur in rapid time. The glucocorticoid receptor acts similarly to mineralcorticoid receptors, activating both transcriptional (genomic) and nongenomic effects but respond to glucocorticoids only.

1.1.4.6 Corticosterone as a signalling molecule in the SCN

Glucocorticoid production is controlled by the hypothalamic-pituitary-adrenal (HPA) axis and has a distinct circadian rhythm, with peak production just before awakening and lowest at the end of the day (Gomez-Sanchez and Gomez-Sanchez, 2014). Both MR and GR are found in the hippocampus and for MR, the high

affinity binding site, corticosterone and aldosterone have the same binding affinity. Both MR and GR are important for the long-term adaptation to stress (Gomez-Sanchez and Gomez-Sanchez, 2014). Under normal conditions, glucocorticoids activate MRs in hippocampal neurons to mediate arousal and trophic effects dampened by high stress levels (Gomez-Sanchez and Gomez-Sanchez, 2014). Interestingly, it is speculated that despite low GR levels in the SCN, glucocorticoid timing messages reach clock cells by glucocorticoid sensitive projections to the SCN. GR and MR are implicated in the differential regulation of serotoninergic receptors within different parts of the hippocampus. At basal corticosterone levels, MR binding occurs, serotoninergic neurons are downregulated, and serotonin activity is suppressed within the raphe-hippocampal system. During stress, GR activates and increases hippocampal neuron responsiveness to serotonin 1A receptors (5-HT1A) and facilitates stress induced serotonin release (Gomez-Sanchez and Gomez-Sanchez, 2014).

Corticosterone is the main hormone released by rodents during stress through the HPA axis. Corticosterone is released spontaneously at different concentrations throughout the circadian cycle, revealing an additional role independent of homeostasis. In central neurons, corticosterone acts on membrane receptors and nuclear receptors, in different time scales and concentrations (Maggio and Segal, 2019). Intracellular MR has a high affinity to corticosterone and is highly expressed in the hippocampus and other areas in the brain. Intracellular GR on the other hand, possess a low affinity to corticosterone and are more widely distributed throughout the brain, expressed in both neurons and glial cells (Maggio and Segal, 2019). It was suggested that the fast response to stress is coordinated by intracellular GR after corticosterone levels pass a threshold, whereas intracellular MR rarely participates, as there is potential of inappropriate MR activation. It was identified that corticosterone release is important for circadian control located in the medial anterior hypothalamic region. Studies using selective antagonists demonstrate that MRs are crucial for response to repeated stress, through negative feedback (Gomez-Sanchez and Gomez-Sanchez, 2014). This reveals how corticosterone alters the circadian clock to orchestrate a response to predict stress. Ono *et al* revealed that corticosterone acts within the PVN, a region regulated by the SCN (Ono *et al.*, 2020). Corticotrophin-releasing factor (CRF) neurons in the hypothalamic paraventricular nucleus were implicated to mediate circadian rhythms in the SCN and regulate wakefulness. The PVN is important for tuning body temperature, feeding behaviour, and metabolism (Ono *et al.*, 2020). CRF neurons in the PVN regulate blood corticosterone concentration, which is a critical factor for regulating the peripheral clock and stress response. Thus, CRF neurons in the

PVN are implicated to receive circadian information from the SCN to transmit to other brain areas and/or other organs to regulate physiological functions (Ono *et al.*, 2020).

Restricted feeding of mice was found to alter the circadian regulation involved in energy storage and lowered corticosterone, thus showing corticosterone is influenced by non-photic environmental cues (Richards and Gumz, 2013). Diurnal corticosterone rhythms are linked with prefrontal cortex dysfunction, which regulates emotional and physiological response to stress (Woodruff *et al.*, 2016). Corticosterone was observed to entrain *Per1, Per2,* and *Bmal1* expression in the male rat prefrontal cortex and the SCN. In these experiments, Adrenalectomy altered the PFC rhythm, which was reversed by in-phase corticosterone treatment. Anti-phase corticosterone treatment eliminated *Per1* and *Bmal1* rhythms and dampened the Per2 rhythm. It was speculated corticosterone entrains the PFC by rapid induced *Per1* expression that was observed in various cell lines and tissues such as the PFC and by GR activation via daily pulses of corticosterone (Woodruff *et al.*, 2016).

Sage *et al* identified rats that lack of plasma corticosterone resynchronise to the internal clock in a shortened time, proving that corticosterone is partially involved in maintaining the internal circadian rhythm (Sage *et al.*, 2004). Corticosterone rhythm did not show any synchronising effect in constant darkness, revealing that corticosterone participates in photic entrainment. It was suggested the rhythmic expression of AVP is regulated by the same transcriptional machinery involved in clock cell regulation and circulating glucocorticoid levels in the SCN that alter locomotor activity (Sage *et al.*, 2004). Forced exercise during the inactive period entrains locomotor activity and peripheral circadian clock rhythm in mice (Sasaki *et al.*, 2016). This entrainment was found to be a form of stress response, depicted with increased levels of corticosterone and noradrenaline in peripheral tissues. Interestingly, peripheral clock entrainment by forced exercise was independent of the SCN, proving that stress can act as an alternative entrainment mechanism (Sasaki *et al.*, 2016). This is due to the possible inability to predict this onset of stress by daily rhythms. If exercise is performed at a set time in form of a routine however, this could be another circumstance corticosterone can be used to entrain the SCN to predict stress.

1.1.4.7 Melatonin

Melatonin is a versatile molecule with multiple functions throughout the body and has adapted as part of evolution (Boutin and Jockers, 2021). Melatonin is synthesised from tryptophan within the Pineal gland by 4 separate enzymes (Figure 7). The first is tryptophan-5-hydroxylase which converts the amino acid into 5-hydroxytryptophanthen converted into serotonin. Next, arylalkylamine-*N*-acetyltransferase (AANAT), the rate-limiting enzyme for melatonin synthesis, converts serotonin into *N*-acetylserotonin. *N*-acetylserotonin is then converted by acetylserotonin O-methyltransferase (ASMT) to form melatonin (Tordjman *et al.*, 2017; Amaral and Cipolla-Neto, 2018). Of note, the AANAT step of melatonin production is promoted heavily by noradrenaline via adenyl cyclase, indicating a possible use of melatonin as part as the stress response.

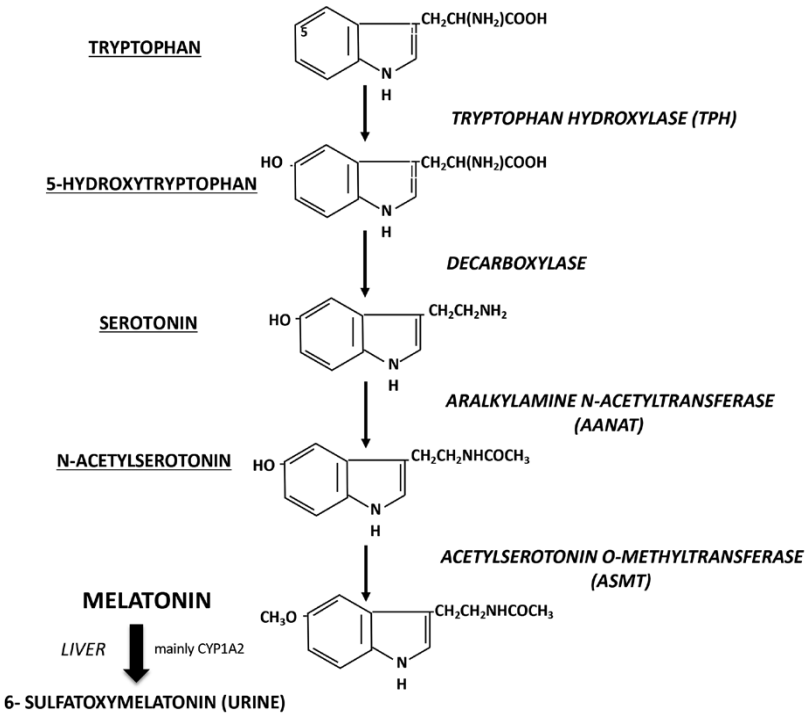

Figure 7 - The Melatonin synthesis pathway and metabolization by the liver. Figure taken from Amaral and Cipolla-Neto, (Amaral and Cipolla-Neto, 2018).

Melatonin synthesis begins with the amino acid tryptophan that, under the action of tryptophan hydroxylase, is transformed in 5-hydroxytryptophan then, is decarboxylated into serotonin. From there, arylalkylamine N-acetyltransferase (AANAT), the rate limiting step of melatonin synthesis converts serotonin to N-acetylserotonin (NAS) that is further converted to melatonin by acetylserotonin O-methyltransferase (ASMT) formerly called hydroxy-indole-O-methyltransferase (HIOMT).

The pineal gland is the predominant source of melatonin and contributes to the nocturnal peak of melatonin. It was discovered recently that there are other areas such as the retina that produce melatonin in a circadian manner (Boutin and Jockers, 2021). Components of the innate immune system such as lymphocytes and phagocytes also produce melatonin, which interestingly produce melatonin outside of the circadian rhythm. A previous misconception of melatonin was that it was produced in the cytoplasm. Reiter *et al.* speculated that the mitochondria could also be a site for melatonin synthesis, due to the ability of melatonin to alleviate ROS. The mitochondria have been shown to generate ROS as a by-product to metabolism and melatonin could be generated to protect the cell from ROS accumulation and damage (Boutin and Jockers, 2021). Alternatively, melatonin can transport into the mitochondria as melatonin can readily travel through biological membranes due to its amphiphilic properties. One consideration however is that the mitochondrial outer membrane is a double membrane which could slow melatonin transport.

Melatonin's best-characterized molecular targets are the G protein-coupled receptors MT1 and MT2 (Boutin and Jockers, 2021). In mammals, these receptors are expressed in the pars tuberalis, the hypothalamus and in the retina, but trace levels of its expression are found in other organs (Boutin and Jockers, 2021). Melatonin has been seen to bind to different molecules, not just MT receptors. Calreticulin, a multifunctional soluble protein for example has a similar affinity of 1nM as MT receptors, implicating melatonin has a potential role in signal transduction and genomic regulation. Melatonin also binds to enzymes NQO2 (found to be the binding site MT_3) and pepsin at higher concentrations (1 µM for NQO2 and 1mM for pepsin). Boutin and Jokers review also speculated that melatonin could target nuclear receptors as it can travel freely through biological membranes. The most proposed yet unconfirmed target is the retinoic acid receptor-related orphan receptor-β (RORβ) (Boutin and Jockers, 2021). Melatonin is speculated to bind to other receptors such as GLUT1, PEPT1 and 2 and mitochondrial permeability transition pores (mtPTP) which could explain the role for melatonin within the mitochondria as explained prior (Figure 8). Melatonin has been detected in bile and cells from the gastrointestinal (GI) tract, reaching 10-100 times the levels recorded in plasma (Boutin and Jockers, 2021). This evidence leads to the theory that melatonin is produced locally within the GI tract. It was later found that melatonin can be synthesised in enterochromaffin cells; melatonin being found to increase during the time of tryptophan intake (Tordjman *et al.*, 2017). It is also theorised that melatonin secretion by enterochromaffin cells could be an anti-inflammatory response as EC proliferation and increased ASMT expression occurs in acute phases of ulcerative proctitis and colitis (Chojnacki et al., 2013).

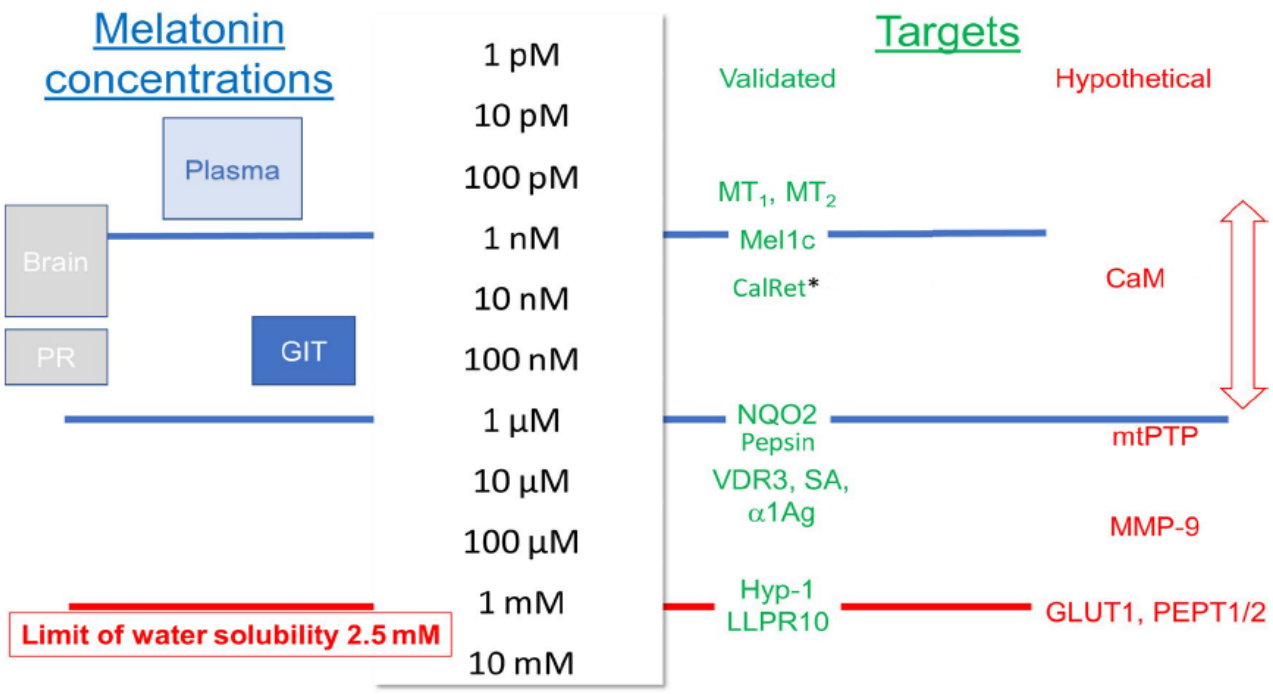

Figure 8 - The physiological concentrations of melatonin and melatonin target proteins, taken from Boutin and Jockers, (Boutin and Jockers, 2021).

Melatonin concentration thresholds are presented in a logarithmic scale from 10^{-12} to 10^{-2} M. The brain has been shown to be highly sensitive to melatonin which is needed to induce circadian activity. Higher concentrations are found in the pineal gland (PR) to evoke activity which may mean to regulate melatonin production. A similar concentration has been identified in the gastrointestinal tract (GIT). The validated targets (green) are target proteins that have been demonstrated to interact and bind to melatonin whereas the hypothesised (Red) targets have been speculated to bind to melatonin but there is no confirmed evidence of this currently. * The information for Calreticulin was obtained by binding 2-iodomelatonin onto the pure recombinant protein.

Abbreviations

CalRet; Calreticulin, CaM; Calmodulin, Mel1c; nonmammalian G protein-coupled melatonin receptor, MT1/ MT2; G protein-coupled melatonin receptor, mtPTP; mitochondrial permeability transition pore, NQO2; NRH: quinone oxidoreductase 2, SA; serum albumin, VDR3: Vitamin D receptor 3; PR; pineal recess, SA; serum albumin, GIT; gastrointestinal tract.

1.1.4.8 Melatonin as a signalling molecule in the SCN

Melatonin acts as the primary circadian pacemaker, acting as a synchroniser to attune the internal environment to the light-dark cycle (Mirick and Davis, 2008). Melatonin rhythm begins to set at around 3 months in typical development, and infants begin to have regular sleep–wake cycles with nighttime sleep lasting 6-8 h (Tordjman *et al.*, 2017). The relationship between the SCN and pineal gland is considered reciprocal. In mammals, melatonin acts as the biological signal for darkness as melatonin release from the pineal gland occurs during the length of night. Light has been shown as a powerful melatonin suppressor, as indicated with studies of nocturnal illumination or light-at-night (LAN) (Mirick and Davis, 2008). The

distinct secretion of melatonin implies the pineal gland may act as a coordinator of the circadian rhythm, adjusting the biological clock by melatonin increase at night (Barriga *et al.*, 2001). Tight regulation of AANAT, the rate limiting enzyme, is a major factor that generates the melatonin circadian rhythm (Boutin and Jockers, 2021). Melatonin is known as a chronobiotic agent, meaning melatonin activity can directly or indirectly affect the phase and period of the circadian clock (Carrillo-Vico *et al.*, 2005).

Melatonin has been known to relay the central circadian rhythm to the peripheral organs. The systemic variation of melatonin throughout the day conveys a message to inform organs of the circadian time and promotes the onset of sleep. In addition, retinal physiology is regulated as a result of melatonin (Boutin and Jockers, 2021). It was suggested that melatonin supplementation can increase total sleep time in individuals with sleep restriction or altered sleep schedules, possibly benefitting individuals that undergo shift work. It could also relieve daytime fatigue with jet lag, reduce time required to induce sleep in individuals with delayed sleep syndrome and can reset the body's sleep-wake cycle, showing further promise (Costello *et al.*, 2014). Melatonin's rhythm has been assessed to uncover whether static or dynamic lighting can alter the melatonin rhythm and function upon sleep and cognitive performance (Stefani *et al.*, 2021). Static light was found to attenuate the evening rise of melatonin, indicated by the significant reduction of melatonin AUC prior to bedtime under static light. Under dynamic lighting, volunteers felt less vigilant with shorter sleep latency while retaining sleep quality and cognition, showing that dynamic lighting promotes melatonin secretion and sleep initiation. Light has a significant impact on the circadian clock as electric LAN can delay the internal clock, risking desynchronization of circadian rhythms from the sleep wake rhythm (Stefani *et al.*, 2021). This desynchronization caused by increased LAN has been associated with negative wellbeing and reduced quality of sleep. Blue light, specifically within the wavelengths of 446-477nm attenuates melatonin secretion and sleepiness via activating intrinsically photoreceptive retinal ganglion cells (Stefani *et al.*, 2021). It is through this knowledge of light and melatonin modulating the circadian clock at day and night respectively, that both are essentially two sides of the same coin, both enforce and attune the clock but at opposite ends of the day-night cycle.

Melatonin is considered a useful biomarker to indicate circadian dysregulation. Dysregulated levels of melatonin are associated with nightshift work and found in studies that induce LAN exposure in both laboratory-based and field studies (Mirick and Davis, 2008). Melatonin is considered the most robust circadian marker as others are more easily influenced by external stimuli. Although, light has been shown as the most powerful synchroniser of the circadian clock and can completely suppress melatonin when exposed at night. Melatonin can be reliably measured directly and indirectly through its metabolites in

urine, blood and saliva (Mirick and Davis, 2008). Urinary melatonin is the most stable over time and has been used to study long latency cancers and used to indicate adaption to shift work which could lead to physiological and mental pathophysiology. Melatonin can influence circadian biology by modulating body temperature. Rhythms of body temperature are involved in sleep regulation and in sustaining mammalian peripheral clocks (Hardeland et al., 2012). Experiments in mice indicated that peripheral clocks are highly sensitive to small variations in temperature that are still within the physiological range. The neurons of the SCN however remain in phase with the light-dark cycle. In Diurnal animals, it has been found that Melatonin induces a reduction in core body temperature through an increased heat loss due to a selective increase in distal skin blood flow (Hardeland et al., 2012).

The daily rhythm of melatonin synthesis, as observed in rats, was found to be controlled by the central clock through a multisynaptic pathway; including PVN neurons, sympathetic preganglionic neurons of the intermediolateral cell column of the spinal cord and noradrenaline containing sympathetic neurons of the superior cervical ganglion (Perreau-Lenz *et al.*, 2005). The SCN utilises daytime inhibitory and night-time stimulatory signals by GABA and Glutamate respectively, such as the PVN-pineal pathway which modulates melatonin synthesis. Glutamatergic signalling within the PVN-pineal pathway was detected during the light period and dark period. However, during normal light conditions, this effect is overwhelmed by GABAergic inhibition, causing an overall decline in Glutamatergic signalling (Perreau-Lenz *et al.*, 2005). Activity of α and β adrenergic receptors by noradrenaline in the pineal gland also promotes melatonin synthesis, associating daily melatonin rhythms with sympathetic activation within the SCN. This sympathetic activity is a potent method of melatonin induction, causing an immediate 10-fold increase of melatonin by enhancing gene expression and enzymatic activity of AANAT (Perreau-Lenz *et al.*, 2005). It was also found that artificially extending α-adrenergic and β-adrenergic receptor activation can prolong melatonin levels even after light onset (Perreau-Lenz *et al.*, 2005). Perreau-Lenz *et al* investigated early morning decline in melatonin synthesis and suggested this decline might be due to increased GABA signalling and decreased Glutamate signalling on pre-autonomic PVN neurons. GABA inhibition also explains the arrest in sympathetic input via noradrenaline signalling at the end of night but noted GABA is not the sole causal factor. Further experiments utilising bicuculline; a GABAA receptor antagonist, did not prevent the melatonin morning decline. They concluded that additional factors other than GABA inhibitory and Glutamate stimulatory signals are involved in the circadian regulation of melatonin.

Despite the information uncovered from studies in rats, studies on melatonin and circadian clock have been found to be more complex in other mammals (Ebihara *et al.*, 1986). Most strains of inbred mice, including the more popularly used C57BL/6J, that are used in biomedical research are deficient in producing melatonin, with small levels detected in plasma and melatonin rhythms displaying either a low peak or are even absent (Zhang *et al.*, 2018; Kennaway, 2019). C57BL/6J mice exhibit a deficiency in endogenous melatonin rhythm but still possess functional melatonin receptors. Signal transduction pathways downstream of these receptors were also found to be intact in the SCN and the pineal gland in these mice (Pfeffer *et al.*, 2022). Loss of melatonin production is due mutations in the *Aanat* and *Asmt* genes that produces enzymes with little or no enzyme activity (Kennaway *et al.*, 2002; Zhang *et al.*, 2018). The presence of functional melatonin receptors means that C57BL/6J mice have retained the capacity to interpret and induce a response of exogenous melatonin (Zhang et al., 2018). For example, melatonin-deficient mice were found to display restored rhythms of dopamine release in the retina similar to melatonin proficient mice when injected with melatonin in extra ocular region (Zhang et al., 2018). Similarly, melatonin supplementation to C57BL/6 mice reduces nocturnal activity and alters sleep onset, which is similar to the behaviours of melatonin proficient strain (C3H/HeN) (Kim *et al.*, 2024). Additionally, creating a congenic strain from C57BL/6J and CBA/CaJ mice, melatonin production in the pineal gland was fully restored (Zhang et al., 2018). Finally, it was identified chronic melatonin treatment improved long-term recognition via binding to MT2 receptors (Pistono *et al.*, 2021).

1.1.5 Circadian clock, Health and disease

The circadian clock has been proven essential for survival and well-being. Desynchrony between the organism and the external environment such as suffering with jetlag and shift work has been associated with health complaints like impaired cognitive function with altered hormone levels and function (Vitaterna, Takahashi and Turek, 2001). Absence of the light-dark cycle has previously been shown to induce chronic sleep deprivation, impairing cognition and mental state. Night shift workers have been found to be vulnerable to mental conditions. Using artificial lighting system has been theorised to actively entrain the circadian clock, which could alleviate implications to prolonged night shift work and help increase performance and cognition during night shifts (Crepeau *et al.*, 2006). As the human circadian system responds to short wavelength light in the blue light spectrum, Crepeau *et al* speculated that yellow light could induce “circadian darkness” to prevent circadian disruption (Crepeau et al., 2006; Brainard et al., 2008). This theory was also considered for submariners to actively re-entrain to shift schedules.

Evidence that immune parameters change with the time of day links the circadian clock to health and disease, with circadian rhythm disruption being linked with inflammatory pathology (Curtis et al., 2014). The circadian clock-controlled immune system allows an organism to anticipate risk of infection. Daily changes in activity such as feeding, opens the body to the risk of infection so the ability to predict infection will optimise the immune system. Studies highlight the extent the molecular clock, most notably core clock proteins BMAL1, CLOCK, and REV-ERBα, influence the immune response. Examples include BMAL1: CLOCK heterodimer regulating toll-like receptor 9 and inflammatory monocyte chemokine ligand (CCL2) and REV-ERBα suppressing the induction of interleukin-6 (Curtis et al., 2014).

Circulating circadian messengers such as Glucocorticoids, catecholamines, prolactin and melatonin, influence the immune system, attuning different immune components in phase with each other (Curtis et al., 2014). There are records depicting temporal increases in chemoattractants, leukocyte trafficking, proinflammatory cytokines and phagocytic activity preceding wake and activity. This has been speculated to be a clock-controlled mechanism, to increase immune sensitivity and immunosurveillance ahead of activity to predict the risk of infection (Curtis et al., 2014). In contrast, constant darkness conditions or jet lag impaired clock can affect immune function adversely. Under jet lag conditions, BMAL1 levels suffer sustained reduction which suppresses anti-inflammatory properties (Curtis et al., 2014). Mice exposed to LPS during jet lag show a more severe inflammatory response, like macrophages at ZT12. Both show BMAL1 as a central gatekeeper that modulates the circadian inflammatory response.

BMAL1 binds onto chemokine ligand 2 and 8, which mark histones to repress transcription. In one study, BMAL1 attenuates ly6C_{hi} monocyte numbers and exacerbates metabolic disease caused by myeloid deletion of *Bmal1*, leading to inflammation. This information supports that BMAL1 acts an anti-inflammatory molecule in monocytes, and a similar response was identified in macrophages (Curtis et al., 2014). Previous studies reveal BMAL1 binding is reduced during the activity phase, therefore when animals transition to activity, it would promote a more robust inflammatory response, with improved pathogen clearance. PER2 is implied to promote inflammation as it inhibits BMAL1: CLOCK activity as the animal enters the active phase, as found with peak mRNA levels in macrophages at that time (Curtis et al., 2014). PER2 also inhibits REV-ERBα when in abundance which induces inflammation. CRY1 + CRY2 have similar effects as an absence within fibroblasts leads to increased expression of proinflammatory cytokines such as TNFα and IL-6 (Curtis et al., 2014). CRY1 binds to Adenyl cyclase to limit cAMP production, therefore a shortage of CRY1 results in increased cAMP activity, which elevates PKA activity and phosphorylation of p65 and NF-κB activation.

It was previously stated BMAL1 can attenuate the CLOCK ability to enhance NF-κB related transcriptional expression. This is theorised to be one method BMAL1 limits inflammation because NF-κB influences multiple genes that encode components of the immune system (Curtis et al., 2014). However, further evidence analysing CLOCK knockouts for specific immune compartments could elucidate the full function of CLOCK in the immune system. REV-ERBα and RORα have also been seen to affect immunity. A REV-ERBα agonist limits IL-6 releases in macrophages as well as *ccl2* and *IL-19* mRNA expression (Curtis et al., 2014). As BMAL1 induces *Rev-erbα* transcription, it was suggested the anti-inflammatory effects of BMAL1 are caused by direct influence on REV-ERBα, as supported by evidence that REV-ERBα represses ccl2. However, BMAL1 and REV-ERBα bind to different sequences to suppress C*cl2*, binding to the E-box element and the RORE sequence respectively. This has led to the notion that BMAL1 and REV-ERBα work together through the circadian cycle to suppress *Ccl2* transcription during inflammation (Curtis et al., 2014). BMAL1 also influences the expression of RORα, which in turn upregulates IκBα and downregulates NF-κB.

CLOCK also has been shown to impact immune signals (Curtis et al., 2014). Unlike its binding partner BMAL1, C*lock* mRNA is not altered throughout the day in macrophages. However, histone acetyl transferase activity of CLOCK was found to be essential in circadian gene expression (Curtis et al., 2014). CLOCK also acetylates the glucocorticoid receptors, suppressing Glucocorticoid receptor binding and activity. CLOCK also enhances transcriptional activity by phosphorylating and acetylating NF-κB subunit p65. Interestingly, *Bmal1* attenuates this effect of CLOCK on NF-κB by sequestering CLOCK, revealing BMAL1 can both limit and promote expression (Curtis et al., 2014). SCN activity towards the PVN and the ARC and their activity in turn induce the immune response (Curtis et al., 2014). Noradrenergic signalling from the PVN for example acts upon the spleen and the noradrenaline released from the PVN mediates the activity of natural killer cells. Noradrenaline also enhances neutrophil recruitment to skeletal tissues by inducing the expression of ICAM-1. Glucocorticoids are also under circadian control, with a rhythmic secretion of glucocorticoids driven by the SCN. This is achieved through the SCN controlled ACTH release from the pituitary gland which triggers the release of glucocorticoids from the adrenal gland (Curtis et al., 2014). The adrenal clock, also influenced by the SCN, dictates the sensitivity of ACTH on the adrenal gland, showing two methods of the SCN influencing glucocorticoid release. In terms of function, Glucocorticoids exudes broad anti-inflammatory effects and influence cytokine production and leukocyte trafficking. Overall, the circadian system is predicted to induce the immune system into two states of activity, one of heightened activity and increased immune cell sensitivity to anticipate infection and the second during

the resting phase, suspected to provide an opportunity for tissue repair and inflammation to be resolved (Curtis et al., 2014).

Inflammatory diseases such as asthma and rheumatoid arthritis (RA) are influenced by the circadian clock and in turn the disease onset influences the circadian clock. Proinflammatory cytokines TNF-α and IL-6 for example, peak at 3am and 6am respectively in healthy humans, but patients of RA show dysregulated rhythms of these cytokines, with peaks levels observed later into waking (Curtis et al., 2014). These irregularities induce the stiffness and pain observed in RA patients in the early hours of the morning, suggesting that perturbed clockwork could be the cause of inflammatory conditions. Disruption of light cues that influence the circadian and molecular clock such as those undergoing shift work, have been found to influence the inflammatory response and increases the susceptibility to numerous metabolic diseases with an underlying inflammatory factor. Immune components in mice have been found to be enforced under a circadian rhythm (Curtis et al., 2014). Leukocyte levels for example have a strong variability throughout the light-dark cycle, reaching a peak at ZT5 and highest recruited at tissues at ZT13. In addition, circadian variation has been found in mouse macrophages and their ability to ingest particles, showing a pre-emptive increase in ability prior to the transition of activity (ZT12) in murine models (Curtis et al., 2014). Bacterial clearance is also enhanced during this timeframe, as indicated in mice when infected with LPS, which showed the induction of proinflammatory cytokines (IL6 and IL12) and chemokine ligands (CCL2 and CCL5) being greater at ZT12 than at ZT0 (Curtis et al., 2014). These mechanisms are part of the TLR4 pathway and thus indicate that these mechanisms are under tight circadian control. The information also anticipates that the circadian clock prepares the immune system for an integrated response as a pre-emptive measure to times to which risk of infection is greater.

In summary, circadian biology has a heavy influence on health and wellbeing. The above information has noted various immune components that are affected by circadian influence and dysregulation of the clock has been implicated in numerous immune related pathology and disease. This thesis addresses this notion, taking interest in a specific disease of ischaemia reperfusion injury. The next section will discuss Ischaemia reperfusion injury and divulge the possibility of circadian influence on the onset of pathology.

1.2 Section 1.2 Ischaemia Reperfusion Injury

1.2.1 Ischaemia Reperfusion Injury, an overview

Ischemia-reperfusion injury (IRI) is a pathophysiological term to describe structural and functional tissue damage, accompanied by blood flow restoration (reperfusion) after a period of ischemia (Soares et al., 2019). Ischemia-reperfusion injury is a multiple step multifactor inflammatory condition that include; hypoxia, metabolic stress, leukocyte extravasation, cellular death pathways, and activation of the immune response (Fernández *et al.*, 2020). Ischemia-reperfusion injury is commonly associated with multiple conditions such as myocardial infarction, stroke and organ transplantation (Peng *et al.*, 2012). For organ transplantation in particular, ischaemia-reperfusion is an inevitable condition and thus impacts both short term and long-term graft survival. Although all parts of the body are susceptible to IRI, this research will focus on the kidney. Renal ischaemia-reperfusion injury is characterised by inflammatory tubular damage which leads to acute kidney injury (Poluzzi *et al.*, 2019). Ischemia-reperfusion injury is a frequent causal factor of acute kidney injury (AKI), which contributes to high morbidity and mortality rates in various injuries (Malek and Nematbakhsh, 2015; Burek *et al.*, 2020). Acute kidney injury is characterized by a sudden decline in kidney function, increased systemic inflammation, oxidative stress, and dysregulation of sodium, potassium, and water channels. In renal transplants, IRI causes delayed graft function (Fernández *et al.*, 2020). The renal microvasculature is serially organised, with blood flow from efferent arterioles of the juxtamedullary glomeruli and moves to the descending vasa recta (Legrand *et al.*, 2008).

Renal ischemia-reperfusion injury not only can cause localized damage to renal tissue but further systemic damage to other organs. One consequence of renal IRI is the accumulation of uremic toxins in the blood, due to the decreased glomerular filtration rate (GFR) by decreased glomerular perfusion and tubular obstruction (Burek *et al.*, 2020). Burke *et al* also describes that renal IRI induces changes in drug transporter expression. The study identified that glucose transporters experience an increased expression in stressed cells and cranial capillaries (Burek *et al.*, 2020). This is speculated to respond to IRI factors as a precaution to maintain the metabolic needs of the brain.

1.2.2 Ischaemia and metabolic stress

The initial step of ischaemia reperfusion injury is hypoxia (Fernández *et al.*, 2020). Low oxygen levels caused by hypoxia induce a switch to anaerobic cellular metabolism after ATP levels are depleted. Oxygen depletion affects mitochondrial coupling directly during ischaemia by inhibiting electron transport in the mitochondrial respiratory chain, leading to a decline in ATP levels (Malek and Nematbakhsh, 2015). This

impaired ATP synthesis reduces calcium pumping out of the cell by Na^+/Ca^{2+} antiporter channels, therefore leading to intracellular calcium overload in the cell. The impaired calcium excretion and calcium overload activates NADPH oxidase generating reactive oxygen species (ROS) (Fernández *et al.*, 2020). ROS generates superoxides by driving the reverse action of the electron transport chain complex 1 due to succinate accumulation, a consequence of reduced ATP (Fernández *et al.*, 2020). During IRI, excessive ROS produced by damaged tissue causes oxidative stress, mitochondrial oxidative phosphorylation, ATP depletion, increased intracellular calcium and activation of membrane phospholipids proteases (Malek and Nematbakhsh, 2015). Depleted ATP levels cause a failure in the sodium potassium ATP pump, Ca^{2+} accumulation and membrane depolarization, thus inducing vasoconstriction in endothelial cells. Increase in intracellular Ca^{2+} relates to the increased influx into the mitochondria, which causes an accumulation of AMP, ADP and phosphate from the ATP lysis and a rise to irregular permeability of the internal mitochondrial membrane (Soares et al., 2019).

Calcium accumulation also occurs by redistributing calcium within the endoplasmic reticulum stores (Malek and Nematbakhsh, 2015). This increase in cytosolic calcium levels induces the activation of calcium-dependent phospholipase A2, as well as endonucleases and proteases within the cell that in turn induce cellular apoptosis (Malek and Nematbakhsh, 2015). Ischaemia reperfusion injury induces lactate accumulation, which decreases the intracellular pH and thus causes conformational changes in proteins as well as the activation of proteases, lipases, phospholipases and ATPases (Soares et al., 2019). Reactive oxygen species are also responsible for lipid peroxidation and calcium accumulation inside the mitochondria, causing the release of cytochrome C and membrane instability. The metabolic stress caused by the accumulation of ROS and calcium within the cell leads to activation of cellular injury pathways and cellular death (Fernández *et al.*, 2020).

During normal physiological conditions, the respiratory chain creates a small amount of ROS, which is removed by potent antioxidant mechanisms. In pathophysiological conditions however, the enhanced ROS production creates an imbalance between the two mechanisms resulting in oxidative stress (Soares et al., 2019). Impaired mitochondria function leads to the metabolic demand exceeding ATP production and ATP consequently falls to critical levels (Slegtenhorst *et al.*, 2014). Reactive oxygen species oxidise proteins, lipids, and DNA and damage the cell cytoskeleton. Cells have evolved several defence mechanisms to cope with oxidative damage, which autophagy plays an important role (Banaei, Rezagholizadeh and Azimian, 2019). In renal IRI, severity of the injury depends heavily on the extent of ischaemia, meaning autophagy can be protective or detrimental to the kidney, depending on the damage

(Poluzzi *et al.*, 2019). Autophagy acts as a modulator to protect mitochondria, which is responsible for cellular recycling to protect the cell from oxidative damage. Autophagy utilises the Beclin-1/PI3K, AMPK/mTOR and PI3K/Akt/mTOR pathways to break down and remove damaged proteins and organelles. Chaperone mediated autophagy and mitochondrial degradation are suggested to reduce oxidative injury caused by mitochondria dysfunction (Banaei, Rezagholizadeh and Azimian, 2019). Heat shock proteins HSP25 and HSP27 act as molecular chaperones to enhance tolerance to oxidative stress and are potentially also involved in anti-apoptotic mechanisms. Reperfusion can contribute to autophagy and cause excessive degradation of essential components leading to autophagic cell death (Soares et al., 2019). During reperfusion, oxygen is reintroduced to the mitochondria and complexes 3 and 4 resume pumping protons, which promotes further dysregulation of ROS modulation.

1.2.3 Cellular death pathways and apoptosis

Ischaemia and reperfusion induced cell death can either be regulated by apoptosis or non-regulated by necrosis (Fernández *et al.*, 2020). In addition, newly discovered pathways such as necroptosis, which is a speculated form of regulated necrosis and ferroptosis, an iron-dependent apoptosis that results in lipid peroxidation are also involved in IRI. In apoptosis, caspases mediate both intrinsic and extrinsic pathways. Extrinsic pathways are dependent on extracellular molecules such as proinflammatory cytokines like TNFα, which bind to death receptors within the cell membrane (Fernández *et al.*, 2020). Activation of death receptors triggers a conformational change of the death-induced signalling complex (DISC) which coordinates the conversion of procaspases into caspases. Caspases 3 and 8 were implicated in IRI as animal studies targeting these caspases resulted in resistance and even the prevention of apoptosis (Fernández *et al.*, 2020). P53 has also been analysed due to its role in facilitating the apoptotic response to hypoxia and oxidative stress. P53 contributes to the progression of IRI, and animal studies indicate that p53 inhibition reduces apoptosis of the proximal tubule epithelial cells of the kidney. Ferroptosis is an iron and ROS dependent cell death that targets the mitochondria, resulting in decreased mitochondria cristae, Condensed mitochondrial membrane and a ruptured outer membrane (Mou *et al.*, 2019). Oxidative stress and lipid peroxidation lead to loss of plasma membrane permeability and inevitably cell death. Cell death of renal cells occurs by ferroptosis, contributing to synchronised tubular necrosis and immune cell extravasation (Fernández *et al.*, 2020).

Cell death invokes the innate immune system by the intracellular communication of proinflammatory molecules. Damaged cells release danger signals such as danger- associated molecular patterns (DAMPs) and DNA into the extracellular environment (Fernández *et al.*, 2020). These danger signals are recognised

by pathogen-associated molecular pattern (PAMPs) that are expressed by the innate immune cells. DAMP signals are sensed by endothelial cells and local macrophages by toll-like receptors (TLR) which have been implicated in IRI. *TLR4* expression for example was associated with tissue damage and seen to act as a sentinel of acute damage, therefore TLR4 deficiency could be protective against IRI (Fernández *et al.*, 2020). TLR4 activation releases NF-κB which induces the transcription of multiple inflammatory genes. One such example is miR-146, a regulator of the innate immune system. It was proposed that miR-146 is transcribed during inflammation, acting as a negative regulator to repress IRAK1, a direct precursor to NF-κB (Fernández *et al.*, 2020). It was found in kidney transplant recipients with decreased miR-146 expression was found to lack protection from reperfusion.

1.2.4 Ischaemia reperfusion injury and complement activation

The next stage of IRI is activation of the complement system, where all three complement pathways are implicated (Fernández *et al.*, 2020). The pathways converge with the cleaving of C3 and C5, proteins that act as powerful inflammatory molecules, which causes the formation of the membrane attack complex and promotes cell injury (Slegtenhorst *et al.*, 2014). It was described that the organ itself affects the role of complement activation in IRI (Fernández *et al.*, 2020). C3 has been found to be a critical component of the complement cascade, which is responsible for binding to danger signals released locally from ischaemic or apoptotic cells (Fernández *et al.*, 2020). Localised C3 in proximal tubule epithelial cells are critical in mediating renal cell injury. Multiple studies have implicated the activation of the complement system in various Ischaemia reperfusion organs. C5a and C5b-9 have been shown to promote the expression of endothelial cell selectins and intercellular adhesion molecule-1 (Malek and Nematbakhsh, 2015).

The complement system consists of a set of effector molecules that have various functions. These include C3b that mediates pathogen opsonization, C3a and C5a that induce local inflammation and cell activation and the membrane attack complex, formed from C5b-C9 that mediates the direct lysis of pathogens and tissue damage (Peng *et al.*, 2012). The complement system is triggered in response to renal IRI, with the small proinflammatory fragments C3a and C5a being shown to contribute to IRI pathogenesis (Peng *et al.*, 2012). C3a and C5a receptor expression was found to be elevated after prolonged cold ischaemia. Deficiency in either or both of C3aR and C5aR protected mice from renal IR injury, with reduced immune cell infiltration as well as lower levels of kidney injury molecule-1 (Kim-1), proinflammatory mediators and adhesion molecules in post ischaemic kidneys (Peng *et al.*, 2012). Absence of these receptors on circulating leukocytes and renal tubular epithelial cells were also demonstrated to attenuate IRI. The data

from Peng *et al* reports the presence of C3aR or C5aR is necessary for renal cell and macrophage production of proinflammatory mediators after renal ischemic insult, particularly observed within the first 24 hours after reperfusion (Peng *et al.*, 2012). In this study, C3a and C5a induced a dose dependent increase of TNF-α, IL-6 and chemokine Receptor CXCR2 Ligand KC in macrophages. C3a/C5a activity also increases proinflammatory cytokine and chemokine production and Kim-1 expression by renal proximal tubular epithelial cells, cells located in the corticomedullary zone that are susceptible to IRI. Kim-1 acts as a marker of renal injury and as an endogenous ligand for leukocyte mono-immunoglobulin-like receptor 5. This is expressed by neutrophils and other myeloid cells and contributes to local inflammation via cell migration and chemokines production in renal IRI (Peng *et al.*, 2012).

1.2.5 Reperfusion and activation of the immune system

The preconceived idea of recovering blood flow after ischaemia is beneficial. However, reperfusion can result in harmful effects such as irreversible cell damage and necrosis, cell swelling and irregular/partial flow restoration (Soares et al., 2019). Reperfusion begins with the mobilisation of innate immune cells such as neutrophils, CD4+ T lymphocytes and circulating platelets into the vascular vicinity in response to damage-associated molecule patterns (DAMPs), giving rise to oxidative and microcirculatory stress thus leading to inflammation and cell death via apoptosis (Figure 9) (Slegtenhorst *et al.*, 2014; Soares *et al.*, 2019). Reperfusion after renal transplantation specifically initiates inflammatory pathways triggering the release of TNF-α, various interleukins, prostaglandins, and adhesion molecules (Banaei, Rezagholizadeh and Azimian, 2019). Ischaemia reperfusion injury is associated with a robust inflammatory response and elevated oxidative stress after hypoxia and reperfusion, the combination leading to organ dysfunction (Malek and Nematbakhsh, 2015). The inflammatory response in reperfusion is considered a double-edged sword, as it not only exacerbates immune injury as found with aggravating secondary brain injury in the acute stage of a stroke but also contributes to brain recovery after stroke (Soares et al., 2019).

Reoxygenation also increases the amount of oxygen free radicals in endothelial, parenchymal and lymphocytic cells that infiltrate the damaged cells. Superoxide anions are produced due to incomplete oxygen reduction by damaged mitochondria and phagocytic activity of neutrophils, parenchymal and endothelial cells (Soares et al., 2019). These processes cause the accumulation of free radicals, unstable molecules that destabilise organic and inorganic chemicals which lead to cell injury (Soares et al., 2019). Free radicals induce Lipid peroxidation and oxidative damage to proteins and DNA, which promotes apoptotic signalling and consequentially cell death (Malek and Nematbakhsh, 2015). In addition, compensatory enzymes of oxidative damage such as catalase superoxide dismutase and glutathione

peroxidase are found to be downregulated which is implicated in ischaemia-reperfusion injury pathophysiology (Malek and Nematbakhsh, 2015). Free radical accumulation has become a target to alleviate IRI as free radical scavengers and antioxidant activity were found to have protective effects in IRI progression, by reducing oxidative stress (Malek and Nematbakhsh, 2015). Renal damage during reperfusion by oxygen free radicals was found to be prevented by the antioxidant and free radical scavenger activity of melatonin (Malek and Nematbakhsh, 2015). In addition, melatonin inhibits sympathetic nerve activity and decreases catecholamine release, which was also speculated to protect the kidney against renal IRI. The increase in circulating ROS and pro-inflammatory factors induces oxidative stress and promotes the expression of endothelial adhesion molecules in distant organs respectively (Soares et al., 2019). An imbalance between endothelin-1 and nitric oxide (NO) production forms as levels decline, leading to expression of adhesion molecules and vasoconstriction. Consequently,

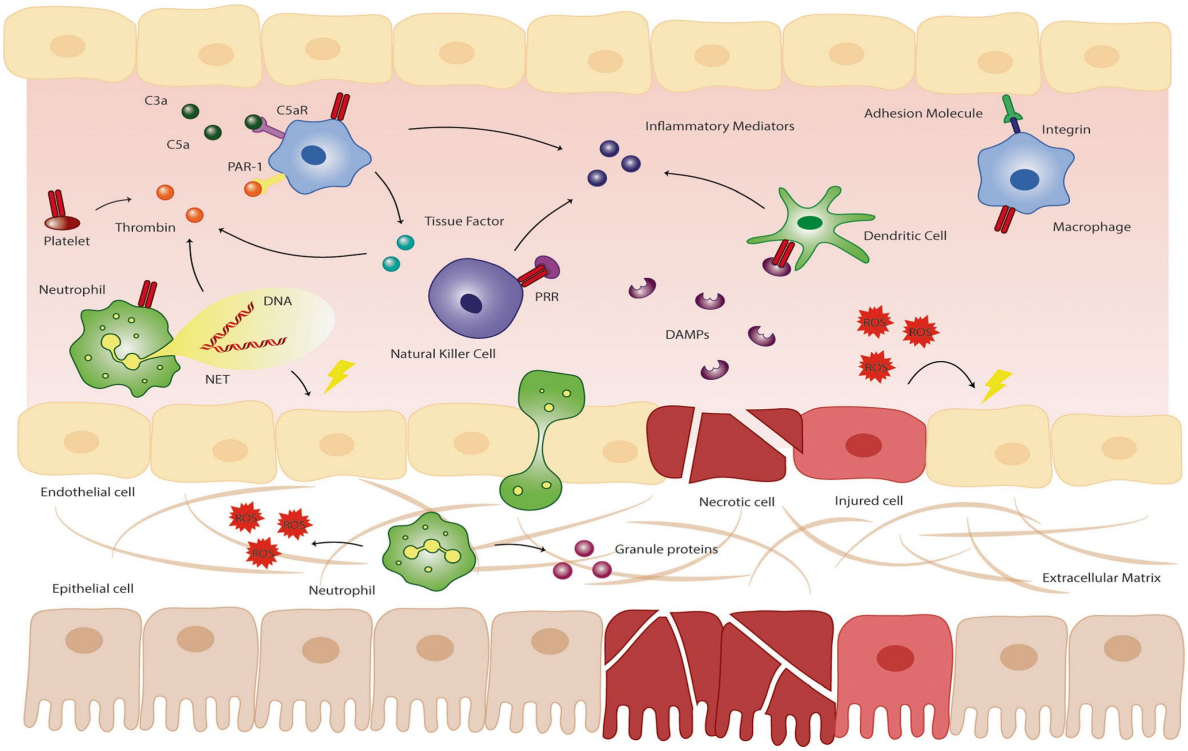

Figure 9 - The detrimental effects of reperfusion in the onset of Ischaemia-Reperfusion Injury, taken from Slegtenhorst *et al.*, (Slegtenhorst et al., 2014).

Damage-associated molecular patterns (DAMPs) are released by injured/necrotic cells and produces inflammatory cytokine and chemokine activating pattern recognition receptors (PRRs) expressed on endothelial, epithelial and innate immune cells. Injured cells produce reactive oxygen species (ROS) that contribute to further damage to local cells. Endothelial cells, once activated, promotes leukocyte adhesion and transmigration. Neutrophils release granular proteins as part of the respiratory burst response, and release neutrophil extracellular traps (NETs) that are pro-thrombotic and cytotoxic. Macrophages and neutrophils both release microvesicles containing thrombin and platelets. Thrombin in turn activates innate immune cells through ligating proteinase-activated receptor 1 (PAR-1), triggering inflammatory cytokine and chemokine production. The complement system is also activated, establishing a chemotactic gradient.

platelets and neutrophils become trapped in the local microvascular structure, promoting ischaemia and aggravate necrosis. The endoplasmic reticulum senses oxidative stress maintains calcium homeostasis and responds to IRI with apoptosis (Soares et al., 2019).

1.2.6 Danger signals and Leukocyte adhesion

In response to DAMPs, innate immune cells coordinate responses such as renal injury and fibrosis. Biglycan is one such danger signal, thought to promote macrophage recruitment in IRI (Poluzzi *et al.*, 2019). Biglycan signalling through Toll Like Receptors 2/4 with CD14 as a co-receptor with macrophages has been observed to regulate inflammation, producing cytokines, chemokines and immune cell recruitment. Toll-Like receptors (TLRs) are pattern recognition receptors like Interleukin-1 receptor, that in response to DAMPs, induces NF-κB, cytokine production, and pro-inflammatory signalling (Figure 10) (Fernández *et al.*, 2020). TLR signalling could be pro and anti-inflammatory, depending upon the co-receptor involved in the signal (Poluzzi *et al.*, 2019). TLR4-CD44 interaction for example was demonstrated to promote macrophage autophagy. CD44 has been implicated in immune cell adhesion and migration, lymphocyte activation and angiogenesis therefore CD44 can be a key signal for the onset of IRI.

The presence of proinflammatory cytokines and chemokines stimulates E-selectin, P-selectin and ICAM-1 on endothelial cells, which promotes leukocyte recruitment and extravasation into the renal interstitium (Soares et al., 2019). The imbalance between ROS production and the antioxidant mechanism is represented by the increase in renal malondialdehyde (MDA) levels and decline in superoxide dismutase and catalase activity (Soares et al., 2019). Studies reveal the beneficial effect of upregulating the glutathione system by administering oxidants or free radical scavengers, showing a reduction of MDA levels in the kidney. Therefore, glutathione depletion can be utilized as a marker of oxidant-induced tissue damage, as seen in experimental models that investigate renal IRI (Ye *et al.*, 2022). Although high concentrations of ROS are implicated in tissue injury during reperfusion after prolonged ischaemia, moderate ROS was shown to aid in ischaemic preconditioning (Soares et al., 2019). Hydrogen sulphide was also observed to attenuate lipid peroxidation and inflammatory events of reperfusion by reducing MDA, NF-κB and ICAM-1 levels. Damage to the actin cytoskeleton is also implicated in the development of AKI as losing the cytoskeleton leads to renal tubular and endothelial apoptosis. Pre-treatment of platelet activating factor antagonists also has been found to reduce renal injury after IRI (Soares et al., 2019).

Multiple molecules are implicated in leukocyte, endothelial cell and platelet communication that induce innate immune response (Fernández *et al.*, 2020). Endothelial cells after IRI express signal molecules such as p-selectin and ICAM1 to facilitate leukocyte adhesion and transmigration into tissues (Fernández *et al.*, 2020). These leukocytes release cytokines and proteases that increase vascular permeability, induce thrombosis and activate cell death. Endothelial cells also secrete platelet-derived growth factor, which promote vasoconstriction to reduce tissue oedema. This vasoconstriction is exacerbated by increased sensitivity caused by increased intracellular calcium and decreased NO due to reduced expression of endothelial nitric oxide synthase. Platelet-activating factor is involved in the acute inflammatory response and has been shown to cause chronic allograft nephropathy and kidney graft rejection in murine models. The combined results prevent microcirculatory reflow of blood which facilitates further tissue damage. C-reactive protein (CRP) is another inflammatory signal and acute phase protein that circulates in the plasma (Fernández *et al.*, 2020). CRP levels play a key role in leukocyte extravasation and tissue damage during renal IRI. Multiple transmembrane proteins establish the paracellular permeability of endothelial cells; such as claudins, junctional adhesion molecules, and VE-cadherin (Burek *et al.*, 2020). Burek *et al*. identified that both *In vivo* and *in vitro* models showed increased expression of efflux pumps such as Abcb1b, Abcc1, and Abcg2, as well as Glut-1 and sodium transporter expression after IRI (Burek *et al.*, 2020).

During reperfusion, Neutrophils stimulate the production of ROS, interleukins, TNF-α and local inflammatory mediators, aggravating tissue damage (Soares et al., 2019). After ischaemia-reperfusion, polymorphonuclear neutrophils exacerbate tissue damage via the release of oxygen radicals, proinflammatory cytokines and cytolytic enzymes and by obstructing the local vessels (Soares et al., 2019). CD4+ T Lymphocytes produce interferon gamma, TNF-β and macrophage stimulating factors which when active releases cytokines and amplifies the activity of local macrophages (Soares et al., 2019). In addition, Ischaemia reperfusion injury stimulates local microvascular responses such as; enhanced oxidative stress, increase Platelet-Leukocyte-Endothelial cell interactions in the cerebral microvasculature and enhanced thrombus formation within cerebral blood vessels, revealing the systemic spread of injury after localized IRI. Macrophages are also key immune cells involved in IRI progression and in IRI repair, depending on the macrophage phenotype (M1 and M2) and the stage of macrophage activation (Poluzzi *et al.*, 2019).It has been established that classically activated M1 phenotype is associated with renal tissue injury, inflammation and subsequent fibrosis whereas M2 mediates kidney repair and regeneration (Poluzzi *et al.*, 2019). In renal IRI, CD14 is a crucial signal for biglycan induced macrophage polarization into the M1 phenotype (Poluzzi *et al.*, 2019). CD44/TLR4 dependent M1 macrophage autophagy also leads to M2

macrophage polarization, triggering repair in later stages. Poluzzi *et al* further hypothesised that imbalance of these 2 pathways promotes disease progression, highlighting how longer ischaemia increases the likelihood M1/M2 and therefore greater damage (Poluzzi *et al.*, 2019). This information reveals biglycan has a narrow window of therapeutic benefit as determined by the extent of ischaemia so may not be a useful therapeutic agent.

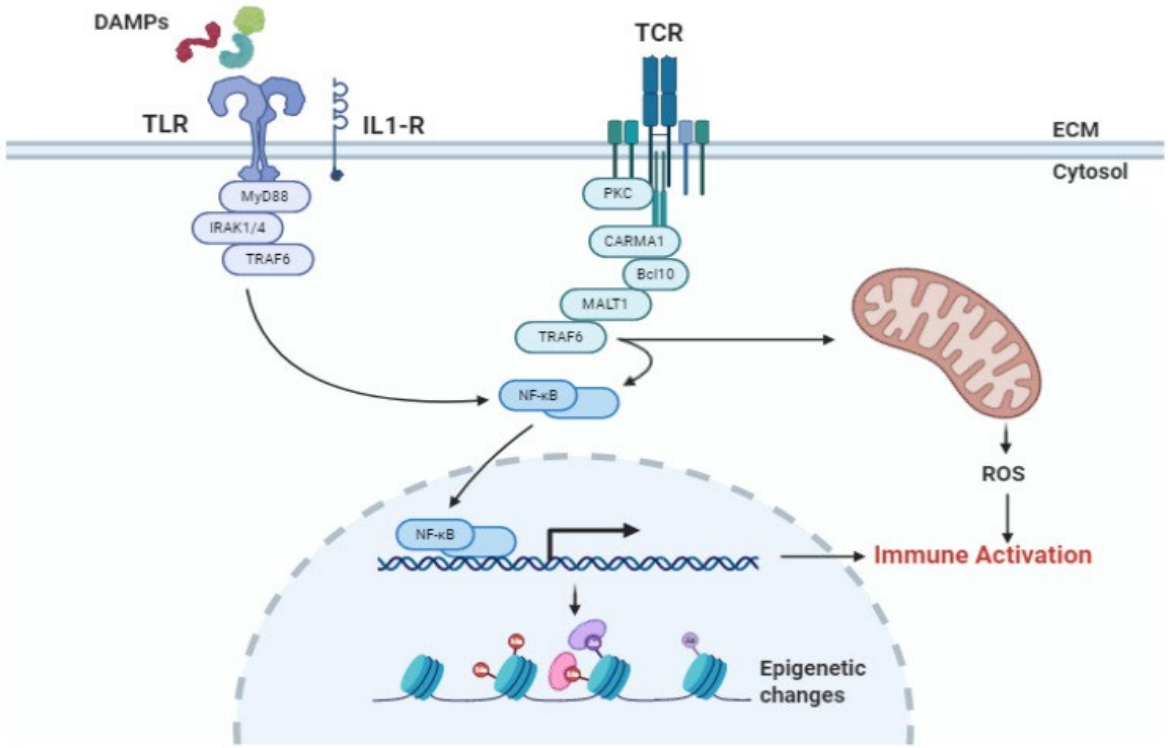

Figure 10 - Signalling pathways identified in ischemia-reperfusion injury (IRI), figure taken from Fernandez *et al*., (Fernández et al., 2020).

Damage-associated molecular patterns (DAMPs) are released by injured and necrotic cells during IRI and are recognized by pattern recognition receptors (PRRs), such as Toll-like receptors (TLR), and Interleukin-1 receptor (IL1-R). This induces nuclear factor-κB (NF-κB), cytokine production, and pro-inflammatory signalling. T cells recognize specific antigens during IRI through T cell receptors (TCR). This activates NF-κB and initiates an immune response without the need of antigen-presenting cells. DAMP or Antigens recognition triggers Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH) oxidase activity in the mitochondria, which leads to reactive oxygen species (ROS) production.

Mineralocorticoid receptor activation also influences the onset of Renal IRI, with activation characterised by intense vasoconstriction and inflammatory cell infiltration, like IRI (Barrera-Chimal and Jaisser, 2020). MR inhibition protects the kidney by mediating NO, produced by microvasculature endothelial cells (Barrera-Chimal and Jaisser, 2020). Recent studies show that non-steroidal MR antagonists provide similar protection from ischaemic AKI, and MR inhibition promotes NO synthase activation in ischaemic kidney injury by reducing Rac1 induced ROS production (Barrera-Chimal and Jaisser, 2020). Persistent

macrophage and inflammatory cell infiltration play a crucial role during repair after ischaemic AKI. Mineralocorticoid receptors play a role in polarization, as MR activity promotes classical M1 polarization, whereas MR deficiency or receptor blockage favours M2 polarization. Although Proinflammatory M1 macrophages were documented to contribute to renal injury and maladaptive repair, M2 polarization contributes to more effective kidney repair (Barrera-Chimal and Jaisser, 2020).

IRI and the innate immune response are also shown to activate the adaptive immune response. In organ transplantation, activated macrophages and dendritic cells sustain the inflammatory cascade via TNFα and IL-1β secretion, and present antigens to effector T cells to modulate transplant rejection (Fernández et al., 2020). Endothelial cells and macrophages express CD40 that have been found to upregulate IRI. CD40 binds to CD40L receptors expressed on activated T cells, increasing microvascular permeability and inflammatory cell infiltration thus promoting chronic graft rejection (Fernández et al., 2020). Myeloid cells have been implicated in modulating T cell activity in acute graft rejection. Myeloid cells release danger signals such as calcium binding protein A9 that modulates the maturation of DCs and suppresses T cells in kidney transplant patients, implicating myeloid cells in promoting the T cell tolerogenic state. Myeloid-derived suppressor cells (MDSCs) have received interest in transplant immunology as systemic inflammation promotes myeloid cell transportation and differentiation (Fernández et al., 2020). Studies of renal IRI found that activated MDSCs secrete arginase-1 that prevents T cell proliferation whereas activation by CRP exacerbates renal damage by IRI, showing unpredictability in the protective effect of activating MDSCs. Hematopoietic stem cells are also suggested to possess unique characteristics to facilitate organ transplant recovery and attenuate IRI damage (Fernández et al., 2020). HSC reverses the delayed recovery of kidney transplants caused by renal IRI by decreasing mitochondrial ROS.

1.2.7 Circadian messengers and the immune system

1.2.7.1 GABA and the immune system

GABAA receptors have been identified on lymphocytes, macrophages, monocytes and dendritic cells (Bhandage, 2016). Neutrophils, macrophages and activated T lymphocytes have been found to synthesize GABA and GAD, implying GABA receptor signalling regulates the immune system. The effect of GABAA receptors on immune cells could be inhibitory or excitatory. For example, GABAA receptor activation can inhibit NF-κB activity, IFNγ production and T cell proliferation in human T cells but also has been shown to depolarise different subsets of immune cells. Fewer studies have identified the presence of GABAB

receptors within immune cells but have suggested that signalling may be responsible for leukocyte migration and neutrophil chemotaxis (Bhandage, 2016).

GABA dysfunction has been implicated in several autoimmune diseases. For example, GABAA TONIC receptors in the airway epithelium cause mucus overproduction in Asthma after IL-13 activation (Xiang *et al.*, 2007). In contrast, APCs and T cells secrete GABA to mediate macrophage activity (Bhat *et al.*, 2010). GABAergic signalling upon Antigen Presenting Cells induced a decrease of MAPK signals and diminishes adaptive inflammatory response. GABA treatment decreased inflammatory cytokine production in peripheral macrophages and Peripheral Blood Mononuclear Cells (PBMCs) and inhibited cytotoxic immune responses. Comparison between individuals with type 1 diabetes (T1D) to non-diseased (ND) revealed that 47 cytokines were inhibited by GABA in stimulated PBMCs from T1D individuals as compared to 16 cytokines from ND individuals, highlighting GABA as a therapeutic agent for autoimmune diseases (Bhandage *et al.*, 2018) GABA also regulates cytokines from CD4+ T cells in a concentration dependent manner and more prominent in responder T cells than non-responders.

In terms of Ischaemia reperfusion injury, GABA is considered a marker of IRI in the brain and CNS. There are multiple causes for IRI, one being prolonged sympathetic nerve stimulation (Kobuchi *et al.*, 2009). This can lead to renal IRI as an adrenaline injection into the renal artery promotes blood overflow to the renal vein, reducing renal blood supply. GABA and GABA agonists inhibit blood pressure by suppressing sympathetic tone by either central or peripheral sympathetic neurotransmission. More specifically they identified GABAB receptors attenuate this type of IRI by suppressing enhanced renal sympathetic nerve activity during ischaemia (Kobuchi *et al.*, 2009). Chen *et al* specified that one mechanism that promotes IRI injury is promoted glutamate activity and a decline in GABA signalling. This suggests GABA preconditioning or post conditioning of GABA could correct this imbalance (C. Chen *et al.*, 2019). Talebi *et al* identified GABA application improves the condition of IRI in ovariectomized rats (Talebi *et al.*, 2016). It was also noted that when preincubated by GABA, the Kidney tissue damage score was lower than without GABA, meaning the GABA could reduce the injury of IRI.

1.2.7.2 Arginine Vasopressin and the immune system

Arginine vasopressin release was found to be part of an inflammatory response and can mediate aspects of the immune system (Mavani, DeVita and Michelis, 2015). Inflammatory cytokines IL-1 and IL-6, which are associated with immune conditions such as pneumonia and encephalitis, have been identified to

induce AVP release. In humans, serum AVP levels were increased within 2h of IL-6 injection, thus implying that IL-6 stimulates AVP-secreting neurons (Mavani, DeVita and Michelis, 2015). Animal experiments show AVP can downregulate the innate immune response when exposed to an antigen showing that AVP has immunomediatory effects (Boyd *et al.*, 2008). AVP was observed to reduce IL-6 levels, further identifying the regulatory relationship between IL-6 and AVP. The immunomodulatory effect of AVP was implicated to occur via V2 receptor activity and that this amelioration is specific to the lungs as systemic levels of IL-6 were unaltered in response to AVP. Arginine vasopressin has also been speculated to alter TR4-mediated signalling which leads downregulates intrarenal inflammation, further suggesting that AVP reduces innate immunity in peripheral organs (Mavani, DeVita and Michelis, 2015).

Arginine vasopressin has been identified to stimulate cytokine and antibody production as well as reduce production (Mavani, DeVita and Michelis, 2015). Both vasopressin receptors and AVP were identified in human peripheral blood mononuclear cells, revealing that AVP induces cytokine production. Cytokines released during inflammation stimulate AVP release from the hypothalamus (Mavani, DeVita and Michelis, 2015). Arginine vasopressin induces cytokine production and increases T helper 1 cell activity, which exacerbates proinflammatory response. V3 receptors induce ACTH release which activates the adrenal gland to release glucocorticoids such as corticosterone, applying further pressure on the immune system. Finally, in rats, AVP expression has been in B cells during arthritis, speculating that AVP can affect humoral immunity in addition to innate immunity.

1.2.7.3 Corticosterone and the immune system

As stated, Glucocorticoid levels peak as an individual enters their active phase, at a time of increased risk of infection and injury (Curtis *et al.*, 2014). Glucocorticoids play critical roles in mediating inflammation by upregulating anti-inflammatory proteins and downregulating pro-inflammatory molecules (Druzd, De Juan and Scheiermann, 2014) As a result, glucocorticoids are utilised in anti-inflammatory/ immunosuppressive therapies, including regimes to suppress organ transplant inflammation. Anti-inflammatory effects by glucocorticoids are found to be mediated by GR. MR have been reported in immune cells but are found to be of little consequence to the anti-inflammatory effect of glucocorticoids and might be associated with proinflammatory effects. Acute administration of glucocorticoids suppresses inflammation by preventing the increased vascular permeability after the initial inflammatory insult (Coutinho and Chapman, 2011). Glucocorticoids also contribute to reducing leukocyte emigration to inflamed tissues, leukocyte distribution, leukocyte cell death and leukocyte differentiation, thus showing glucocorticoids shape the delayed immune response. These effects are induced by genomic

actions of glucocorticoids upon leukocytes by direct or indirect action, altering gene upregulation/downregulation. Glucocorticoids repress transcription of pro-inflammatory cytokines, chemokines and cell adhesion molecules, all of which contribute to chronic, unrelenting inflammation conditions (Coutinho and Chapman, 2011). Many of the immunosuppressive actions of glucocorticoids are mediated by repressing NF-κB and AP-1 transcriptional regulators through an activated glucocorticoid induced Leucine zipper (Coutinho and Chapman, 2011). Glucocorticoids also upregulate the production of IL-10, which is suppress the HPA axis and corticosterone synthesis once inflammation begins to resolve.

Interestingly, glucocorticoids alter T cell maturation within the thymus (Coutinho and Chapman, 2011). Defective T cells, known as double positive cells ($CD4^+CD8^+$), are susceptible to glucocorticoid-induced apoptosis, which occurs during physiological levels. In addition, glucocorticoids can alter the differentiation of progenitor cells (Coutinho and Chapman, 2011). This is utilised for clinical applications of glucocorticoids during incidences of peripheral tolerance induced by an allergenic stimulus. Glucocorticoids influence dendritic cells to promote the tolerogenic phenotype and induce T_{reg} cells to suppress antigen-specific immunity. Glucocorticoids induce IL-10 secreting T_{reg} cells, which regulate the Th1 response and autoimmunity (Coutinho and Chapman, 2011). Coutinho and Chapman speculated that Glucocorticoids induce anti-inflammatory phenotype in macrophages, promoting differentiation to M2 macrophages. Glucocorticoids also promote phagocytosis by 2 mechanisms: either by the induction of S/Mer tyrosine kinase dependent apoptotic cell clearance pathway or by programming blood monocytes into highly phagocytotic anti-inflammatory macrophages, depending upon monocyte differentiation. Corticosterone has also been observed to promote the immune response, primarily via intracellular GR activity altering gene transcription (Lattin and Romero, 2014). It was hypothesised that chronic stress alters corticosterone receptors in a tissue specific manner (Lattin and Romero, 2014). In the brain the negative feedback response to stress is attenuated after prolonged exposure to corticosterone by the change of corticosterone receptors in the hippocampus. Chronic stress reduces GR expression in the hippocampus and prefrontal cortex (Mizoguchi *et al.*, 2003). Lattin and Romero built upon this and investigated MR and GR expression in other tissues to see if levels differ during chronic stress. MR and GR density was altered after stress was induced in the Pectorals muscle, the kidney and the testes (Lattin and Romero, 2014).

Long term use of oral glucocorticoids has been associated with pathological conditions such as increased risk of metabolic disease, cardiovascular disease and inflammatory disease (Coutinho and Chapman, 2011). This could mean that glucocorticoids immunosuppressive effects could be due to acute increase in

concentration, but prolonged exposure can result in an elevated immune system. This notion is shared with Lattin and Romero who note that acute stress allows animals to re-establish homeostasis to cope with stress whereas chronic stress leads to metabolic dysfunction (Lattin and Romero, 2014). For example, chronic elevation of corticosterone caused an increase of infarct size and mean arterial pressure in rats after ischaemia-reperfusion injury (Scheuer and Mifflin, 1997). Blocking Glucocorticoid receptors ameliorated both arterial pressure and infarct size, showing these were due to excessive glucocorticoid concentration.

Increased corticosterone is also implicated to induce inflammation which can lead to other pathological conditions such as hypertension, renal vessel and heart hypertrophy, and fibrosis (Gomez-Sanchez and Gomez-Sanchez, 2014). Because of this, inflammation is considered one of the more detrimental effects of inappropriate MR activation. In the endothelium, MR activation promotes ROS generation and therefore increases the likelihood of oxidative injury (Barrera-Chimal and Jaisser, 2020). MR increases the expression and activity of NADPH oxidase and decreases NO production by endothelium nitric oxide synthase which is linked to endothelial dysfunction. This increased MR activation will induce hypertension and inflammation as observed in vascular and inflammatory diseases in the kidney (Barrera-Chimal and Jaisser, 2020). Glucocorticoids were demonstrated to interact with iNOS in a non-genomic manner to produce RNS and ROS, which induce DNA damage in tumour cells (Flaherty *et al.*, 2017). It was further noted that glucocorticoid reduces DNA repair after damage by ROS/RNS. Vascular inflammation induced by MR is associated with increased adhesion molecules such as ICAM-1by binding to responsive elements in the ICAM-1 promoter region. These adhesion molecules may facilitate immune cell accumulation and infiltration thus increases the inflammatory response in the kidney (Barrera-Chimal and Jaisser, 2020).

It was identified that mice with T cells lacking MR are protected against angiotensin II induced hypertension and show decreased vascular and renal damage caused by interferon-γ producing T cells (Barrera-Chimal and Jaisser, 2020). They also found MR activation may have a role in T-cell activation due to the interaction with nuclear factors of activated T cells and activator protein 1. Circulating proinflammatory cytokines can induce excessive stimulation of the sympathetic nervous system by glucocorticoid activity. The circulating proinflammatory cytokines induce perivascular macrophages to upregulate cyclooxygenase 2 which leads to synthesis of prostaglandin E_2. Prostaglandin E_2 then diffuses across the blood brain barrier and acts upon the PVN to promote MR and NADPH oxidase activity (Gomez-Sanchez and Gomez-Sanchez, 2014).This leads to excitation of the sympathetic nervous system as well as inflammatory cytokine and superoxide free radical production within the CNS. MR antagonists alleviate

hypertension in aldosterone and angiotensin II salt excess models, proving that MR involvement in the pathway. Macrophages and T Lymphocytes have also been implicated in hypertension, inflammation and fibrosis mediated by MR activity (Gomez-Sanchez and Gomez-Sanchez, 2014). MRs in macrophages are responsible for inducing cardiac inflammation and cardiac fibrosis caused by the production of L-NAME. L-NAME inhibits nitric oxide synthase which prevents NO production and therefore causes enhanced vasoconstriction and hypertension.

Finally, corticosterone was shown to induce phagocytosis when investigating melatonin and corticosterone levels to analyse phagocytosis and superoxide level in doves (Rodríguez *et al.*, 2001). At the respective peak concentration of melatonin and corticosterone, there was an observed rise in phagocytosis, with melatonin inducing a decline in superoxide anion levels (Rodríguez *et al.*, 2001). When in presence of both, increased phagocytosis and decreased superoxide anion levels were enhanced in comparison to each hormone separately. This supports that corticosterone acts as a mediator of phagocytosis in some situations of stress, stimulating the innate immune response. Barriga *et al* similarly investigated the relationship between corticosterone and melatonin in response to phagocytic stress found in BALB/c mice (Barriga *et al.*, 2001). BALB/c mice showed a constant level of corticosterone instead of circadian regulation when in response to stress. Melatonin still exhibited a circadian rhythm, but the peak concentration was reduced. It was suggested in this work that melatonin did not act as a stress inducer, rather facilitated reduction of the stressor due to its antioxidant properties to combat the rise of ROS during phagocytosis. In addition, Flaherty *et al* detailed that Glucocorticoids promote cell survival in breast tumours through glucocorticoid receptor-mediated activation of anti-apoptotic genes, thus implicating glucocorticoids and inappropriate GR activation in cancer onset and progression (Flaherty *et al.*, 2017).

1.2.7.4 Melatonin and the immune system

Multiple studies both *in vivo* and *in vitro* have suggested that melatonin can regulate some immune functions and attenuate oxidative species accumulation (Rodríguez *et al.*, 2001; Carrillo-Vico *et al.*, 2005) Multiple pathways in which melatonin regulates certain immune responses are by; inducing anti-oxidative enzymes, upregulating glutathione synthesis, neutralising of nitrogen-based toxicants, and suppressing pro-oxidative enzymes (Boutin and Jockers, 2021). The proposed mechanisms of melatonin on the immune system are summarised in figure 11 (Carrillo-Vico *et al.*, 2005). There is an established functional relationship between the pineal gland and the immune system in mammals, with multiple immune

components influenced by the circadian clock (Barriga *et al.*, 2001). As melatonin is one of the principal hormones to alter the circadian clock, it is assumed that the circadian rhythm of these immune components is due at least partially by melatonin. This notion is supported by Barriga *et al.,* whose work discovered that following surgical removal of the pineal gland and *in vitro* incubation of melatonin can alter phagocyte function as well as the oxidative metabolism of cells involved in non-specific immunity (Barriga *et al.*, 2001).

Different subsets of immune cells have been found to exhibit diurnal rhythms, including mammalian bone marrow, the lymphoid system, lymphocyte subsets, NK activity and cytokine production (Carillo-Vico *et al.,* 2005). Multiple studies that study the relationship between the photoperiod and the immune system state that short day lengths are typically associated with an enhanced immune system. It was later confirmed that night melatonin levels correlate with the immune cell response in humans and rodents (Carillo-Vico *et al.,* 2005). It was discovered that the main immune organs such as the thymus and spleen suffer a loss in mass after surgical and functional pinealectomy. Melatonin administration induces an increase in weight of these organs, so melatonin must help in their development (Carillo-Vico *et al.,* 2005). Neonatal pinealectomy affects immune function as it was found to significantly impair lymphocytes, erythrocytes and leukocytes (Carrillo-Vico *et al.*, 2005). Evidence indicates that pinealectomy affects several immune components in birds such as humoral, cellular and non-specific immunity (Carrillo-Vico *et al.*, 2005). Melatonin administration typically reverses these effects on the immune system. In C57BL6/J mice, absence of the pineal gland significantly reduces NK activity and IL -2 production. As discussed earlier in section 1.1.4.8, this strain is melatonin deficient. Despite this, melatonin injection into these pinealectomized mice restore IL-2 and NK levels, meaning mechanisms that melatonin could influence these levels are intact in this deficient strain, and this influence is likely to occur in melatonin proficient mice (Gobbo *et al.*, 1989; Carrillo-Vico *et al.*, 2005).

Melatonin can enhance antigen presentation, as seen with melatonin increasing the expression of MHC class II molecules in mice (Carrillo-Vico *et al.*, 2005). Chronic administration of melatonin was found to induce Th2 cell response through increasing IL10 and decreasing TNFα. Melatonin also participates in apoptosis regulation of T and B cells. Oral administered melatonin inhibits B cell apoptosis in pre-B-cell stage of B-cell maturation in C3H/HeJ mouse bone marrow, whereas in the thymus, melatonin induces anti-apoptotic activity in T cells at all stages of T cell development in Wistar rats (Yu, Miller and Osmond, 2000; Carrillo-Vico *et al.*, 2005). Melatonin has been found to play an important role in modulating acute and chronic inflammation. Carillo-Vico *et al* commented that exogenous melatonin supplementation

helps improve the survival rate of rats and mice exposed to a lethal dose of LPS. Melatonin mitigates the proinflammatory response, inhibits TNFα and IL6 levels so they don't exceed to levels found in septic shock. Furthermore, melatonin abolishes LPS induced lipid peroxidation and counteracts LPS induced iNOS/mtNOS and NO levels in mice and rats. Finally, melatonin ameliorates allergy inflammation in the lungs by improving immune cell migration from the bone marrow to the bronchoalveolar fluid (Carrillo-Vico *et al.*, 2005).

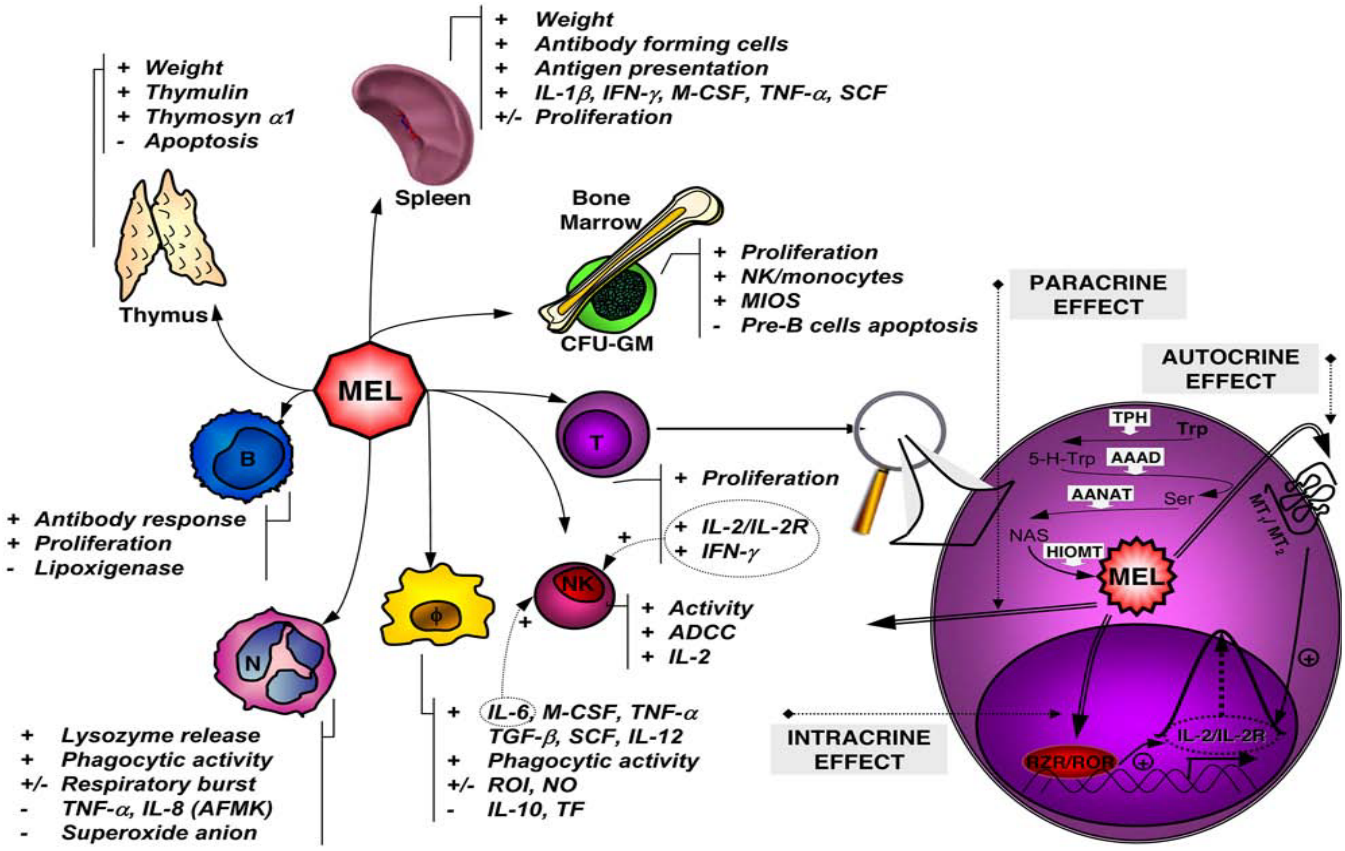

Figure 11 - The proposed effects of melatonin (MEL) on the immune system, taken from Carillo-Vico *et al.,* (Carrillo-Vico *et al.*, 2005)

The + or – refers to whether Melatonin promotes or suppresses the component of the immune system. The review by Carillo-Vico *et al* notes that Melatonin influences activity in the bone marrow, thymus and spleen. Melatonin also influences cytokines/chemokines and cell subsets of the humoral immunity. The enlarged cell depicts signalling pathways of melatonin, showing melatonin acts via autocrine, intracrine and paracrine pathway as well as endocrine.

Abbreviations: Aromatic L-amino acid decarboxylase (AAAD); arylalkylamine-N-acetyltransferase (AA-NAT); aromatic amino acid decarboxylase cytotoxicity (ADCC); hydroxyindole-O-methyltransferase (HIOMT); N-acetyl-serotonin (NAS); N1-acetyl-N2-formyl-5-metoxykynuramine (AFMK); B cells (B); macrophages (ø); antibody-dependent cellular monocyte colony stimulating factor (M-CSF); melatonin-induced opioid system (MIOS); progenitor cells for granulocytes and macrophages (CFU-GM); neutrophils (N); Natrual Killer cells (NK); reactive oxygen intermediate(ROI); stem cell factor (SCF); serotonin (Ser); T cells (T); tissue factor (TF); transforming growth factor-beta (TGF-β); Tryptophan (Trp); 5-hydroxy-tryptophan (5-H-Trp); tryptophan hydroxylase (TPH);

As stated, corticosteroids trigger immunosuppressive properties in lymphocytes, but melatonin has been implied to neutralise these properties (Barriga *et al.*, 2001). This has led to the theory of physiological coupling between melatonin and glucocorticoids and hints at melatonin as an antistressor. Barriga *et al* expanded on the melatonin-corticosterone relationship upon phagocytosis during normal and stress situations. The peak phagocytosis activity occurred between 02:00-03:30hr (Barriga *et al.*, 2001). Melatonin levels peak at 23:30hr and as phagocytes possess melatonin receptors, the time difference could be the time taken for melatonin to induce phagocytosis. The experiment revealed that animals that had undergone stress over a 24-hour period maintained a melatonin circadian rhythm with a similar peak to the control group but did not reach the same concentrations compared to the control group. During stress, phagocytosis induced by macrophages was caused by corticosterone stimulation (Barriga *et al.*, 2001). Multiple theories have considered why the decline of melatonin levels occurs; either due to direct effect of corticosterone on the pinealocytes as corticosteroids is reported to inhibit melatonin synthesis or because melatonin was used up, the latter implicating melatonin as a stress buffer (Barriga *et al.*, 2001). Another immunoregulatory effect of melatonin is as a free radical scavenger (Stiegler *et al.*, 2018). Local elevated levels of melatonin have been detected in immune cells such as dendritic cells, lymphocytes and macrophages, which produce melatonin in response to diverse stimuli such as bacterial insult (Boutin and Jockers, 2021). It is believed that this rise in melatonin occurs to modulate phagocytic activity of these cells. Melatonin has properties that lead to the belief that melatonin is an antioxidant molecule; such as the direct scavenging of reactive oxygen (ROS) and reactive nitrogen (RNS) species, the acceleration of antioxidant enzymes activity, the ability to protect against oxidative damage, synergistic effects with other antioxidants, and improving the electron transport efficiency in the mitochondrial respiratory chain via limiting free radical production by reducing electron leakage (Boutin and Jockers, 2021). Active NQO2 upon binding with melatonin has also been speculated to contribute to melatonin's antioxidative role. The comprehensive review by Stiegler *et al.,* presents a collation of information supporting melatonin can alleviate the detrimental effects of ischemia and reperfusion on organ transplants (Stiegler *et al.*, 2018). This review focussed on multiple organ transplants, including the heart, liver and kidneys. Melatonin improved functional parameters such as cardiac output and aortic/coronary flow, when melatonin supplements are added to the preservation fluid between transplants. Decreased creatinine kinase release and preservation of high energy phosphates was found in the heart tissue, suggesting reduced injury to the heart tissue (Stiegler *et al.*, 2018). High dose melatonin therapy used after heart transplantation was found to reduce lymphocyte proliferation and alleviate inflammatory response and apoptosis, leading to significantly prolonged graft survival. Of note, graft survival was found to be greatest

when melatonin was used in co-therapy with cyclosporine, an immunosuppressive drug used to prevent organ rejection, possibly implying melatonin acts on similar mechanisms (Stiegler *et al.*, 2018).

In liver transplants, multiple studies have used melatonin as an additive to preservation and perfusion solutions, to improve marginal graft quality (Stiegler *et al.*, 2018). Adding melatonin to static, cold preservation solution showed reduced vascular resistance and therefore less susceptible to hypertension (Zaoualí *et al.*, 2011). This correlated with NO production and showed a significant decline in an immune response by preventing oxidative stress and inflammatory cytokine release. Stieger *et al*'s review specified this effect persisted after warm Ischaemia-Reperfusion was induced, and demonstrated the protective effects of melatonin, showing significant decrease in liver necrosis, leukocyte infiltration, necrosis and nitric oxide synthase production (Stiegler *et al.*, 2018). It was theorised hepatoprotective ability of melatonin was likely due to inhibition of the IKK and JNK signal transduction pathways that regulate cell proliferation (Liang *et al.*, 2009). Vairetti *et al* utilised this experimental procedure to investigate liver function after IRI (Vairetti *et al.*, 2005). Melatonin supplementation increased bile production, bilirubin excretion, preservation of liver ATP levels and improved mitochondria function. Melatonin was administered as part of a multidrug cocktail by intraperitoneal injection to both healthy and steatotic livers and both exhibited reduced parenchymal injury and abolished the inflammatory response induced by cold IRI. These two methods were collated by Song *et al.,* who utilised both methods to investigate melatonin's beneficiary effects on small for size (SFS) or partial liver transplants (Song *et al.*, 2018). The ability for livers to regenerate has become an exploited ability to attempt to meet demands by partial liver transplants to allow the partial pieces to regenerate themselves, allowing living donors to donate their liver. For SFS liver transplants, melatonin was administered intraperitoneally before the operation and after reperfusion to the recipient (Stiegler *et al.*, 2018). In addition, melatonin was used within the perfusion and storing solution. The results revealed that melatonin utilised by both methods promoted graft regeneration and improved survival of the recipient. This was identified clearer in IRI models and partial hepatectomy administered to the intraperitoneal injection before the operation and after reperfusion. Mouse models injected with melatonin displayed reduced liver injury and enhanced liver regeneration, with the promotion of interleukins IL6, IL10 and TNFα release via Ly6C+ F4/80+ inflammatory monocytes. Specifically, IL6 was found to significantly improve microcirculation with the liver and thus promoted animal survival.

In terms of renal transplantation, IRI occurs as frequently as with other organs, with transplantation from deceased donors being far more prone to IRI than transplantation from living donors (Stiegler *et al.*, 2018). Melatonin was investigated by using a single concentrated dose, administrated 2 hours before reperfusion to the recipient after 24 hours of cold storage. Results showed that donor preconditioning with melatonin, similarly to the procedure with other organs, improved recipient survival. The kidney itself displayed reduced histological damage to the tubules, induced superoxide dismutase activity in kidney tissue and decreased levels of blood urea nitrogen, creatinine, transaminases and lactate dehydrogenase in the blood after transplantation, thus revealing melatonin preconditioning reduces kidney damages (Stiegler *et al.*, 2018). Molecular levels of NF-κB, p65, iNOS and caspase-3 were downregulated showing reduced apoptotic capacity. In conclusion, studies have found that melatonin has ability to ameliorate IRI in multiple transplanted organs. These studies have either used melatonin in preservation solution or as a drug against IRI either administered as part of organ preconditioning/ after reperfusion and has shown promising results.

1.2.8 Renal ischaemia reperfusion Injury- impact on renal function

1.2.8.1 Renal IRI on Renal physiology

Ischaemia is thought to be a major component in developing acute renal failure in promoting the initial tubular damage (Legrand *et al.*, 2008). Prolonged Ischaemia can occur even after reperfusion due to an imbalance between vasoconstrictors and vasodilators, thus promoting microvascular dysfunction, endothelial damage and endothelial-leukocytes communication (Legrand *et al.*, 2008). The arrangement of the renal microvasculature makes the kidney sensitive to ischaemic injury, due to its high complexity and demands large quantities of energy (Legrand *et al.*, 2008). Murine models show that tubular epithelial cells in the kidney are vulnerable to hypoxia and undergo apoptosis during IRI (Fernández *et al.*, 2020). Renal ischaemia results in epithelial and endothelial injury, which causes a loss of cytoskeleton polarity and loss of the brush border in tubular cells (Ye *et al.*, 2022). Leukocyte-Endothelial cell communication causes endothelial swelling, impedes blood flow and promotes further injury (Legrand *et al.*, 2008; Malek and Nematbakhsh, 2015). The endothelium affects microvascular permeability, blood flow regulation, cell trafficking, and additional immunologic functions. Endothelial and vascular muscle cells undergo structural damage after ischaemic insult. This damage includes cytoskeleton modifications, F-actin modification, endothelial aggregation, endothelial swelling and increased permeability via loss of adherens junction (Legrand *et al.*, 2008). The persistent reduction of regional blood flow and the

consequent reperfusion of renal tissue are thought to play a crucial role in the pathogenesis of acute renal failure via IRI, especially within the outer medulla and the cortico-medullary junction (Legrand *et al.*, 2008).

Direct staining observed a high number of monocytes and macrophages and a few PMNs in the outer medulla, most notably in the early stage of reperfusion. Blocking mononuclear leukocyte infiltration by targeting the B7-CD28 co-stimulation pathway, the pathway that activates T cells, improves renal function after IRI. Activation of the inflammatory cascade induces capillary plugging caused by interaction between leukocytes, platelets and red blood cells, due to the promoted leukocyte infiltration (Legrand *et al.*, 2008). This plugging of the microvasculature leads to local dysfunction and causes the no-reflow phenomenon that follows Ischaemia and reperfusion. IL-6 and IL-8 levels within plasma can act as an indicator to predict mortality of patients with acute renal failure (Legrand *et al.*, 2008). Leukocyte activation induces further endothelial damage by sustaining the inflammatory response, as leukocytes release cytokines and proteases and induces additional oxidative stress (Legrand *et al.*, 2008). Blocking ICAM-1 or ICAM-1 deficiency protects the kidney from moderate injury by ischaemia and reperfusion, therefore targeting intracellular adhesion molecules could be a beneficial target for renal ischaemic damage.

Ischaemic injury is associated with systemic inflammation, due to the increased cytokines and adhesion molecules surrounding endothelial cells and the hypoxic parenchymal (Soares *et al.*, 2019). If severe apoptosis and necrosis occur, inflammation is exacerbated and results in greater damage to renal cells (Ye *et al.*, 2022). This evokes endothelial dysfunction and disrupts vascular tone which both contributes to tubular cell permeability and intravascular coagulation. Reperfusion carries toxic metabolites, systemically damaging distant organs including the lungs, heart, liver and brain (Ye *et al.*, 2022). Monocytes and renal macrophages are also involved in the pathogenesis of renal ischemia-reperfusion injury. Several subsets of monocytes and macrophages localize in the injured tissue, and modulate the immune response observed in renal IRI (Karasawa *et al.*, 2015). CD169+ Monocytes and macrophages for example, play a crucial role in preventing excessive inflammation in IRI via downregulating intercellular adhesion molecule-1 (ICAM-1) expression on vascular endothelial cells (Karasawa *et al.*, 2015). Mice depleted of CD169+ cells exhibit increased ICAM-1 expression along the renal vasculature and experienced irreversible renal damage associated with enhanced neutrophil infiltration (Karasawa *et al.*, 2015). Interestingly, CD169+ macrophages were found to preferentially localise in the renal medulla and these localised CD169+ monocytes and macrophages were shown to interact with blood vessels directly to downregulate ICAM-1.

Renal IRI triggers an inflammatory cascade that promote further renal damage, therefore inhibiting this response can help protect renal tissue (Malek and Nematbakhsh, 2015). Chemokines are major mediators of the expression of proinflammatory cytokines and adhesion molecules. Chemokines also mediate leukocyte activation and infiltration into affected tissues. Pro-inflammatory cytokines mediated by the JAK/STAT pathway such as interleukin 6 (IL6) and TNFα play a major role in renal dysfunction of IRI (Malek and Nematbakhsh, 2015). Inhibiting the JAK/STAT pathway by targeting protein phosphorylation led to reduction of IL6 and TNFα and promoted anti-inflammatory effects. ICAM-1 and P-selectin were implicated in leukocyte recruitment and neutrophil infiltration into the post ischaemic tissue (Malek and Nematbakhsh, 2015). Neutrophils are especially implicated in the renal IRI development as neutrophils were observed to release proinflammatory cytokines, proteases, ROS and other mediators that are involved in lipid peroxidation and tissue injury.

1.2.8.2 Renin-angiotensin system

The Renin-angiotensin system is associated in renal IRI, as angiotensin II levels and RAS activation are considered important risk factors in IRI (Malek and Nematbakhsh, 2015). Angiotensin II contributes to renal injury via multiple mechanisms; including renal vessel constriction, inducing apoptosis, generating ROS and increasing vascular sensitivity to sympathetic nerve stimulation (Malek and Nematbakhsh, 2015). RAS modulates inflammation in renal tissue via two pathways; the angiotensin-converting enzyme (ACE)/Angiotensin2/AT1 receptor and the angiotensin-converting enzyme 2 (ACE2)/(Angiotensin1-7)/Mas receptor pathways which in terms of IRI are deleterious and protective pathways respectively (Malek and Nematbakhsh, 2015). Administration of Mas agonists showed decreased leukocyte infiltration in kidney IRI and therefore attenuated renal tissue damage (Malek and Nematbakhsh, 2015). In addition, promoting ACE2 to convert angiotensin II to angiotensin I have led to therapeutic potential of manipulating the (ACE2)/Mas receptor pathway to alleviate renal IRI.

Low concentrations of Nitric oxide (NO) are considered renoprotective against renal ischaemia to encourage vasodilation, antioxidation and anti-inflammatory properties as well as inhibiting nuclear proteins and induces beneficial cell signalling (Malek and Nematbakhsh, 2015). After renal IRI, nitric oxide synthase is activated and promotes the expression of NOS proteins. There are 3 isoforms of NOS; endothelial NOS, neuronal NOS and inducible NOS. Inducible NOS is considered a toxic agent whereas endothelial and neuronal NOS act as protective enzymes (Malek and Nematbakhsh, 2015). NO is produced in the renal proximal tubules in response to ischaemic injury, with inducible NOS speculated to mediate injury progression. Inhibition of inducible NOS prior to renal IRI has been found to protect the kidneys

from ischaemic injury, supporting that iNOS facilitates renal damage (Malek and Nematbakhsh, 2015). Under ischaemic conditions, nitrite is converted to NO by NOS and xanthine oxidase enzymes and is considered a cytoprotective mechanism in IRI (Malek and Nematbakhsh, 2015).

1.3 Kidney Physiology

1.3.1 Introduction to Kidney physiology and structure

1.3.1.1 Kidney structure

The kidney is arranged in a highly ordered manner to optimise transport of excess materials (Pocock, Richards and Richards, 2017). Visually, the kidney is separated into two regions, the cortex and the medulla. The cortex contains approximately 1.25 million nephrons, the functional units of the kidney (Figure 12) (Coe, 2017). Blood enters the kidney from the afferent arterioles of the renal artery, approaching the glomerulus. The glomerulus is a cluster of capillaries that filter the blood into the Bowman's capsule by ultrafiltration (Pocock, Richards and Richards, 2017; Leatherby, Theodorou and Dhanda, 2021). The fluid enters the proximal tubule, which is optimally designed for reabsorption via active transport. The cells of the proximal tubule wall are rich in mitochondria, impermeable due to its tight junctions and densely covered by microvilli on the apical brush border to maximise the surface area (Pocock, Richards and Richards, 2017). About two thirds of water, sodium and chloride resorption occur in the proximal tubule, which aids in pH maintenance by bicarbonate resorption. Sodium ions are reabsorbed together with organic solute-specific symporters with amino acids, glucose and other constituents. The early region of the proximal tubule is impermeable to chloride ions to build a concentration and biochemical gradient. This gradient induces chloride resorption in the latter region via sodium and water cotransporters, with sodium-hydrogen antiport activity re-establishing the gradient (Pocock, Richards and Richards, 2017). The next section is the loop of Henle, which is separated into the thin and thick loop. The production of ions produces osmotic pressure between the lumen and extracellular space, thus causing water resorption. In the thick ascending loop, sodium, chloride and potassium symporters are the main transport mechanism from the apical surface. Solutes such as calcium and magnesium are resorbed via the tight junctions. This transport dilutes the contents within the tubule due to tight junction permeability to water.

The distal tubule and collecting ducts proceed from the loop of Henle and are the final functional segments of the nephron (Pocock, Richards and Richards, 2017). Resorption prior to the distal tubule occurs regardless of the body's ionic balance. The distal tubule and collecting ducts therefore play a key role in

ion, water and pH balance. Sodium intake occurs early in the distal tubule by chloride symport similar to the ascending loop of Henle. The latter region absorbs sodium by principal cells on the apical surface and passes the basolateral membrane by the typical sodium-potassium-ATPase channel. Approximately 12% of the sodium filtered and reabsorbed, monitored by the juxtaglomerular apparatus. The juxtaglomerular cells secrete renin in response to low sodium to induce the renin-angiotensin-aldosterone pathway. Renin induces angiotensin-I production which is further converted into angiotensin-II by Angiotensin converting enzyme. Angiotensin-II transfers to the zona glomerulosa cells of the adrenal cortex to stimulate aldosterone release. Aldosterone acts back upon the kidney to increase sodium channels and activate the sodium-potassium-ATPase channels, both to increase sodium resorption and potassium excretion. In addition, calcium ions are absorbed within the distal tubule upon stimulation of the parathyroid hormone. The collecting ducts regulate the osmolality of plasma by adjusting the amount of water reabsorbed. The nephron folding over the cortex (outer medulla space) provides an osmotic gradient between the cortex and the medulla. Much of the osmotic gradient is generated by the think ascending loop, facilitating active transport of sodium, potassium and chloride ions while preventing water resorption. This, with the counter-current arrangement of the fluid in the loop of Henle produces a gradient that optimises the resorption of essential ions.

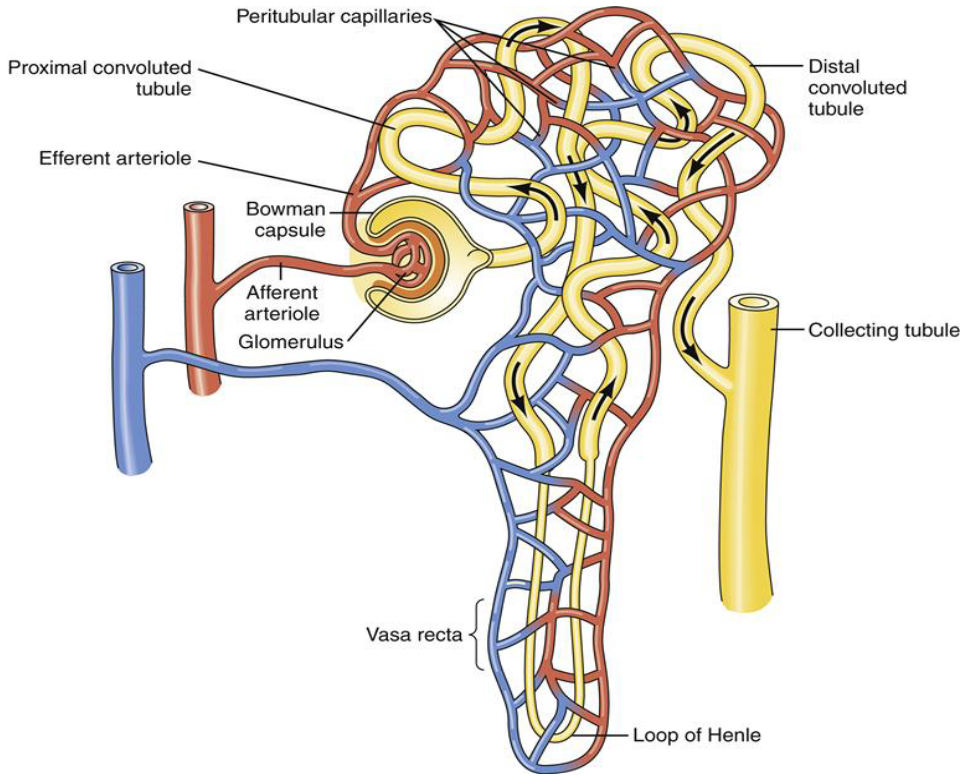

Figure 12 - The structure of a Nephron, Figure taken from Coe, (Coe, 2017)

Blood to the kidney is supplied by the renal artery and plasma is filtered into the Bowman's capsule from the afferent arteriole. The plasma filtrate then travels through the proximal convoluted tubule, the loop of Henle and the distal convoluted tubule where water and ions can be reabsorbed into the peritubular capillaries. The remaining filtrate is concentrated into urine, transported to the collecting tubule, to be sent to the bladder to be removed from the body.

1.3.1.2 Kidney Function

One primary function of the kidney is to regulate filtration and plasma clearance. The kidney receives approximately 20-25% of cardiac output, roughly 1 litre of blood per minute (Leatherby, Theodorou and Dhanda, 2021). Over 90% of this blood flow is supplied to the renal cortex, and the remainder is sent to the medulla. Three factors determine renal homeostatic and excretory functions; adequate renal blood supply, Effective plasma ultrafiltrate production and sufficient modification of the ultrafiltrate composition via tubular reabsorption and active secretion (Leatherby, Theodorou and Dhanda, 2021). Filtration begins between the glomerular capillaries and Bowman's capsule, filtering roughly 20% of renal plasma flow with a Glomerular filtration rate (GFR) of 100-140ml per minute (Leatherby, Theodorou and Dhanda, 2021). This ultrafiltrate travels along the renal tubular where it can be reabsorbed. Blood enters and exits the glomerulus by the afferent and efferent arterioles respectively (Scott and Quaggin, 2015). The glomerular filtration barrier between the glomerular capillaries and the Bowman's capsule has 3

properties that helps filter plasma. The first is the fenestrated capillary endothelium, which filters red blood cells and larger proteins. Next is the glomerular basement membrane, composed of a collagen matrix and proteoglycan fibrillae that prevents plasma protein passage. Last is the epithelial layer with podocytes that surround glomerular capillaries. Plasma ultrafiltrate forms as starling forces formed by capillary and interstitial hydrostatic and oncotic pressures, act on the capillary wall (Leatherby, Theodorou and Dhanda, 2021). The glomerular filtration rate increases by vasoconstriction of the efferent arterioles and the relative relaxation of the afferent arterioles whereas the converse would produce the opposite effect by increase of resistance. This is because the pressure differences between these arterioles determine the hydrostatic pressure upon the capillaries and therefore determines the filtration.

Blood flow through vascular beds is typically determined by the diameter of precapillary resistance vessels and the pressure gradient (Chalmers and Macdonald, 2018). Renal blood flow (RBF) occurs by renal arteries, transferring blood from the afferent arterioles to the glomerular capillaries to the efferent arteries (Chalmers and Macdonald, 2018). RBF is tightly regulated by the arrangement of the renal arterial vasculature and the GFR indirectly. The cortex is less metabolically active than the medulla despite receiving 90% of blood flow. The increased demand of blood flow through the cortex is needed to drive plasma filtration but consequently leaves the medulla vulnerable to ischaemia (Chalmers and Macdonald, 2018). Numerous factors affect the starling forces and therefore GFR: including hormonal secretion, sympathetic nervous system and renal blood flow autoregulation. Renal plasma flow affects the GFR, determined by the pressure gradient of the renal vasculature divided by the renal vascular resistance (Leatherby, Theodorou and Dhanda, 2021). Oral intake of protein has also been indicated to increase the GFR (Palsson and Waikar, 2018). The kidneys can self-regulate their blood flow to maintain a consistent renal plasma flow and glomerular filtration rate that is independent of renal perfusion pressure (Leatherby *et al.,* 2021). This is achieved by adjusting renal vascular resistance, to maintain the systolic blood pressure between 80-180mmHg. Autoregulation is also useful to protect glomerular capillaries and renal parenchyma from the extreme hydrostatic pressures, highlighting a potential renoprotective mechanism for systemic hypertension. There are two feedback mechanisms that influence the autoregulation of renal vasculature: the myogenic response and the tubule-glomerular feedback response (Leatherby, Theodorou and Dhanda, 2021). Both mechanisms work in concert to alter afferent arteriole vascular activity but react to different changes in renal vasculature (Burke *et al.*, 2014). Under normal conditions, contributions of each mechanism to autoregulation are; 50% by myogenic, 35-50% by tubular glomerular feedback (TGF) and the final 15% by a hinted mysterious third mechanism (Chalmers and Macdonald, 2018). The third mechanism is currently poorly understood and may reflect upon how myogenic mechanisms and TGF

interact. The myogenic response is activated by stretching smooth muscle cells of the arterial wall which activates voltage gate calcium channels and leads to vasoconstriction. The tubule-glomerular feedback response is the slower feedback mechanism and involves macula densa cells in the junction between the distal collecting tubule and the preceding loop of Henle. In the process of TGF, the macula densa cells in the ascending limb of the loop of Henle detect increased concentrations of sodium and chloride (Chalmers and Macdonald, 2018). As a result, juxtaglomerular cells activate and reduce renin secretion. The reduced renin secretion causes afferent arterioles to constrict and efferent arterioles to dilate, reducing glomerular capillary hydrostatic pressure and decreased GFR as a result. Recent evidence indicates the myogenic response is considered the most important mechanism to protect the glomerular capillaries against rapid levels in the mean arterial pressure (MAP), whereas TGF acts to sustain reduction in MAP.

The kidneys regulate blood pressure primarily by the Renin-Aldosterone-Angiotensin-System (RAAS) (Figure 13). The kidney is the primary source of renin, which is tightly regulated at the juxtaglomerular apparatus by renal baroreceptors and sodium chloride delivery to the macula densa (Sparks *et al.*, 2014). Numerous environmental stimuli increase renin in the glomerulus and afferent arteriole; such as prolonged adrenergic stimulation, sodium depletion and chronic ischaemia. Renin expression and release is controlled by intracellular mediators' cAMP and calcium ions that increase and inhibit renin release respectively (Sparks *et al.*, 2014). Mediators like Nitric oxide also inhibit renin release, acting through the cGMP intracellular pathway. Sympathetic stimulation induces renin secretion and modulates renal blood flow and tubular function. Angiotensin-I is converted to vasoactive peptide angiotensin II via angiotensin converting enzyme. Angiotensin II then binds to angiotensin receptors to induce vasoconstriction. Angiotensin II has been implicated to exert an inhibitory effect on renin release as part of a short feedback mechanism (Sparks *et al.*, 2014). Endothelial cells play an important role in regulating RBF through their response to paracrine vasoactive mediators (Chalmers and Macdonald, 2018). The most notorious is angiotensin II, the vasoactive mediator of RAAS which induces vasoconstriction, preferentially in the efferent arterioles. Endothelial cells of the renal arterioles also act upon renal vasculature and renal plasma flow by the local production of hormones. Endothelin is produced locally and induces vasoconstriction of afferent and efferent arterioles and thus reduce renal plasma flow and GFR (Leatherby, Theodorou and Dhanda, 2021). Nitric oxide is a potent vasodilator that acts on endothelial cells to increase renal plasma flow and GFR. Coordination of vasoconstriction of the renal vasculature and therefore renal plasma flow and glomerular filtration rate are controlled by a specialised type of cell called

mesangial cells. Mesangial cells are a type of cell known as pericytes, cells that encompass capillaries and control vasoactivity of the microvasculature.

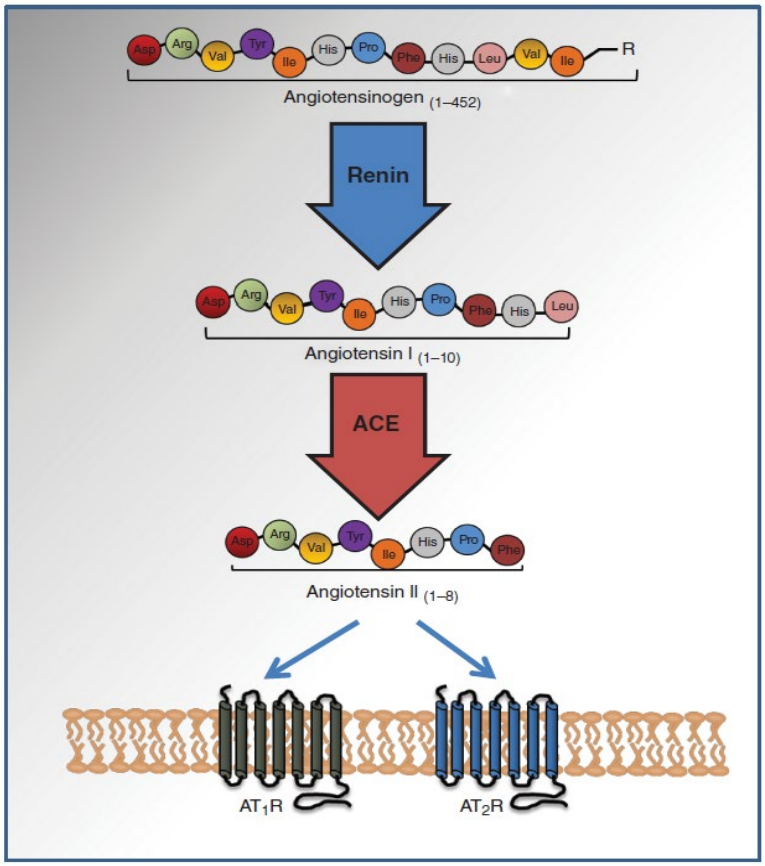

Figure 13 - Diagram of the classical Renin-Angiotensin-System, taken from Sparks *et al,* (Sparks *et al.*, 2014)

Angiotensinogen is initially cleaved by Renin to form Angiotensin-I. The second component of RAS is Angiotensin-converting enzyme (ACE) which converts angiotensin-I to angiotensin-II by removing 2 amino acids from the C terminal. The vasoactive Angiotensin-II then acts upon angiotensin AT1 and AT2 receptors, GPCRS that activate intracellular pathways to induce distinct functions.

Sympathetic nerve stimulation also regulates blood pressure. The importance of renal sympathetic nerve activity (RSNA) is associated with many pathophysiological conditions such as hypertension and chronic kidney disease, with continuous overactivity causing a decline in renal function (Sata *et al.*, 2018). Increased RSNA contributes to increased blood pressure by increased tubular reabsorption of water and sodium ions, reduction in glomerular filtration rate and renal blood flow and activating RAAS via renin release. Moderate sympathetic stimulation reduces renal plasma flow without altering GFR, suggesting the primary effect acts upon efferent arterioles. Increased sympathetic stimulation reduces RPF and GFR

which suggests vasoconstriction in both afferent and efferent arterioles (Leatherby, Theodorou and Dhanda, 2021). Sympathetic nerve stimulation in the kidney induces alpha-adrenoreceptors, which mediates vasoconstriction of both afferent and efferent arterioles (Chalmers and Macdonald, 2018). B1-adrenoreceptors are also implicated in RBF as these receptors induce renin secretion (Sparks *et al.*, 2014). The renal afferent and efferent nerves allow bidirectional communication between the CNS and kidney (Linz et al., 2018). Central and peripheral inputs alter renal sympathetic nerve activity of efferent arterioles, to alter structural and functional components of the kidney, such as RBF, GFR, tubular water and sodium handling and renin secretion from the juxtaglomerular apparatus (Linz et al., 2018). This efferent activity is also regulated by afferent input in response to renal chemo and mechanoreceptors. Abnormal renal efferent sympathetic activity contributes to hypertension and renal disease. Linz *et al* identified the association between increased renal sympathetic activation and pathophysiological conditions such as renal ischaemia and hypoxia through afferent fibre signals from the kidney to the brain (Linz et al., 2018). The current information suggests pericytes may play a role in renal and immune function. As both can potentially tie to Renal Ischaemia Reperfusion Injury, pericytes shall be discussed in greater detail in section 1.4.

1.3.2 The Circadian clock and the Kidney

As stated previously, circadian rhythms coordinate the peripheral clocks including the kidney (Carney, 2016). Regulating renal physiology and function by the circadian clock has become apparent, as urine excretion is reduced in the night (Birder and Van Kerrebroeck, 2019). Various renal functions have been shown to exhibit circadian rhythms, such as glomerular filtration and electrolyte excretion (Hara et al., 2017). Macroscopic bioluminescence imaging performed by Hara *et al* revealed that strong and robust circadian clock oscillations are observed in the renal medulla. In the inner medulla, vasopressin receptors (V1a + V2 Receptors), urea transporter *UT-A2* and water channel (aquaporin2) show diurnal variation (Hara et al., 2017). *Bmal1* deficiency impairs circadian variations of osmotic pressure in the inner medulla, suggesting the cortico-medullary osmotic pressure gradient is under circadian influence, thus contributing to the day/night rhythm of urination. Recent data suggests the circadian clock acting upon renal tubular cells controls multiple homeostatic and metabolic processes, including drug entry and exit (Carney, 2016; Nikolaeva *et al.*, 2016). Renal Blood flow, GFR, renal cortico-medullary osmotic gradient and tubular transport of water and electrolytes all show circadian rhythms and are expected to be driven by the internal kidney clock (Costello *et al.*, 2022). Human studies reveal the GFR circadian rhythm is independent of circadian oscillations of blood pressure and sympathetic regulation, leading to the hypothesis that

kidney clocks are involved (Firsov and Bonny, 2018; Costello et al., 2022). Nikolaeva *et al* investigated mice with tubule specific knockout of *Bmal1* and found a significant decrease in systolic blood pressure and increased plasma urea and creatinine (Nikolaeva et al., 2016). This suggests tubular impairment, whilst maintaining normal levels of glomerular filtration rates and circadian renal water, sodium and potassium levels. Gene expression of metabolic pathways and anion transport were found altered in *Bmal1* knockout mice (Carney, 2016). A similar result was found with whole body inactivation of clock, showing dramatic changes in expression of circadian regulated genes in the distal convoluted tubule and cortical collecting duct (Nikolaeva et al., 2016). Whole body inactivation of different components of the molecular clock in mice caused a loss in circadian rhythms in plasma aldosterone levels, abnormal circadian patterns or urinary excretion sodium and potassium excretion and induced significant changes to arterial blood pressure. In humans, similar effects are speculated, as growing evidence suggests a link between dysregulation of renal circadian rhythms with the development of hypertension and chronic kidney disease (CKD) (Nikolaeva et al., 2016).

Gumz *et al* identified that PER1 is responsible for diverse effects on renal handling of sodium (Gumz et al., 2009). Ion transporters in the kidney are regulated by the circadian clock, as illustrated by table 1 (Stow and Gumz, 2011). The first evidence that illustrated circadian control of a renal gene was found with the sodium hydrogen antiporter (NHE3), which was found to be promoted by CLOCK/BMAL1 binding onto the E-box binding domain (Solocinski and Gumz, 2015). PER1 alters ENaC expression via decreased action on aldosterone. In addition, gene expression and protein levels of Sodium-phosphate cotransporters have been found to exhibit circadian variation. In the loop of Henle, it is theorised that ALMS1 gene is a circadian clock target speculated to enforce the regulation of NKCC2 (Costello et al., 2022). Stow and Gumz noted that transcription repressor Kidney ischaemia developmentally regulated gene 1 (Kid-1) was found to undergo rhythmic expression, providing a link between Renal signal transduction and circadian controlled genes (Stow and Gumz, 2011). In another study, circadian clock ablation in renin-secreting granular cells leads to increased GFR, decreased plasma aldosterone and low blood pressure, further reiterating the circadian clock on renal function (Nikolaeva et al., 2016). It has been suggested that the tubular circadian clock is involved in intrarenal and systemic metabolisms, xenobiotic drug elimination and renal compound secretion into the blood. The renal NAD+: NADH ratio was also found to be significantly decreased which implies increased anaerobic activity within the renal tissue, which could possibly lead to ischaemic conditions within the renal tissue (Carney, 2016; Nikolaeva *et al.*, 2016).

Gene	Function	RNA Source
Slc9a3 (NHE3)	Sodium/Hydrogen exchange	Whole kidney
Gilz	Leucine zipper protein/regulator of sodium transport	DCT, CNT, CCD, Whole kidney
Usp2	Ubiquitin specific protease/ regulator of sodium transport	DCT, CNT, CCD, Whole kidney
V1aR	Vasopressin receptor/regulation of water balance	DCT, CNT, CCD, Whole kidney
V2R	Vasopressin receptor/regulation of water balance	DCT, CNT, CCD, Whole kidney
Slc6a6	Taurine transporter	CCD
Slc6a9	Glycine transporter	DCT/CNT
Aqp2	Water channel	CCD
Aqp4	Water channel	CCD
Scnn1a (αENaC)	Alpha subunit of epithelial sodium channel	Cortex, outer medulla and inner medulla
DCT, distal convoluted tubule; CNT, connecting tubule; CCD, cortical collecting duct		

Table 1 - A list of Clock-controlled transport genes in the kidney, taken from Stow and Gumz, (Stow and Gumz, 2011)

The Kidneys have been shown to be sensitive to light cues and food cues. Diurnal rhythms of urine, sodium and potassium reveal peak levels in the morning and the lowest levels at night (Solocinski and Gumz, 2015). A study investigating light cue effect on the kidney clock found that reversing the light-dark cycle for Wistar rats induced a 4-hour phase delay in *Per1*, but no changes in *Clock* gene expression, suggesting that the kidney clock are self-sustained (Costello et al., 2022). SCN lesioning also did not abolish the renal peripheral rhythm but caused phase desynchrony, reiterating the role of the central clock to coordinate the peripheral clocks. In addition to the SCN influencing the renal peripheral clock, the Kidney clock has been suggested to possess a feedback mechanism to the SCN. This was found in a recent study that utilised an adenine-induced CKD model in mice and found impaired behavioural rhythms despite having an intact SCN (Costello et al., 2022). The timing of food is a major zeitgeber that influences blood pressure homeostasis and therefore would influence the renal clock. Restricted feeding of mice during their inactive period was found to invert the blood pressure rhythm, independent of BMAL1 activity and without changing the rhythm of renal sodium excretion (Costello et al., 2022). Restricted feeding was also

found in induce a phase shift in PER2 in the renal medulla but not in the SCN, revealing that food availability affects peripheral clocks only. A study revealed that changes in core body temperature has been shown to induce phase shifts in peripheral clocks including the kidney clock, revealing temperature as an output of the clock and an input signal to the peripheral clock like the kidney (Costello et al., 2022). Finally, it was suggested that the timing of afferent and efferent nerve activity influences the expression of clock-controlled genes (Costello et al., 2022).

The circadian clock can also influence renal physiology indirectly via hormonal rhythms (Costello et al., 2022). The mineralocorticoid aldosterone for example plays an important role in water regulation in the distal nephron via RAAS. In humans, there is evidence that urinary aldosterone exhibits a circadian pattern, with a peak in the morning and higher levels during the day compared to the night. There is evidence that aldosterone is influenced by CRY1 and CRY2, with KO mice exhibiting salt sensitive, non-dipping hypertension with higher levels of plasma aldosterone, ameliorated by Mineralocorticoid receptor blockers (Costello et al., 2022). Costello *et al.,* identified *Per1* regulates aldosterone, with decreased expression causing reduced aldosterone in both plasma and urine and increase sodium excretion. Cortisol glucocorticoids, cortisol and corticosterone in humans and rodents respectively, also exhibit a 24hr rhythm, peaking similarly in the early active period. Circadian control of glucocorticoid secretion is influenced by the central clock. Glucocorticoids play a role in renal function by increasing renal blood flow and GFR (Costello et al., 2022). They are also speculated to influence renal vascular resistance and water/electrolyte metabolism. Glucocorticoid activity influences the diurnal variation of sodium chloride cotransporter activity and NHE3 channel accumulation in the proximal tubule (Costello et al., 2022). Disruption of glucocorticoid release was suggested to dysregulate the circadian clock as it affects renal function and blood pressure rhythm. Arginine vasopressin, which is synthesised in the hypothalamus, influences renal sodium handling, blood pressure regulation and the cortico-medullary osmotic gradient (Costello et al., 2022). Evidence speculates arginine vasopressin is influenced by the circadian clock, with higher levels observed late in the inactive phase of the circadian rhythm. However, evidence has not been consistent, possibly due to the high clearance rate of AVP in the blood. In mice lacking folate, the rhythms of vasopressin were found to be decreased compared to normal mice (Solocinski and Gumz, 2015). Folate is a co-factor to CRY1 and CRY2 so could have an impact on circadian rhythms. This is supported by rats lacking folate experience decreased amplitude in melatonin secretion. Interestingly, folate is found to decrease in availability with age so it could be involved in the reduced efficacy of the circadian clock with age (Solocinski and Gumz, 2015). Melatonin is a key regulator of the sleep/wake cycle of the circadian clock and is used as a messenger to regulate physiological function. There was a suspected correlation

between low levels of melatonin with a decrease in renal function (Costello *et al.,* 2022). Rats with CKD for example have decreased intrarenal RAAS activity and exhibit a decreased burden of kidney injury when treated with melatonin (Costello et al., 2022). This information provides promise of a link between renal clock and melatonin, but more research is needed.

1.3.3 Circadian messengers and the kidney

1.3.3.1 GABA and the Kidney

Expression of GABA and related enzymes were first discovered in kidney tubular cells and isolated brush border vesicles (Erdö and Wolff, 1990). Since then, GABA immunostaining was identified in epithelial cells of the thin and thick ascending limb of the loops of Henle, the connecting tubules, the collecting ducts, the distal tubules, the juxtamedullary cortex and the medulla (Wildman *et al.*, 2014). Expressions of a1, a5, b1, c1 and c2 GABAA receptor subunits were identified in the rat cortex (Takano *et al.*, 2014). GABAA subunits β2 and β3 were identified in humans, rats and rabbits, primarily in the proximal tubule (Sarang *et al.*, 2001). The review by Takano *et al* reveals the presence of GABAB receptors R1 and R2, GABA enzymes GAD65 and GAD67 and GAT2 transporter within the cortex (Takano *et al.*, 2014). Because GABA producing enzymes, transporters and degrading enzyme were all detected, it is suggested of the existence of a local renal GABAergic system with an autocrine/paracrine mechanism(Kobuchi *et al.*, 2009). GABA immunostaining found by Párducz *et al* suggests GABA involvement in the tubular transport process, potentially modulating Cl^- and Ca^{2+}/K^+ ion transport (Parducz *et al.*, 1992). GABA receptors and GABA signalling may play important roles in renal physiology. Glutamate receptors have been identified in the kidney as an excitatory signalling mediator. Specifically, NMDA expression is found in the renal cortex and medulla, associated to play a role in regulating renal blood flow, glomerular filtration and proximal tubule reabsorption (Dryer, 2015). As GABA is typically the inhibitory counterpart to glutamate in the CNS, it is assumed GABA plays a role in the kidney. Sustained activation of NMDA receptors induces oxidative stress so GABA could reduce oxidative stress. The primary action of GABA upon the kidney is to mediate blood pressure and nutrient retention in humans and rodents, hinting at GABAs antihypertensive role (Takano *et al.*, 2014; Wildman *et al.*, 2014). GABA has been reported to regulate blood pressure by increasing excretion of water and sodium ion in the kidney ex vitro (C. Chen *et al.*, 2019). GABAA receptor agonist muscimol and GABAB agonist baclofen induced increased water and sodium excretion in isolated rat kidney perfusion (Takano *et al.*, 2014).

GABA's role in communication between the CNS and the Kidney must also be considered. The sympathetic nervous system influences renal regulation of arterial pressure and the composition of body fluids (Nishi, Bergamaschi and Campos, 2015). Sympathetic nerve activity has been associated with renal tubular water and sodium reabsorption through the nephron, changes in renal blood flow and Glomerular filtration rate by changing the RAAS via renin release and regulating renal vasculature constriction. GABA is associated with sympathetic and parasympathetic activity and therefore blood pressure homeostasis. In terms of efferent signals to the kidney, the PVN is one of the areas that innervate sympathetic preganglionic neurons. GABA is considered an output signal, coordinating sympathetic and parasympathetic signal by communicating from the SCN to the PVN, thus GABA could modulate efferent signals to the kidney. Glutamergic input to the rostral ventrolateral medulla is seen to be increased during hypertension and inhibition of this input by GABA ameliorates hypertension. This is confirmed by Kobuchi *et al.,* who found central administration of GABA and GABAB agonists decrease blood pressure by decreasing sympathetic tone (Kobuchi *et al.,* 2009). GABA and GABAB agonists also inhibit vascular constriction induced by noradrenaline release. Interestingly, increased renal sympathetic nerve activity and noradrenaline release are associated to the progression of renal IRI (Wildman et al., 2014). Intravenous treatment of baclofen attenuates sympathetic nerve enervation and noradrenaline release observed in ischaemia induced acute kidney injury in rats. GABA Treatment attenuates the progression of glycerol-induced acute renal failure, highlighting nerve activity as a critical component to prevent renal dysfunction.

Finally, GABA is implicated in the regulating renal medullary blood flow via pericyte vasoactivity. As stated previously, contractile pericytes are known to regulate capillary diameter in a similar way to vascular smooth muscle cells in larger vessels (Wildman et al., 2014). Whether GABA acts as a vasoconstrictor or vasodilator is up to debate. Uptake of GABA into astrocytes-initiated vasoconstriction of blood vessels in the nerve layer of the olfactory bulb (Wildman et al., 2014).) but vasodilation in cerebral arteries *In vivo* (Edvinsson and Krause, 1979). Wildman *et al* specified GABA induces vasoconstriction in the afferent arteriole in the rat kidney and their work found that GABA induced pericyte mediated vasoconstriction of vasa recta, thus identifying that GABA regulates medullary blood flow (Wildman et al., 2014)..

1.3.3.2 Arginine Vasopressin and the kidney

The interplay of the sympathetic nervous system, the renin angiotensin aldosterone system (RAAS), and AVP all can contribute to the blood pressure (BP) regulation (Mavani, DeVita and Michelis, 2015). In normal circumstances, AVP does not play a significant role in the BP maintenance. However, human experiments reveal vasopressin deficiency contributes to the vasodilatation of septic shock. This was

considered depletion of vasopressin stores thus implicating vasopressin as a crucial safety mechanism in BP maintenance. It is also suggested that a higher level of vasopressin is required for sodium conservation and sodium absorption occurs when AVP levels exceed a threshold. A study investigating acute stimulation of V2 receptors in men by Desmopressin showed reduced urine volume and an increase capacity of epithelial sodium channel (ENaC) receptors to reabsorb sodium in the distal tubule (Mavani, DeVita and Michelis, 2015).

Vasopressin is well known to coordinate blood pressure so plays an important role in the kidney. V1 receptors are identified in the kidney, with high density on medullary interstitial cells, vasa recta blood vessels and epithelial cells of the collecting duct (Holmes, Landry and Granton, 2003). Vasopressin acts on V1 receptors to reduce blood flow to the inner medulla without affecting the outer medulla(Rostron *et al.*, 2007). V1 receptors activity also selectively induces constriction of the efferent arterioles. This tends to increase glomerular filtration, an effect that can be paradoxical. For example, when ADH is increased in response to vasodilatory shock, the urine output increases due to selective increase in the efferent arteriole which is counterproductive. Action of V1 receptors in the collecting ducts decreases the glomerular filtration while inducing states of hypertension. This opposing action on the glomerular filtration rate by V1R activity within different regions of the kidney could compensate and promote water retention when during vasodilatory shock. V2 receptors present the most known antidiuretic effect of vasopressin increasing the osmotic water permeability of the renal collecting duct (Holmes, Landry and Granton, 2003). The increased cAMP activity in the kidney by V2R activity triggers aquaporin-2 expression on the apical plasma membrane of the collecting ducts, which promotes water reabsorption. In addition, Vasopressin increases the upregulation of aquaporin2 mRNA and promotes aquaporin 2 vesicle transport to the cell surface. AVP interacts with V2 receptors to cause direct and indirect stimulation of renin production and release (Mavani, DeVita and Michelis, 2015).

1.3.3.3 Corticosterone and the Kidney

It has become apparent glucocorticoids display similar influence on kidney physiology and function as mineralocorticoids. It was revealed that deficiencies in the conversion between active and inactive glucocorticoids in the kidney can lead to hypertension (Usa et al., 2007). This is possibly due to the reduced competition of glucocorticoids like corticosterone to MRs, with a decline in active glucocorticoids causing unrestricted binding of aldosterone. This theory is supported by evidence that 11-HSD inhibitor carbenoxolone induces renal medulla induced hypertension. 11β-HSD1 expression was found to be higher in the medulla than in the cortex, whereas 11β-HSD2 was most enriched in the outer medulla, maybe

revealing most of the glucocorticoid activity residing within the medulla (Usa *et al.*, 2007). Usa *et al* commented that 11-HSD2 deficiency can lead to the development of mineralocorticoid excess syndrome, which causes hypertension and hypokalaemia due to prolonged MR activity. The resulting corticosterone increase in the renal medulla could mediate or contribute to the concomitant hypertension (Usa et al., 2007). Despite predominantly responsible for blood vessel vasoconstriction and increasing vascular resistance, there has been evidence that glucocorticoids can cause vasodilation (De Matteo and May, 1997). This response seems exclusive to the kidney, where glucocorticoids increase renal blood flow and the glomerular filtration rate. Analysis by De Matteo and May demonstrated that this effect was induced by NO production at the endothelium.

Corticosterone is implicated in the development of the RAAS, as maternal corticosterone exposure has been suggested to programme RAAS in their offspring (Cuffe *et al.*, 2016). Short term administration of maternal corticosterone mid-gestation reduces nephrons in both male and female mice offspring. Analysis in the sex differences in RAAS in 6-month offspring revealed that in untreated mice, female offspring had higher intrarenal renin and aldosterone concentrations, but males possessed higher intrarenal angiotensin II. When exposed to maternal corticosterone, male offspring experienced a reduction in both water intake and urine excretion, but females were unaffected (Cuffe *et al.*, 2016). Molecularly, male offspring also experienced elevated expression of Ren1 and Ace2, encoding renin and angiotensin II converting enzyme as well as aldosterone receptor (MR) gene Nr3c2 and renal sodium transporters. In adulthood, excess glucocorticoids/mineralocorticoids and renal Nr3c2 signalling impairs renal function and blood pressure regulation. Male offspring analysed by Cuffe *et al* displayed impaired renal function and blood pressure regulation as well as reduced nephron number.

Renal MR activation regulates transcription of multiple factors responsible for sodium, potassium and fluid homeostasis, including ATPase α1 subunit and sodium channels on the epithelium (Cuffe *et al.*, 2016). MR activation in the kidney can occur at other sites than the aldosterone sensitive distal nephron including the endothelium, smooth muscle and inflammatory cells (Barrera-Chimal and Jaisser, 2020). Overstimulation through MRs in the kidney contributes to renal pathophysiology. MR activation increases ROS production and induces oxidative injury by increased NADPH oxidase activity and inhibiting endothelial nitric oxide synthase activity which is linked to endothelial dysfunction. The increased activity of endothelial NADPH oxidase may further activate MRs through increasing active glucocorticoids, which may inadvertently inhibit aldosterone binding by competitive inhibition. Within vascular smooth muscle, MR activation induces proliferation and oxidative injury through a similar method to endothelial cells.

Persistent MR activation induces vasoconstriction, oxidative stress and increased myogenic tone, all of which have detrimental effects on long term blood pressure control, a likely method for the physiological hypertension with aging. MR activation promotes expression of genes involved in MR-dependent vascular remodelling and calcification in VSMCs (Barrera-Chimal and Jaisser, 2020). Glucocorticoid activity is also suggested to modify the progression of chronic renal failure (Quan, Walser and Hill, 1992). This was suggested due to the correlation between urinary `7-hydroxycorticosteroids and the rate of progression of chronic renal failure. In addition, other work found ketoacid supplements slow this progression and suppress glucocorticoid production. Despite this evidence, corticosterone was not identified to be involved in the progression of renal failure with the model utilised by Quan *et al* as amelioration of CKD by adrenalectomy was not reversed with supplementation with low or high corticosterone levels (Quan, Walser and Hill, 1992). It is suspected another molecule is responsible, probably aldosterone because of its clear role in kidney function.

MR activation of inflammatory cells in the kidney can lead to kidney injury. MR has already been implicated in T cell development in the thymus. It was found that mice that lack MR show a decrease in vascular and renal damage due to a decline of IFNγ producing T cells (Barrera-Chimal and Jaisser, 2020). MR is also involved in inducing M1 Macrophage phenotype. Pharmacological inhibition of MR has been seen to ameliorate the effects of inflammatory cell infiltration and vasoconstriction such as in ischaemia/reperfusion injury (Barrera-Chimal and Jaisser, 2020). This MR inhibition prevented or reduced disease onset by reducing oxidative stress by reduced Rac1 induced ROS production in vascular smooth muscle cells and the increase of NO production. L-type calcium channel activity in vascular smooth muscle is also reduced which prevents vascular calcification. Overall effects of MR activation that contribute to renal injury is illustrated in figure 14 (Barrera-Chimal and Jaisser, 2020)**.**

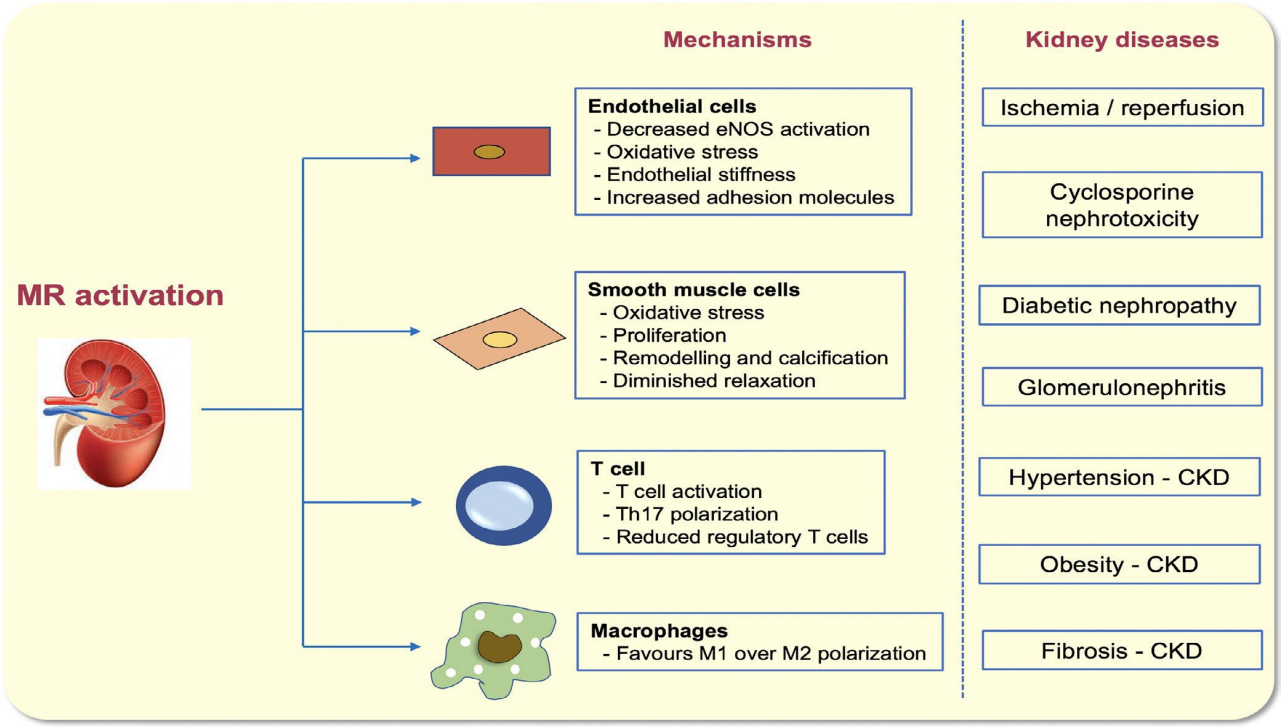

Figure 14 - The Pathological mechanisms caused by MR activation in endothelial cells, smooth muscle cells, T cells and macrophages that promote kidney injury and contribute to kidney disease. Figure taken from Barrera-Chimal and Jaisser, (Barrera-Chimal and Jaisser, 2020)

1.3.3.4 Melatonin and the Kidney

Melatonin plays a prominent role in many physiological processes, including regulating circadian and endocrine rhythms, influencing immune function and preventing adverse effects of antibiotics, including renal failure (Tavakoli, 2014). A key role of melatonin in the kidney is as an antioxidant. The kidney works with the liver to coordinate xenobiotic metabolism and excretion. Therefore, kidneys have a greater load of free radical activity and thus are more prone to oxidative damage (Tavakoli, 2014) It was already discussed that melatonin is an efficient neutraliser of free radicals, which is dependent on the ability to act as an electron donor (Nava et al., 2000). The ability of melatonin to cross morphophysiological barriers unrestricted and easy access to subcellular compartments also aid in ameliorating ROS activity. In addition, melatonin stimulates antioxidant enzymes such as superoxide dismutase, GSH and glutathione reductase. *In vivo* and *in vitro* melatonin protected tissues against oxidative damage generated by various toxic agents and metabolic processes, including ischemia reperfusion injury in kidney (Tavakoli, 2014). Nava *et al* specified that melatonin markedly alleviated renal histological damage and reduced ROS induced apoptosis of renal tissue by inhibiting apoptosis transcription factors such as NF- kB (Nava et al., 2000). The beneficial effects of melatonin only occurred when melatonin was administered at least 30 minutes before administration of mercuric chloride, a toxin utilised to study nephrotoxic acute renal

failure (Tavakoli, 2014). Therefore, prevention of intense oxidative stress requires pre-existing availability of antioxidants like melatonin. Thus, melatonin has a therapeutic capability, best utilised in anticipated ROS accumulation such as in under ischaemic conditions i.e. Ischaemia reperfusion injury.

Melatonin's potential as a therapeutic agent was expanded upon with a recent study that assessed drug induced nephrotoxicity, a frequent adverse condition and major complication of drug therapy. 66% of renal failure in the elderly population is due to drug induced nephrotoxicity, with high incidence or morbidity and mortality in 40-70% of all renal injuries (Raza and Naureen, 2020). This nephrotoxicity occurs when therapeutic doses of several drugs are increased over time due to drug resistance. Increased doses reach toxic levels, and nephrotoxicity occurs as the kidney cannot compensate. A way to reduce the risk of drug induced toxicity is by using these drugs with others known as therapeutic adjuncts to compensate for the toxic levels. Melatonin's potent antioxidant action by receptor mediated and receptor independent means has made it an ideal candidate as a therapeutic adjunct. Melatonin attenuates nephrotoxicity of various drugs, including anticancer, antibiotic and immunosuppressant drugs. The receptor mediated pathway of melatonin increases the expression of antioxidant enzymes, modulates various inflammatory cytokines and reduces apoptotic signalling to promote cell survival. For example, melatonin stimulates hemeoxygenase-1 (HO-1) and anti-inflammatory cytokine IL-10 to mitigate apoptosis and CD19 B cell activity as identified in idiopathic membranous nephropathy (Raza and Naureen, 2020). The receptor independent mechanism of melatonin is the ability to neutralise free radicals which protects against cellular and subcellular oxidative damage. Free radicals if not neutralised induce an inflammatory cascade by increasing RNA expression of chemoattractant and adhesion molecules such as ICAM-1, MCP-1 and CSF-1, thus promoting immune infiltration. The ability to scavenge free radicals is speculated to be associated with melatonin's indole ring accepting electron donation (Raza and Naureen, 2020).

Melatonin has an additional role in renal function i.e. suppressing the RAAS system in the Kidney (Ohashi, Ishigaki and Isobe, 2019; Rahman, Hasan and Kobori, 2019). RAAS is implicated with renal pathophysiology, being a major contributing factor to CKD and hypertension as it induces renal inflammation and fibrosis. Recent studies have shown an association between impaired night-time levels of melatonin and the presence of urinary angiotensinogen excretion, a surrogate marker of intrarenal RAAS activation and renal damage in CKD patients (Ohashi, Ishigaki and Isobe, 2019). Exogenous melatonin ameliorates intrarenal RAAS activation and renal injury in chronic CKD animal models. Emerging evidence suggests the irregular increase in RAAS triggers increased ROS production, an imbalance of

sodium homeostasis and hypertension, conditions that can lead to the progression of CKD. In addition, sleep disorders are highly prevalent in patients with CKD and sleep disorders in turn contributes to CKD through direct intrarenal renal hypoxia induced by elevated RAAS (Rahman, Hasan and Kobori, 2019). This combined information interlinks melatonin, RAAS and CKD, speculating melatonin could regulate RAAS activity and alleviate CKD. Melatonin levels are dysregulated in CKD patients, which would explain the sleep disorders and increased ROS damage. Melatonin also maintains the bioavailability of nitric oxide (NO) by action on MT2 receptors which promotes endothelium dependent vasodilation and thus alleviates hypertension (Rahman, Hasan and Kobori, 2019). Moreover, melatonin administration to nephrectomised rats caused a significant reduction of urinary angiotensinogen and expression of angiotensin 1 receptors and angiotensin 2 in the kidney suggesting that melatonin suppresses intrarenal RAAS and could be a potential therapeutic agent to ameliorate CKD. It was also demonstrated that melatonin supplementation improves objective sleep onset latency, sleep time/efficacy and subjective sleep parameters (Rahman, Hasan and Kobori, 2019). Of note, intrarenal RAAS was found to trigger obstructive sleep apnoea thus melatonin supplements could improve obstructive sleep apnoea by relieving intermittent hypoxia and cognitive impairment generated by obstructive sleep apnoea (Wei *et al.*, 2025). Supporting this, melatonin was suggested to manage sleep disturbances in chronic dialysis therapy patients (Kalra, Agrawal and Sahay, 2012).

Overall, the effect of melatonin on renal function is still being discovered with few pharmacological studies conducted. Melatonin acts through multiple pathways, as an antioxidant, a modulator of apoptosis and a circadian modulator of vascular function within the kidney (Kalra, Agrawal and Sahay, 2012). Melatonin rhythms inversely correlate with ambient blood pressure which implies melatonin regulates blood pressure. Majority of kidney studies focus on melatonin as a therapeutic agent for CKD. The combined studies show melatonin can reduce proteinuria and inflammation as well as restore normal sleeping patterns in patients with renal dysfunction.

1.4 Pericytes physiology and circulation

1.4.1 Pericyte overview

Pericytes are mural cells of microvasculature embedded in the vessel basement membrane, wrapping around and surrounding endothelial cells (Dessalles *et al.*, 2021). Pericytes are found in almost all capillaries, as well on some smaller arterioles and venules (Hamilton, Attwell and Hall, 2010). Pericytes morphologically differ depending upon the position along the arterio-venous axis and on the vascular bed

as illustrated in figure 15 (Attwell *et al.*, 2016). Pericyte share morphology similar to fibroblast-like cells with a pronounced nucleus (soma), little cytoplasm and multiple projections extending from the soma around the microvasculature (Gökçinar-Yagci, Uçkan-Çetinkaya and Çelebi-Saltik, 2015). Pericytes are characterised as spatially isolated cells with a 'bump-on-a-log' morphology along microvasculature (Sweeney *et al.,* 2016). Pericytes are present at regular intervals along the walls of capillaries, pre-capillary arterioles and post-capillary venules (Attwell *et al.,* 2015). Smaller blood vessels like capillaries comprise of endothelial cells surrounded by basal membrane and pericytes (Gökçinar-Yagci, Uçkan-Çetinkaya and Çelebi-Saltik, 2015). Arteries and veins are composed of three layers; tunica intima, tunica media and tunica adventitia with pericytes if found are located between the tunica intima and tunica media layers, around the endothelial layer as found in other vessels (Gökçinar-Yagci, Uçkan-Çetinkaya and Çelebi-Saltik, 2015).

Pericytes exist in two states; Quiescent, where pericytes are structurally characterised with a central protruding soma with elongated branches that surround endothelial cells, and active pericytes, where the pericytes removes itself from the basement membrane (Dessalles *et al.*, 2021). In the quiescent state, Pericytes send primary projections along the blood vessel to cause secondary and tertiary processes that further project around the vessel (Hamilton, Attwell and Hall, 2010). Quiescent pericytes are further divided into 3 subtypes: ensheathing pericytes (ePCs) on precapillaries, thin-stranded pericytes (tsPCs) on capillaries and stellate pericytes (sPCs) on post capillaries (Dessalles *et al.*, 2021). The first two subtypes differ slightly, with ePCs having short primary projections and secondary processes that fully encircle the vessel whereas tsPCs the primary processes are longer and secondary processes only partially wrap around the vessel. Stellate pericytes however, lose the orthogonal organisation of the primary and secondary processes and are situated in a fractal branching pattern (Dessalles *et al.*, 2021). Regardless of subtype, the processes co-localise with endothelial cell junctions, with ePCs and sPCs having high coverage and tsPCs with low coverage along the vessel respectively. Active pericytes break from the basement membrane and have 2 action stages; pericyte retraction and detachment from the vessel wall and the pericyte migration into the parenchymal tissue (Dessalles *et al.*, 2021). This active phase of pericytes can be observed in angiogenesis where pericytes detach, allowing endothelial cells to migrate and proliferate into new vessels. A way to distinguish between quiescent and active pericytes is in how they migrate. Quiescent pericytes migrate via "crawling" along the vessel wall, whereas active migration involves retracting and "escaping" the vessel (Dessalles *et al.*, 2021).

Pericytes interact with the vessel basement membrane, endothelial cells and other pericytes (Dessalles *et al.*, 2021). Pericytes enable direct contact to endothelial cells through multiple methods as illustrated in figure 16 (Dessalles *et al.*, 2021). One method is by forming peg-socket invaginations, microstructures that allows the pericyte to protrude inside the endothelial cell. Another method is by the formation of Gap junctions, forming transmembrane channels for rapid cytosolic exchange between endothelial cells and pericytes. The third method is via adherens junctions, composed of transmembrane N-Cadherens that form a direct link between the actin cytoskeleton of both cell types (Dessalles *et al.*, 2021). Pericytes also interact indirectly with endothelial cells by adhesion plaques, fibronectin patches within the basement membrane that link with adjacent endothelial cells. In addition, endothelial-pericyte communication is facilitated by several signalling pathways to coordinate proliferation and differentiation of both cell types (Dessalles *et al.*, 2021). One pathway is the angiopoietin 1/Tie 2 pathway/ angiopoietin 2/Tie 2 pathway. Pericytes express angiopoietin 1 which interact with the Tie2 receptor on endothelial cells to promote vessel stabilisation and maturation (Gökçinar-Yagci, Uçkan-Çetinkaya and Çelebi-Saltik, 2015). Angiopoietin 2 on the other hand is expressed dominantly in endothelial cells and induces pericyte detachment and vascular destabilisation.

NG2 proteoglycan is one of the prominent pericyte markers and has been used to accurately quantify pericyte: endothelial cell ratio in different vascular beds and reveal pericyte participation within all stages of blood vessel formation(Birbrair, 2018). NG2 influences pericyte proliferation and motility by acting as an auxiliary receptor to enhance integrin and tyrosine kinase growth factor receptor signalling. PDGF receptor β (PDGFRβ) is another commonly used marker for pericytes and is used with NG2 to reliably identify mural cells during the early stages of microvessel formation (Birbrair, 2018). Endothelial cells also interact with pericytes by PDGF-B release, a molecule proven critical for pericyte function (Hamilton, Attwell and Hall, 2010). PDGFRβ activates in response to PDGF-B and is responsible for promoting pericyte migration and expansion along the vessel during angiogenesis (Birbrair, 2018). CD146 is another marker to detect pericytes, expressed by perivascular endothelium, pericytes and smooth muscle cells. All pericytes express CD146 so it is used to isolate pericytes from heterogeneous cell suspensions from human tissues (Gökçinar-Yagci, Uçkan-Çetinkaya and Çelebi-Saltik, 2015). As most markers for pericytes are not exclusive to this cell type, double labelling with markers such as NG2, PDGFRβ or CD146 are required to confirm the identified cell is a pericyte (Birbrair et al., 2015; Navarro et al., 2016; Birbrair, 2018). Pericytes form discontinuous layers, a characteristic that discern pericytes from smooth muscle. Pericytes appear to not have physical contact with neighbouring pericytes, but their distribution may allow transient contact to detect local pericytes. A research article that analysed brain pericytes supports this,

finding the Brain pericytes were observed to constantly extend and retract its processes to probe surroundings, which could be a method pericytes use to remodel after injury (Dessalles *et al.*, 2021).

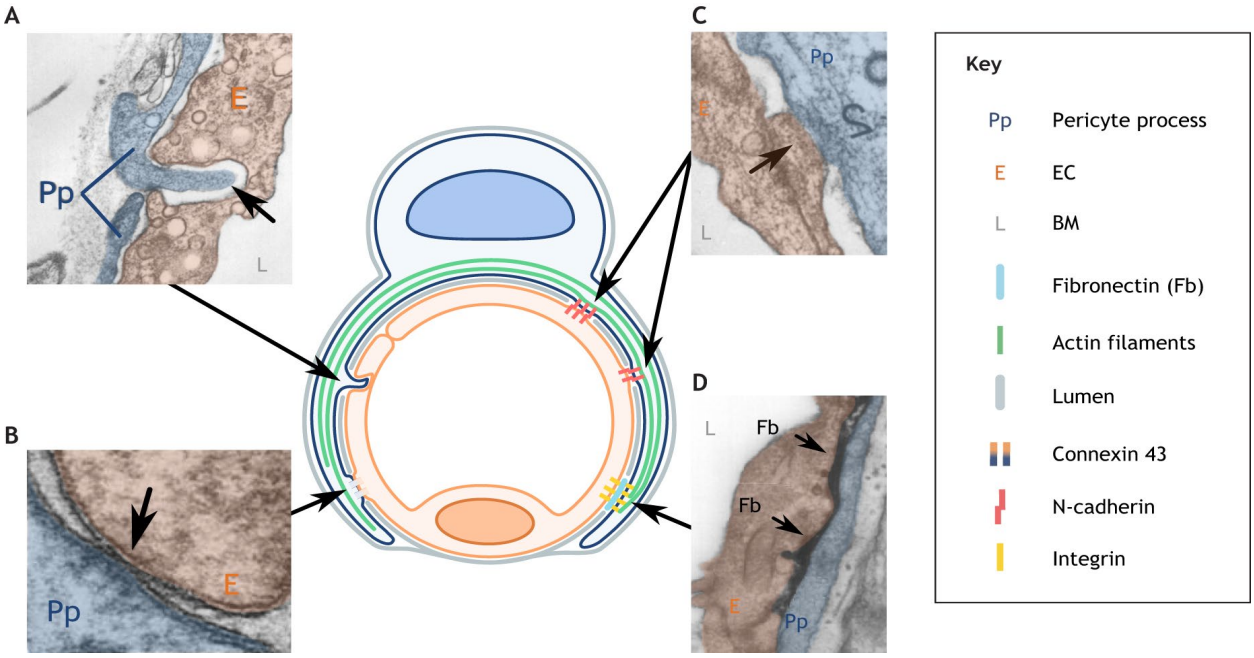

Figure 16 - Illustration of different Pericyte–Endothelial cell interactions (A-D), taken from Dessalles *et al.,* (Dessalles *et al.*, 2021)**.**

(A) Pericytes form peg and socket invaginations that protrude into the endothelial cell. (B) Gap junctions, Pericytes and endothelial membranes are aligned and possess connexin 43 channels that allow direct transfer of ions and small molecules between cells. (C) Cell-cell contact via transmembrane proteins allows formation of adherens junctions between cell types. (D) via adhesion plaques, Pericytes are linked to the fibronectin patches in the basement membrane which indirectly link pericytes to endothelial cells.

Pericytes have been associated with a range of functions such as angiogenesis, vessel formation and stabilisation, endothelial cell regulation, blood flow regulation and blood brain barrier maintenance (Hamilton, Attwell and Hall, 2010; Dessalles *et al.*, 2021). An essential function of mural cells like Pericytes is the ability to regulate blood flow. Pericytes have been found to be contractile, containing similar machinery to smooth muscle function (Hamilton, Attwell and Hall, 2010). Pericyte contraction and relaxation generates changes in vessel diameter of up to 20% which translates to a huge impact on the flow rate. An example of pericytes impact on blood flow is demonstrated in the no-reflow phenomenon. Loss of tsPCs is implicated in no-reflow that occurs after cerebral and cardiac ischaemia thus highlighting the importance of pericytes in blood flow control (Dessalles *et al.*, 2021). There are multiple theories that describe how pericytes constriction regulates blood flow (Figure 17) (Dessalles *et al.*, 2021). One

hypothesis theorises the pericyte constriction increases the vessel wall stiffness, reducing the ability to dilate in response to higher blood pressure. The second hypothesis describes pericyte constriction pulling the endothelial cells out of shape, causing it buckle. Consequently, fenestrations of endothelial cells are forced open, allowing enhanced fluid exchange between the blood vessels and the tissues. The final method applies for pericytes with processes that fully encircle the endothelial cell and describes the conventional concept of constricting blood vessels by compressive forces (Dessalles *et al.*, 2021).

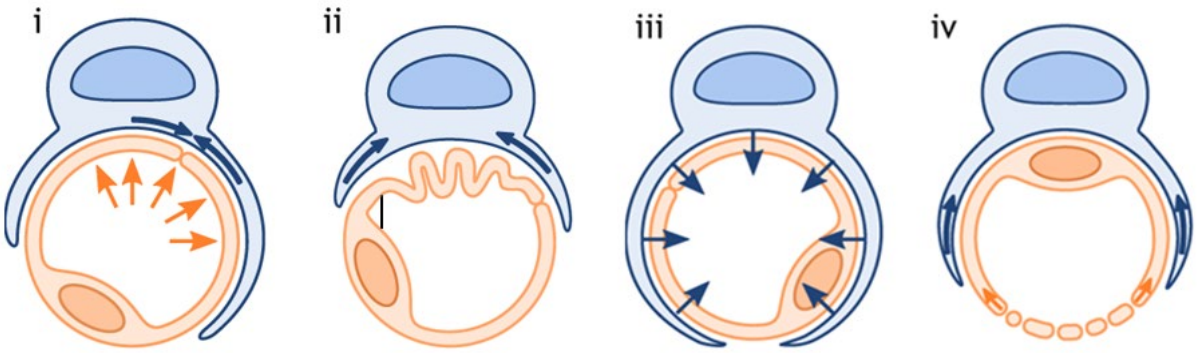

Figure 17 - The effects of pericyte contraction onto endothelial cells, taken from Dessalles *et al.*, (Dessalles *et al.*, 2021)**.**

(i) Pericytes strengthen the endothelial cell junctions to resist the luminal pressure. (ii) Pericytes cause tangential pulling, which in turn induces narrowing and buckling of the underlying endothelial cells. (iii) Depicts the typically characterised constriction of capillaries by pericytes (iv) Fenestrations of the endothelial cell are forced open due to tangential pulling, allowing enhances fluid exchange.

As specified prior, pericytes are implicated in vasculogenesis and angiogenesis which are the terms for generating new blood vessels in embryogenesis and generating new blood vessels from pre-existing vessels respectively (Gökçinar-Yagci, Uçkan-Çetinkaya and Çelebi-Saltik, 2015). For Vasculogenesis, TGF-β1 and PDGF-B stimulate differentiation and expansion of pericytes and endothelial cells to form a primary plexus. In angiogenesis, the vessel plexus is remodelled to become functional by promoting endothelial sprouting and bridging. Once stimulated by angiogenic factors, endothelial cells proliferate and migrate to form the new vessel lumen and in turn recruit new pericytes to the vessel. This way, pericytes support vessel maturation and aids in transferring angiogenic signals along the vessel (Gökçinar-Yagci, Uçkan-Çetinkaya and Çelebi-Saltik, 2015). Pericytes are proven integral to maintain the integrity of the Blood-Brain-Barrier (BBB), by controlling the expression of endothelial tight and adherens junctions with brain microvasculature (Sweeney, Ayyadurai and Zlokovic, 2016). Endothelial-Pericyte communication are manipulated to open the blood brain barrier “on demand” to allow delivery of neuro-pharmaceuticals and to reverse BBB breakdown. Degeneration of Pericytes and blood brain barrier breakdown has been

identified in complex neurological disorders. Pericytes have also been found to possess multipotent precursors for several different cell types, allowing it to form neurons and glial cells within the CNS (Hamilton, Attwell and Hall, 2010). Multiple studies have uncovered that perivascular cells like pericytes are counterparts of mesenchymal stem cells (Gökçinar-Yagci, Uçkan-Çetinkaya and Çelebi-Saltik, 2015).

1.4.2 Pericytes as a vascular unit

There is a known correlation between high neural activity and increased regional blood flow, with increased blood flow is required to meet the increased metabolic demand within the vicinity of the active neurons (Hamilton, Attwell and Hall, 2010). Multiple studies describe capillaries to regulate this blood flow via pericyte activity within the capillary wall. The ratio of pericytes to endothelial cells differs among different tissues according to the function and blood pressure/blood flow requirements of the tissue (Gökçinar-Yagci, Uçkan-Çetinkaya and Çelebi-Saltik, 2015). Pericytes are most abundant in the CNS, implying that pericytes may have an important role in the nervous system, particularly where the regional blood flow is important (Hamilton, Attwell and Hall, 2010). There are neuronal terminals near capillaries and pericytes such as terminals containing GABA, vasoactive intestinal peptide and nitric oxide. This information suggests that capillary blood flow is controlled at least partially by neuronal input.

Pericytes in the brain, retina and spinal cord capillaries express αSMA, with expression higher where pericytes are found in branching points from arterioles and venules, supporting the notion of pericytes as a contractile cell (Hamilton, Attwell and Hall, 2010). This is supported with electron micrographs of brain pericytes, revealing these pericytes contain actin and myosin- like microfilaments. Cultured pericytes constrict in response to vasoactive agents such as Endothelin-1 and angiotensin 2 and also dilate to others such as Prostaglandin I_2 and Nitric Oxide. Neural release of messengers to pericytes such as noradrenaline and serotonin also induce vasoconstriction. High metabolism also affects vasoactivity, as ADP and a more acidic pH induces vasodilation in arterioles and cultured pericytes, indicating a more direct response to high metabolism (Hamilton, Attwell and Hall, 2010).

Lactate has an interesting relationship with pericytes as lactate production within retinal pericytes induces constriction in a high oxygen microenvironment but dilation in low oxygen metabolism (Hamilton, Attwell and Hall, 2010). This response is dependent on endothelial communication with the pericytes. Endothelial cells possess Sodium-Calcium exchange channels that in normal oxygen conditions increase intracellular calcium concentration transport via gap junctions which result in constriction. Hypoxia causes these gap junctions to close, therefore inhibiting this intracellular calcium influx. PDGF-B, has a similar effect upon

Pericytes, instructing pericytes to constrict in normal oxygen conditions but in ischaemic conditions evokes dilation. This occurs due to the way Pericytes induce constriction and dilation. Similar to vascular smooth muscle, Pericyte constriction is controlled by intracellular calcium concentration (Hamilton *et al.,* 2010). This was elucidated by the work of Peppiatt *et al.,* who proved that pericytes can be electrically blocked by removing extracellular calcium, preventing constriction (Peppiatt et al., 2006; Hamilton, Attwell and Hall, 2010). When exposed to vasoconstrictors like PDGF-B, the cell membrane is depolarised, activating voltage-operated calcium channels (VOCC), triggering calcium influx. Calcium entry triggers calcium activated chloride channels to open, thus triggering chloride ions to exit the cell due to its high intracellular chloride concentration which further depolarises the cell and therefore maximises VOCCs activation (Hamilton, Attwell and Hall, 2010). In ischaemic conditions however, PDGF-B does not activate Chloride or any other cation channels and instead activates Potassium ATP channels, which induces vasodilation as a result (Sakagami *et al.*, 2001) These channels are also metabolically influenced as ATP decline and ADP increase promotes channel activation. Pericyte constriction is also influenced by the Rho Kinase pathway, as over expression of Rho-GTPase in pericytes increases constriction (Hamilton, Attwell and Hall, 2010).

As speculated earlier, a key mechanism of pericyte dilation is via voltage sensitive potassium channels, including K-ATP channels, inward rectifying K channels and big and small conductance calcium-potassium channels (Sakagami, Kodama and Puro, 2001; Hamilton, Attwell and Hall, 2010). Activation of these channels induces hyperpolarisation and reduced VOCC activation. Interestingly, some vasoconstrictors such as IGF-1 activate potassium channels in addition to calcium channels, which could be a mechanism to limit constriction. Other vasoconstrictors like ET-1 inhibit potassium channels to increase pericyte tone. The vasodilator NO uses an alternative method to achieve pericyte dilation, partially via inhibition of VOCC and Calcium activated Chloride channels. Nitric oxide also activates soluble guanylate cyclase which activates protein kinase G. Protein kinase G induces dilation by activating potassium channels, inhibiting calcium activated chloride channels and by activating myosin light chain phosphatase, which inhibits MLC-αSMA constriction (Hamilton, Attwell and Hall, 2010). A diagram is used to illustrate the constriction and dilation of pericytes in Figure 18.

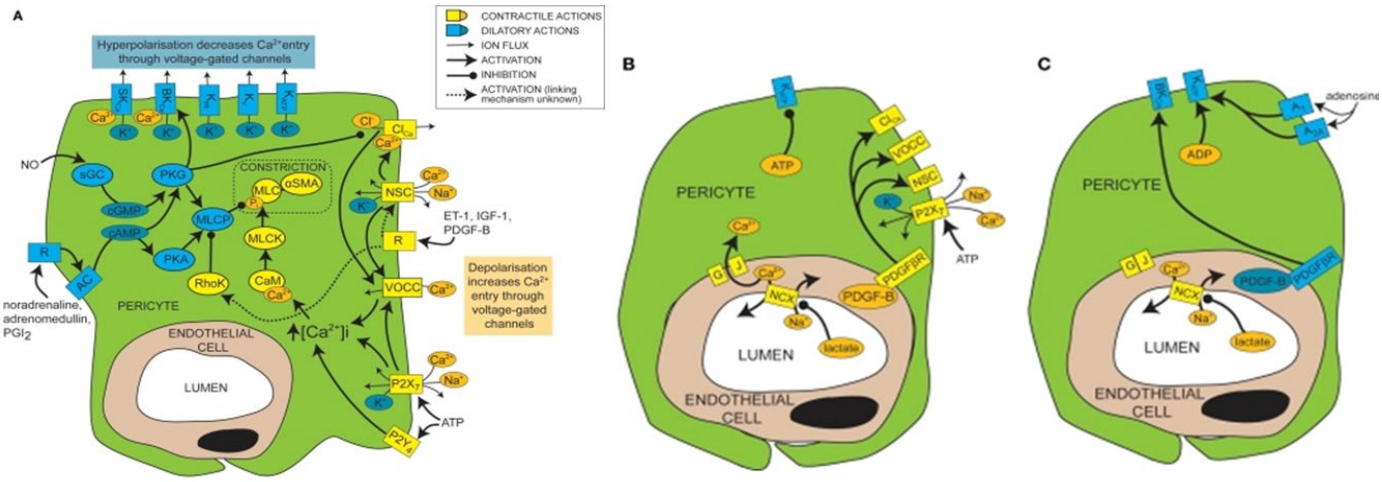

Figure 12 - Mechanisms of action that control capillary tone, figure taken from Hamilton *et al*., (Hamilton, Attwell and Hall, 2010)**.**

(A) Molecular mechanisms of vasoactive molecules acting upon receptors (labelled "R") that lead to pericyte constriction/dilation (constriction pathways are depicted as yellow/orange and dilation as blue). Rectangles represent membrane proteins; ovals are components in the cytosol. (B-C) depict the actions of molecules under different conditions. **(B)** Under the physiological conditions, with plentiful supply of O_2 and ATP. Under these conditions, levels of lactate, PDGF-B and intracellular ATP favour pericyte constriction. **(C)** Under hypoxic conditions, lactate, PDGF-B and high intracellular ADP induce dilation.

Key: SK_{Ca}, small conductance, calcium-activated potassium channel; BK_{Ca}, large conductance, calcium-activated potassium channel; K_{IR}, inwardly rectifying potassium channel; K_V, voltage-gated potassium channel; K_{ATP}, ATP-sensitive potassium channel; Cl_{Ca}, calcium-activated chloride channel; NSC, non-specific cation channels; R, ligand-binding receptor; VOCC, voltage-operated calcium channel; MLC, myosin light chain; αSMA, alpha smooth muscle actin; MLCK, myosin light chain kinase; CaM, calmodulin; RhoK, Rho kinase; sGC, soluble guanylyl cyclase; PKG, protein kinase G; AC, adenylyl cyclase; PKA, protein kinase A; MLCP, myosin light chain phosphatase; ET-1, endothelin-1; IGF-1, insulin-like growth factor 1; PDGF-B, platelet-derived growth factor-B; PGI_2, prostacyclin GJ, gap junction; PDGFβR, PDGF-B receptor.

1.4.3 Pericytes and the immune system

Pericytes are found to influence the immune response. For example, pericytes differentiate into collagen type-1 producing fibroblasts which facilitate inflammatory processes and wound healing. Pericytes also secrete several cytokines and chemokines that modulate the inflammatory process (Gökçinar-Yagci, Uçkan-Çetinkaya and Çelebi-Saltik, 2015). Pericytes have been identified to exhibit macrophage-like activity, suggesting a possible role of pericytes in the immune system (Hamilton, Attwell and Hall, 2010). In addition, Pericytes are capable of phagocytic activity and express CR3 complement factor, CD4, MHC class I and II molecules (Gökçinar-Yagci, Uçkan-Çetinkaya and Çelebi-Saltik, 2015; Navarro et al., 2016). Pericytes can perform antibody-dependent phagocytosis using Fc receptors.

Pericytes have diverse effects that influence ischaemia, such as ischaemic stroke (Yang *et al.*, 2017). Ischaemic stroke describes the sudden loss of cerebral blood flow to an area of the brain and thus results

in the loss of neurological function. In addition to being an important component of the BBB, pericytes have a central position in the neurovascular unit among neurons, astrocytes and endothelial cells. Pericytes receive and process signals from neighbouring cells and maintains brain tissue homeostasis. This includes regulating capillary haemodynamics and BBB permeability, clearing of toxic metabolites, stem cell activity and angiogenesis (Yang *et al.*, 2017). Recently, Pericytes that control capillary diameter were implicated in ischaemic stroke (Yang *et al.*, 2017). Initially, arterioles were thought to be responsible for brain blood flow regulation, but now it is clear that it can be regulated by capillaries (Peppiatt *et al.*, 2006; Khennouf *et al.*, 2018). The work of Peppiatt *et al* demonstrated pericytes constrict capillaries *in situ* in the retina and cerebellum (Peppiatt *et al.*, 2006). In ischaemia, pericytes induce capillary constriction, trapping blood cells, and therefore preventing microcirculatory reperfusion, causing cell death and loss of vascular integrity (Sweeney, Ayyadurai and Zlokovic, 2016). The pericytes remain constricted during ischaemia despite the main feeding artery reopens. This is considered the major cause of the no-reflow phenomenon to ischaemic tissue and results in pericyte cell death and loss of vascular integrity. Persistent constricted pericytes have also been seen to be enforced partially by peroxynitrite formation and oxidative/nitrative stress, as suppressing both reversed pericyte constriction and restored blood flow (Yang *et al.*, 2017). Lack of ATP may have a role in persistent pericyte constriction as ATP inhibits ion pumping and hinders actin-myosin separation to relieve constriction. Pericyte death during ischaemia is caused at least partly to excitotoxicity, as removing external Ca^{2+} has been found to significantly reduce pericyte death (Yang *et al.*, 2017).

Pericyte function have been implicated to contribute to ischaemic stroke when dysregulated. Firstly, pericytes detach from microvessels after cerebral ischaemia, causing a breakdown of tight junctions between endothelial cells and pericytes (Yang *et al.*, 2017). Loss of tight junctions leads to a loss of Blood brain barrier integrity and leakage occurs via transcellular and paracellular pathways, contributing to stroke progression. Accumulation of basal membrane proteins and pericyte atrophy thickens the basement membrane and disrupts tight junctions and this with neuroinflammation all compromise vascular integrity (Majerova et al., 2019). It is suggested that brain pericytes contribute to the inflammatory signal communication within the neurovascular unit. For example, PDGFRβ signal transduction in pericytes promotes the expression of monocytes chemoattractant protein-1, NO, IL-1, IL-6, IL-12 and TNF-α in the BBB thus mediates the proinflammatory response (Sweeney, Ayyadurai and Zlokovic, 2016). Interestingly, IL-17 activation was found more in human pericytes than endothelial cells (Navarro *et al.*, 2016). IL-17-stimulated pericytes overexpress CXCL8 and induce neutrophil expression of CXCL8, TNF-α, IL-1α, and IL-1β. Pericytes also induce a similar effect with monocytes and NK cells by CCR2

and CXCR3 respectively. TNF-α, IL-1β, and IFN-γ are implicated in pericyte upregulated expression of inducible nitric oxide synthase (iNOS) which in turn induces pericyte vasodilation to promote immune cell trafficking. Pericytes increases inflammatory prostaglandin production and generates reactive oxygen/nitrogen species via cyclooxygenase-2 (Navarro *et al.*, 2016).

Brain pericytes express damage-associated molecular pattern (DAMP) receptors such as TLR4 which respond to microenvironmental cues (Yang *et al.*, 2017). Under ischaemic conditions, DAMPS induce non-infectious immune responses. Brain pericytes in response to TLR4 induces the secretion of proinflammatory cytokines and chemokines IL-6, IL-8, CXCL1-3 and CCL2 which activates an inflammatory response in response to ischaemia. Brain pericytes also respond to IFN-γ, which enhances ICAM-1 and VCAM-1 expression to promote leukocyte recruitment (Yang *et al.*, 2017). Studies show pericytes control endothelial-mediated leukocyte adhesion and transmigration to the CNS, with pericyte deficiency enhancing leukocyte trafficking to the tissue (Sweeney, Ayyadurai and Zlokovic, 2016). Pericytes also support neutrophil crawling and transmigration through the venular walls in inflamed tissues. This transmigration through venular walls is a key part of innate immunity. Neutrophils crawl along pericyte processes to gaps between adjacent pericytes via binding to ICAM-1, Mac-1 and LFA-1 adhesion molecules (Proebstl *et al.*, 2012). Pericytes specifically induces abluminal crawling of neutrophils to enlarged gaps between pericytes in inflamed tissues to allow entry. Arteriolar and capillary NG2+ pericytes have also been implicated to instruct neutrophils and monocytes to undergo extravasation upon reaching postcapillary venules (Navarro *et al.*, 2016).

In addition to phagocytic activity, pericytes perform pinocytosis and receptor mediated endocytosis, suggesting pericytes contribute to removing toxic molecules from the microcirculation (Navarro *et al.*, 2016).This has led to the speculation that pericytes act as resident macrophages and are involved in the first line of immunological defence in the brain (Yang *et al.*, 2017). In addition, transgenic pericyte-deficient mice have shown increased levels of leukocyte trafficking occur in regions of pericyte lost. Therefore, it is suggested that pericyte detachment, possibly due to pericyte activating macrophage like properties, contribute to leukocyte infiltration in ischaemic stroke. Majerova *et al* found similar results in the pathology of tauopathies with ischaemic stroke, as they found gene dysregulation related to inflammation, leukocyte influx, angiogenesis and vasoconstriction (Majerova et al., 2019). Pericytes have an additional immune function tied with adaptive immunity (Birbrair et al., 2015; Navarro et al., 2016). Pericytes are referenced as non-professional antigen presenting cells, as compared to macrophages, they do not express co-stimulatory molecules such as CD80 and CD86, in addition to MHC class 2

molecules (Navarro *et al.*, 2016). Despite this, pericytes still induce antigen presentation specific immune response such as T cell activation. Navarro *et al* commented that pericytes upon IFN-γ exposure induce MHC class II mRNA and protein expression. An example of pericytes in adaptive immunity has been pericytes expressing higher levels of PD-L1 and PD-L2 to act as an inhibitory checkpoint for activated T cells (Navarro *et al.*, 2016). It has been speculated that pericytes affect T cell trafficking to modulate T cell activation. This has been seen in retinal pericytes, as co-culture experiments decreased T cell proliferation and production of IFN-γ and TNF- α, with both cell-cell contact and soluble factors implicated.

1.4.4 Pericytes and renal function

In the kidney, pericytes have been identified on the peritubular capillaries of the cortex and medulla and the descending vasa recta in the medulla (Kennedy-Lydon *et al.*, 2013; Peppiatt-Wildman, 2013; Freitas and Attwell, 2021). Pericytes differs morphologically between the medulla and cortex, with medulla DVR pericytes showing more circumferential processes around capillaries and cortical pericytes with longitudinal processes that run along the capillary with little circumferential processes around the vessel (Freitas and Attwell, 2021). Pericyte populations in the kidney, particularly in the descending vasa recta, express α-SMA similar to brain and retina pericytes (Freitas and Attwell, 2021). The pericyte density within the kidney are found to be greater than the CNS and retina, with greater concentrations in the outer medulla region than inner medulla (Crawford et al., 2012; Kennedy-Lydon et al., 2013). Renal Pericytes are associated with regulating medullary blood flow, essential to maintain adequate oxygen and nutrient delivery (Kennedy-Lydon *et al.*, 2013; Peppiatt-Wildman, 2013). It is also necessary to enforce adequate metabolic clearance and maintain cortico-medullary gradients of NaCl and urea. Blood flow is supplied to the medulla by the descending vasa recta (DVR) and arises from efferent arterioles of the juxtamedullary glomeruli (Kennedy-Lydon *et al.*, 2013). The DVR capillaries consist of continuous endothelium, with pericytes positioned at regular intervals, becoming less frequent as the DVR descends deeper to the inner medulla. Pericyte constriction of the DVR redirects blood towards the outer medullary inter-bundle capillaries and dilation directs away. Pericyte density within the capillary bed, need to match the functional demands of the tissue to optimise blood flow (Peppiatt-Wildman, 2013). High energy demands and the low oxygen tension in the renal medulla, makes the area borderline hypoxic so requires a tight regulation of renal blood flow within the medulla to preserve function and prevent regional hypoxia /ischaemia. Pericyte constriction induces reduced blood flow by increasing vascular resistance and leads to vessel occlusion (Figure 19) (Freitas and Attwell, 2021). Vasa recta pericytes induce vessel constriction

and dilation within studies using isolated perfused DVR in response to endogenous molecules found in the medulla; Angiotensin-II and endothelin-1 inducing constriction and nitric oxide, adenosine and prostaglandin E2 inducing dilation respectively (Kennedy-Lydon *et al.*, 2013). Reactive oxygen species were also shown to induce vasoconstriction in the medulla and is associated with hypertension and nephropathy (Kennedy-Lydon *et al.*, 2013). ROS induced constriction is modulated by peroxynitrite formation because of superoxide interacting with localised NO. This vasoconstriction is known to be attenuated by antioxidants like superoxide dismutase which enhance medullary blood flow.

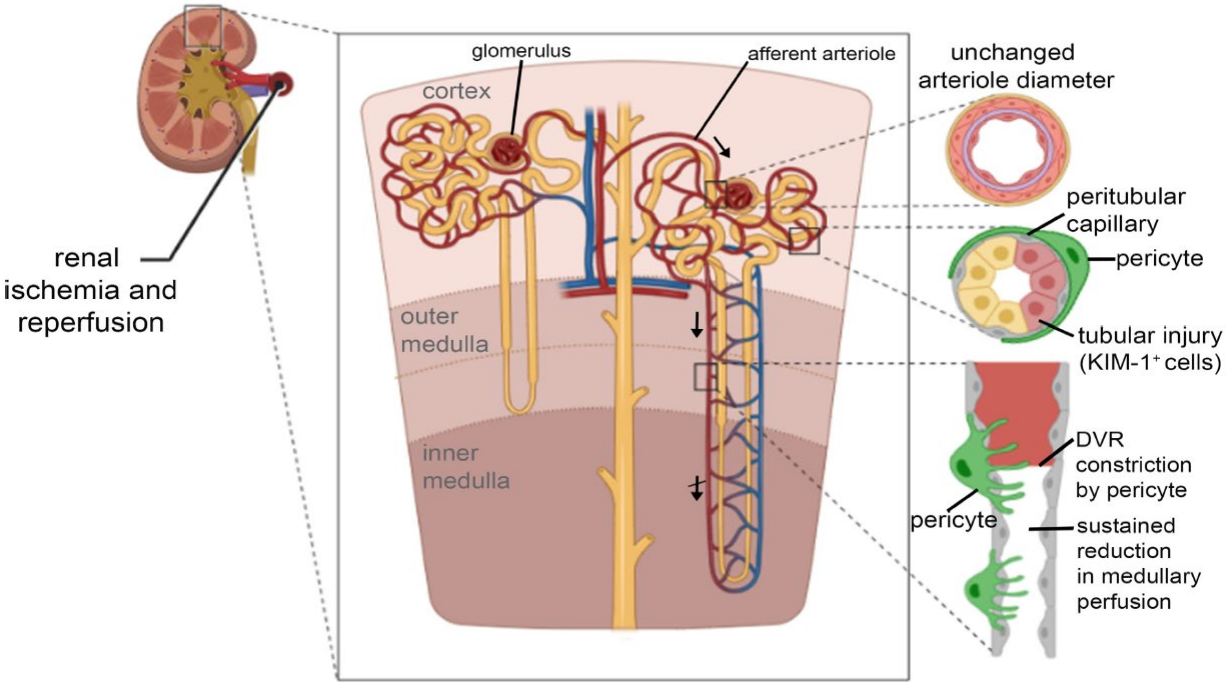

Figure 19 - Diagram of the reduced blood flow caused by renal Ischaemia reperfusion injury, taken from Freitas and Attwell, (Freitas and Attwell, 2021)**.**

Pericytes on the peritubular and descending vasa recta show a significant reduction of blood flow, with constriction of capillaries causing blockages as demonstrated at the bottom right of the figure. The resulting Ischaemia causes renal damage as observed by Kim-1 detection. The afferent and efferent arteriole facilitates blood flow to the glomerulus but show little change in blood flow due to IRI.

Renal pericytes have been identified to produce renin in human kidneys therefore controlling vasoactivity by manipulating RAAS (Stefanska *et al.*, 2016). Immunohistochemistry reveals renin is localised in CD146 and NG2 expressing pericytes, specifically surrounding juxtaglomerular and afferent arterioles, revealing an indirect method pericytes affect blood pressure (Stefanska *et al.*, 2016; Shaw *et al.*, 2018). In addition, specialised pericytes known as mesangial cells have been identified in kidneys to provide physical support to glomerular capillaries (Shaw *et al.*, 2018). Mesangial cells help form the increased surface area to

increase blood ultrafiltration by adapting their morphology to be more compact and rounded (Gökçinar-Yagci, Uçkan-Çetinkaya and Çelebi-Saltik, 2015).

Renal ischaemia is a typical cause of acute kidney injury (AKI), a condition found to be typical and inevitable following cardiac surgery, renal transplantation or after a severe haemorrhage (Freitas and Attwell, 2021). In AKI via ischaemia, reduced blood flow or "no-reflow" has been shown to exacerbate renal injury even after the compromised blood supply is removed. Renal no-reflow induces further complications such as impaired erythrocyte movement, increased intratubular pressure and leukocyte accumulation within the renal capillaries. Freitas and Attwell analysed IRI and how it affects blood flow in the renal medulla and cortex (Freitas and Attwell, 2021). They observed that perfusion of the cortical region could be completely restored after ischaemia, but medullary flow remained compromised, highlighting compromised medullary blood flow as the cause of injury in renal IRI. Within mice, kidney tubule damage and the urinary kidney injury molecule-1 (Kim-1) expression was observed even after 30-60 reperfusion within the renal medulla. Analysis found that the cortical peritubular and medullary DVR capillaries were constricted near the pericyte somatas, causing capillary blockage. Other areas like glomerular arterioles were unaffected by ischaemia, which suggest the reduced renal blood flow was caused by pericyte constriction (Freitas and Attwell, 2021). By blocking Rho Kinase, medullary blood flow improved, with a lower percentage of blocked capillaries and reduced kidney injury. This reveals the extent of ischaemia induced renal no-reflow, and pericytes facilitate AKI due to prolonged constriction of the descending vasa recta and peritubular capillaries (Freitas and Attwell, 2021). Rho kinase inhibits myosin phosphatase, causing increased phosphorylation of the myosin light chain and results in enhanced vasoconstriction (Hamilton, Attwell and Hall, 2010; Freitas and Attwell, 2021). Rho kinase additionally inhibits endothelial nitric oxide synthase (eNOS) thus halting NO production. This pathway occurs during ischaemia which provides insight to how irreversible pericyte constriction can cause capillary collapse.

Pericytes have been shown to have regenerative capabilities and are regarded as a local reservoir of regenerative cells in ischaemic heart, damaged skeletal muscle and others (Stefanska *et al.*, 2016). However, within toxic microenvironments pericytes utilise their multipotent capability and differentiate into fibroblasts/myofibroblasts leading to tissue damage and scarring. Kidneys are particularly susceptible to this due to filtering toxic molecules from the body. Renal pericytes give rise to mesenchymal cells which prove essential in protecting the kidney from AKI and chronic kidney disease (CKD) (Shaw *et al.*, 2018). Renal pericytes are shown to play a role in glomerular and interstitial fibrosis and the development of glomerular sclerosis. In tissue fibrosis, pericytes detach from capillaries, migrate into the renal interstitial

space, proliferate and differentiate into collagen-I–producing myofibroblasts (Fligny and Duffield, 2013). When pericytes proliferate to myofibroblasts they lose pericyte function, resulting in loss of capillary stability, renal tissue scarring and deleterious consequences for the kidney. Pericyte-Endothelial communication is necessary to maintain vessel stability, such as PDGF-B/PDGF receptor-β and angiopoietin/Tie2 required for pericyte recruitment and expansion during angiogenesis and TGF-b and VEGF to regulate stabilisation for existing blood vessels (Fligny and Duffield, 2013). Thus, pericyte detachment results with increased permeability and an unstable proliferating endothelium, leading to a loss of microvasculature in a toxic microenvironment. Stefanska *et al* identified that pericyte number declines in both the cortex and the medulla with age (Stefanska *et al.*, 2016; Shaw *et al.*, 2018). Pericyte loss has already been shown to be a causal factor for acute kidney injury, thus age-linked loss of pericytes is associated with declining kidney function. In addition to contributing to the fibroblast/myofibroblast population, as described in section 1.4.3, pericytes have an active role in amplifying kidney inflammation and leukocyte recruitment to renal tissue through the TLR pathway activation and the generation of components of the complement pathway. Renal Pericytes play a role in salt-sensitive hypertension in addition to renal fibrosis (Peppiatt-Wildman, 2013). Medullary oxidative stress was already implicated in hypertension and leaves the kidney susceptible to vasoconstrictor agents. NAD(P)H oxidase, superoxide and hydrogen peroxide were all found to contribute to oxidative stress-mediated tubular damage. Carbonyl stress was also implicated as a potential causal factor of medullary tubular damage and together with ROS attenuates local production of nitric oxide, thus rendering the microcirculation susceptible to vasoconstrictor agents.

1.5 Identifying the link between the circadian clock and ischaemia reperfusion injury

1.5.1 Is there a link between the circadian clock and ischaemia reperfusion injury?

The culmination of data in section 1.1-1.4 addresses one question; whether the onset and pathogenesis of ischaemia- reperfusion injury is influenced by the circadian clock. Circadian transcription factors BMAL1 and rev-erbα have already been documented to influence immune cell trafficking by directing chemokine expression involved in mobilisation, achieved by regulating active enhancers in association with chromatin modifiers (Orozco-Solis and Aguilar-Arnal, 2020). There is emerging evidence that speculates the circadian clock has the capacity to regulate IRI pathogenesis. Regulation of mitochondrial stability presents a promising method in treating renal IRI. Furthermore, BMAL1 has been implicated in mitochondrial biogenesis and mitochondria-mediated apoptosis in IRI via the regulation of PGC-1α, which in turn regulates silent information regulator 1 (SIRT1) and thus mitochondrial biogenesis (Ye et al., 2022).

Recent research describes BMAL1 to regulate renal function, but it is unknown to what extent. BMAL1 overexpression has been shown to alleviate ischaemia-reperfusion injury. In contrast, under ischaemic conditions, reduced BMAL1 levels exacerbates mitochondrial damage and subsequent apoptosis, with the decline of SIRT1 levels implicated with this effect (Ye et al., 2022). The SIRT1/PGC-1α pathway induces the anti-apoptotic response and encourages mitochondrial repair, restoring mitochondrial electron transport chain proteins damaged by ischaemia (Y. Zhou *et al.*, 2018; Liang *et al.*, 2020). Nephron specific BMAL1 knockout mice were found to cause abnormal pharmacokinetics and mitochondria dysfunction (Ye *et al.,* 2022). Similar results in the renal granular cells and collecting duct indicates increased urine volume, decreased plasma aldosterone and a decrease in blood pressure (Ye et al., 2022). Ye *et al* revealed that using SIRT1 agonist resveratrol partially alleviates cell injury in Human kidney-2 cells.

The NRF2/ARE pathway affects oxidative stress as the pathway regulates ARE-regulated antioxidant stress proteins such as NQO1 and HO1 (Sun *et al.*, 2021). At the circadian rhythm nadir of NRF2, NQO1 and HO1 expression is at its weakest, leading to greater renal oxidative stress injury after IRI. The recent study concludes that the circadian rhythm of the nuclear erythroid 2 related factor 2 (NRF2)/ antioxidant response element (ARE) pathway mediates oxidative stress in renal IRI by regulating antioxidant mechanisms, therefore hinting at a therapeutic strategy of targeting the diurnal variation of human renal disease (Sun *et al.*, 2021). The data states that BMAL1 plays a vital role in activating NRF2 in the kidney, activating the signalling pathway to promote antioxidant proteins and promote resistance to oxidative stress. BMAL1 was found to bind to the E-Box element of NRF2 and circadian regulation was observed in both normal kidneys and kidneys exposed to ischaemia and reperfusion (Sun *et al.*, 2021).

1.5.2 Potential therapeutic targets of Ischaemia-Reperfusion Injury

1.5.2.1 The link between circadian clock and ischaemia reperfusion injury

The study that influenced this thesis to investigate the therapeutic potential of the circadian clock in IRI was the work of Montaigne *et al*. This study speculated a diurnal variation underlying cardiovascular disease, with specific pathology of ST elevated myocardial infarction being more prevalent to occur in the early morning rather than the evening (Montaigne *et al.*, 2018). With this association in mind, the study investigated whether cardiac surgery had different clinical consequences throughout the day for aortic valve replacements and identified that the incidence of perioperative myocardial ischaemic injury was reduced if surgery was scheduled in the afternoon. Transcriptional analysis indicated that myocardial ischaemia reperfusion tolerance was due to the absence of REV-ERBα as this tolerance was recreated in

mice with the pharmacological inhibition and gene deletion of REV-ERBα during the sleep to wake transition (Montaigne *et al.*, 2018). This discovery has the capacity of changing surgical procedure if the results translate to other types of surgeries. Alternatively, if these results occur in other surgical procedures, this could lead to using methods to shift patient's circadian clock to reduce post-surgery complications.

Although there is promise, there is much research needed to elucidate the pathogenesis of IRI, to uncover therapeutic targets to relieve onset and severity of the condition. As stated in section 1.2, Ischaemia reperfusion injury is a condition that impacts organ quality and overall transplant outcomes, which is why it is heavily researched today. During transplantation, the donor organ is inevitably subjected to interruption of the blood supply during the harvest and reperfusion occurs after the organ is transplanted to the recipient (Fernández *et al.*, 2020). In consequence, IRI is a common condition that affects organ transplantation and can induce deleterious effects on the graft and the recipient overall. As mentioned prior, the kidney is the most frequently transplanted organ and shown to have a high susceptibility to Ischaemia-reperfusion injury (Stiegler *et al.*, 2018). In addition, the kidneys were found to undergo injury during IRI of different organs such as the liver, highlighting how systemic susceptibility of renal tissue to ischaemic damage (Soares *et al.*, 2019). This is because kidneys filter of blood and thus are particularly vulnerable to exposure of toxic metabolites.

Renal transplantation is considered an ideal treatment for patients with end stage renal disease, a common cause of acute renal failure (Banaei, Rezagholizadeh and Azimian, 2019). Ischemia insult that occurs during renal transplantation results with impaired graft function and loss of renal grafts. The kidney is known to have an independent biological clock system that exhibits diurnal variability and regulates renal physiological function including blood pressure, glomerular filtration rate, and urinary sodium excretion (Sun *et al.*, 2021). The rhythm of *Clock* genes becomes dysregulated following renal IRI, accompanied by oxidative stress and functional and physiological impairment thus suggesting an endogenous mechanism in which renal oxidative stress occurs. Better understanding of the cellular pathophysiological mechanisms underlying kidney injury via ischaemia-reperfusion will hopefully result providing more targeted therapies to prevent and treat IRI (Malek and Nematbakhsh, 2015).

1.5.2.2 Therapeutic targets for Ischaemia-Reperfusion Injury

Currently, multiple processes are being investigated to limit IRI during transplantation. To help reduce damage via IRI, organs are preserved in hypothermic preservation solutions free from blood (Koo *et al.*, 1998). For renal transplants, Collins solution and University of Wisconsin solution are used, with recent advances adding signal pathway blockers like endothelin blockers and ICAM-1 antisense as well as antioxidants (Y. Chen *et al.*, 2019). Cold ischaemia has been seen as an ideal method to reduce the severity of IRI in organ transplants. Despite this, prolonged cold ischaemia is associated with decreased graft survival in renal transplantation despite perfect histocompatibility matching of the donor and recipient (Fernández *et al.*, 2020). Delayed renal graft function requires the patient to undergo dialysis for the first week after transplantation, with complications in managing immunosuppression for the organ recipient (Koo *et al.*, 1998). Increased cold ischaemia was a major factor for the degree of organ injury, potentially leading to prolonged hospitalisation and detrimental effects on graft function and transplant survival (Koo *et al.*, 1998; Slegtenhorst *et al.*, 2014). Hypothermic machine perfusion is another method theorised to help IRI, where perfusion reduces ROS accumulation. Comparison of hypothermic machine perfusion to cold static storage found both reduce DGF in kidney transplants within a single centre. This was supported by a systematic review but although it was perceived that perfusion would reduce DGF, the difference was non-significant, so further work is required (Wight *et al.*, 2003; Bellini *et al.*, 2019). Ex vivo pharmacological conditioning and targeting the innate immune response, are also thought to be beneficial. Both methods could be utilised to exploit tolerogenic innate immune properties to promote immunosuppression.

Implications have led into the research of organ preconditioning in a bid to reduce early allograft injury, thus IRI onset and severity. Ischaemic preconditioning is a process utilised to encourage organ/tissue tolerance to damage due to ischaemia. One target is to reduce metabolic stress during ischaemia, via extracorporeal organ perfusion. This has been experimented in rat and human liver transplants which show promising results with perfusion preserving liver function and allowed livers to repair from injury. Testing this notion on the kidney is required as the liver's high capacity to regenerate and repair may be responsible for the promising result (Fernández *et al.*, 2020). The kidney is theorised to be preconditioned by a non-lethal period of ischaemia, allowing it to tolerate subsequent ischaemia injury. The preventative effects of ischaemic preconditioning in the kidney are theorised to be via the reduction of adhesion molecules and inflammatory responses or via action of A1 adenosine receptors (Malek and Nematbakhsh, 2015). The current therapeutic target for IRI includes reducing impairment on endothelial relaxation,

scavenging free radicals and blocking leukocyte adhesion and activation (Soares *et al.*, 2019). Fernández *et al*. suggests that the increased cell death during IRI represents a promising target to reduce/prevent IRI which in terms of organ transplantation will improve organ quality (Fernández *et al.*, 2020). Past studies have shown different agents can aid in the combat of IRI. For example, leptin was observed to decrease TNF-α and nitrite levels, levosimendan acts upon antioxidant and NO-related mechanisms, iloprost suppress lipid peroxidation and ascorbic acid promotes free radical scavenging and antioxidant mechanisms (Malek and Nematbakhsh, 2015).

All agents that are potential targets for IRI act as agonists or antagonists for key cellular events, either utilising naturally occurring cell substances like Glutathione and melatonin, or mimicking these substances like with trimetazidine (Soares *et al.*, 2019). Characterised pathways targeted for IRI is the Reperfusion injury salvage kinase (RISK), Survivor activating factor enhancement (SAFE), cGMP/PKG and other inflammatory, mitochondrial factor and metabolic pathways. The RISK pathway is linked to a group of anti-apoptotic protein kinases that involve either the PI3K-Akt or MAPK, ERK1/ERK2 pathways, both activated during reperfusion (Soares *et al.*, 2019). Both pathways culminate in inhibiting the opening of the mtPTP, found to have anti-apoptotic and cardioprotective effects. Pharmacological interventions during IRI shows reduced tissue damage but exhibit undesirable side effects (Singhanat *et al.*, 2018). The decline of antioxidant mechanisms and the subsequent overload of reactive oxygen species and reactive nitrogen species is a crucial factor in the development of renal IRI and ischaemic acute renal failure (Banaei, Ahmadiasl and Alihemmati, 2016). Therefore, radical scavengers and antioxidant mechanisms has become an ideal target for IRI therapy. One free radical scavenger that has therapeutic potential is Erythropoietin, a hypoxia-inducible hematopoietic factor that is predominantly expressed in the kidney (Banaei, Ahmadiasl and Alihemmati, 2016). Erythropoietin is recognised as a multifunctional cytokine that promotes anti-inflammatory properties and has a significant effect in acute and chronic inflammation models (Silva *et al.*, 2021). Erythropoietin is involved in antioxidant, anti-apoptotic, and anti-inflammatory effects (Banaei, Ahmadiasl and Alihemmati, 2016). Erythropoietin also has pleiotropic properties, acting as a potent stimulator of erythroid progenitor cells which inhibits NF-κB to promote anti-inflammatory effects (Mateus *et al.*, 2017). The biological effects of erythropoietin are mediated by binding to erythropoietin cell surface receptors which have been identified in renal mesangial and tubular epithelial cells. Following renal IRI, it was found that renal EPO levels decreased, hinting at a potential role for erythropoietin in protecting the kidney (Banaei, Ahmadiasl and Alihemmati, 2016).

Another example is cortisol, a hormone released in response to stress and shown to be expressed rhythmically with circulating cortisol levels reaching a peak in the morning and low at night (Banaei, Rezagholizadeh and Azimian, 2019). Patients with kidney impairment had reduced cortisol secretion, with administration of exogenous cortisol increasing renal blood flow, thus increasing glomerular filtration rate, natriuresis and diuresis. Cortisol enhances the renal capacity of regulating fluid and electrolyte homeostasis by raising GFR and transferring more sodium and water to the distal nephron to greater modulate reabsorption (Banaei, Rezagholizadeh and Azimian, 2019). During chronic kidney disease, subjects displayed disrupted cortisol circadian rhythms with increased inflammation markers. Glucocorticoids restored adrenal function and restored blood pressure in patients with end stage renal disease. Cortisol has two anti-inflammatory effects; blocking early stages of inflammation before onset and causing inflammation to resolve swiftly and promote rapid healing (Banaei, Rezagholizadeh and Azimian, 2019). Cortisol prevents inflammation by multiple actions: stabilising lysosomal membranes, decreasing capillary permeability, decreasing white blood cell migration into the inflamed site and reducing interleukin release by white blood cells (Banaei, Rezagholizadeh and Azimian, 2019). Therefore, cortisol could protect renal tissue from ischaemia reperfusion injury via anti-inflammatory effects.

Melatonin is a hormone produced by the pineal gland and plays an important role in regulating many physiological functions in humans (Singhanat *et al.*, 2018). Accumulating data suggests melatonin can relieve cardiac I/R by increasing cell survival and reducing cell death. Melatonin can increase p-JAK and p-STAT of the JAK/STAT pathway which results in reduced mitochondrial oxidative stress and thus reduced apoptosis. STAT inhibits Bax translocation and enhances Bcl2 expressions which are proapoptotic and anti-apoptotic proteins respectively, reducing cell apoptosis. Melatonin also activates reperfusion injury salvage kinase (RISK) and Notch1 pathway which results in increased Akt function, a survival protein that reduces oxidative injury and thus apoptosis. The results indicate that melatonin attenuates myocardial I-R injury via reducing oxidative stress, and inflammation, and ameliorating cardiac apoptosis (Singhanat *et al.*, 2018). Melatonin is also implicated to play a role in hormone signalling and IRI due to its role in immune function.

The traditional role of melatonin is regulating sleep, the circadian rhythm and immune function (Banaei, Ahmadiasl and Alihemmati, 2016). In addition to sleep regulation and the circadian rhythm, melatonin is a potent ROS/RNS scavenger due to its ability to be an electron donor (Banaei, Rezagholizadeh and Azimian, 2019). Melatonin can also stimulate antioxidant enzymes, stabilise cell membranes and decrease

apoptosis and lipid peroxidation. Melatonin pre-treatment evokes protective effects against IRI in the kidney, as indicated in the study by Banaei *et al*. The study shows melatonin pre-treatment to improve the kidney as shown by the absence of the bowman capsule membrane thickening and attenuated increase in hyaline cast, both characterised in renal Ischaemia and reperfusion. In addition, Haemoglobin and Haematocrit values in kidneys were found to be significantly greater in melatonin pre-treated mice compared to those exposed to ischaemia and reperfusion only (Banaei, Ahmadiasl and Alihemmati, 2016). Corresponding research collated from Banaei *et al* states melatonin has greater nephroprotective effects than erythropoietin for rat renal IRI and melatonin could reverse the histopathological changes caused by renal IRI (Banaei, Ahmadiasl and Alihemmati, 2016). Finally, nuclear melatonin receptor RORα was observed to inhibit mitochondria mediated apoptosis via reduction of ER and mitochondrial stress (He *et al.*, 2016). RORα activity also suppresses oxidative/nitrative stress and restores autophagy function. RORα was implicated to mediate the beneficial effects of melatonin in myocardial IRI, highlighting RORα in protecting against cardiac pathology. Furthermore, RORα transcription factor is documented to possess a potent antioxidant effect in hepatocytes and neurons (He *et al.*, 2016).

1.5.3 Targeting immune cell entry for ischaemia Reperfusion injury and the potential of using pericytes

One approach of targeting IRI is by reducing the innate immune response that accompanies reperfusion into the tissue. The innate immune response is a crucial component that determines the function of the recently transplanted organ and the long-term survivability of the graft (Slegtenhorst *et al.*, 2014). Understanding the mechanisms of immune cell entry into the graft tissue will provide insight for an effective therapy to optimise transplantation (Slegtenhorst *et al.*, 2014). Anti-inflammatory and anti-oxidative hormones such as erythropoietin, cortisol and melatonin, could protect against IR damage and improve transplant function and the overall outcome after kidney transplantation (Banaei, Rezagholizadeh and Azimian, 2019).

Endothelial cells and pericytes are two main cell populations in capillaries, with physical and paracrine interaction driving and regulating vessel sprouting. There is evidence that suggests pericytes are essential players in regulating leukocyte extravasation, so pericytes can be a good target to analyse immune cell entry(Rudziak, Ellis and Kowalewska, 2019). The link between the circadian clock and pericytes is a relatively new field of exploration. Studies analysing the presence and role of circadian rhythms in pericytes and whether the molecular clock affects the endothelial-pericyte interactions is

relatively unexplored (Mastrullo *et al.*, 2021). Circadian clock components are found to alter the phenotypes of endothelial pericytes (Zhang *et al.*, 2022). In addition, Pericytes were found to exhibit an endogenous self-sustained molecular clock (Mastrullo *et al.*, 2021, 2022). Interestingly, endothelial cells did not share this rhythmicity unless synchronised by pericyte contact, leading to the speculation that pericytes regulate endothelial cell molecular clocks as part of secondary regulation (Mastrullo *et al.*, 2021). Further supporting this, when the pericyte clock was disrupted, pericyte capacity to support endothelial microvessel tube forming was impaired, whereas clock disruption of endothelial cells had no effect on Angiogenesis. 3D tissue scaffold model showed that development of vascular-like structures at the scaffold pores were influenced by clock synchronisation between the two cell lines whereas the non-synchronised experiments displayed disorganised structures. The results from Mastrullo *et al.,* demonstrate that pericytes play a critical role in regulating circadian rhythms in endothelial cells, and silencing this system via *Bmal1* knockout prevents their pro-angiogenic function (Mastrullo *et al.*, 2022). Disruption of the clock reduced lactate production and release in endothelial cells whereas lactate formation in pericytes was unaffected. Thus, clock disruption in pericytes affects the rhythmicity of lactate production and reuptake, theorised to be a mediator influencing endothelial cell clock synchronisation and angiogenesis (Mastrullo *et al.*, 2022).

The implication of the circadian clock affecting angiogenesis was identified with the discovery that Jet lag inhibits blood reperfusion after ischemic injury (Zhang *et al.*, 2022). For normal blood vessel function, mature pericytes must be recruited and covered with parietal cells. After this, pericytes attach to endothelial cells and regulates extracellular matrix deposition and forms endothelial tight junction proteins that stabilise the new lumen. The progressive loss of pericytes observed in *Bmal1*$^{-/-}$ mice contributes to the blood–brain barrier hyperpermeability and weakened angiogenesis (Zhang *et al.*, 2022). Recent studies further explored the potential mechanism of circadian clocks in pericytes. First, human primary pericytes were found to display a rhythm and induced human umbilical vein endothelial cell circadian rhythmicity in the EC–pericyte contact co-culture environment (Zhang *et al.*, 2022). Also, *Bmal1* was implicated in the maturation and morphology of vascular structure (Mastrullo *et al.*, 2022). Pericyte marker, PDGF-β is also worth considering, as PDGF-B regulates pericyte proliferation and migration, and induces recruitment by interaction with PDGF receptor β (Zhang *et al.*, 2022). PDGF and circadian genes were found to interact, both regulating cell proliferation (Zhang *et al.*, 2022). PDGF-BB increased *Bmal1* mRNA and protein expression in a time-dependent manner, upregulating other components of the circadian clock such as *Clock, Per1/2* and *Cry1/2*. *Bmal1* also regulates PDGF-BB, and *Bmal1* knockdown impairs proliferation induced by PDGF-BB.

1.5.4 Experiment protocol and the null hypothesis

1.5.4.1 Hormone preconditioning

Current information on hormone preconditioning for IRI analyses the hormone as an antioxidant molecule. However, another vital component of IRI is immune cell entry into organs/tissues and microvascular blockage. Immune cell entry blockers are already used in organ preservation solutions, but another target is to reduce the expression of adhesion molecules in the vessels surrounding the target organ. Another aspect of IRI is the no reflow phenomenon, triggered by pericyte-endothelial vasoconstriction. Both these aspects of ischaemia reperfusion injury are influenced by pericyte function therefore it highlights pericytes as a target to alleviate IRI. This information has led to the proposed research for this thesis to combine all the small aspects of the circadian influence upon IRI. The idea is to analyse renal microvasculature i.e. pericytes to investigate how It is response to circadian messengers. Pericyte vasoactivity will be observed by utilising the live kidney slice model described by Crawford *et al* (Crawford et al., 2012). The work of Kennedy-Lydon *et al* has previously investigated a multitude of vasoactive mediators (Kennedy-Lydon *et al.*, 2013). However, this type of research has only started to be explored so the effects of circadian mediators remain unknown. The hormones that shall be investigated in this thesis are: GABA, arginine vasopressin, corticosterone and melatonin.

1.5.4.2 GABA

GABA was selected to be analysed because GABA can reset/entrain the circadian clock (Liu and Reppert, 2000). GABAA activation was predicted to reset the SCN and thus the circadian clock and inhibits the ability of light to reset the clock during the night cycle (Ehlen *et al.*, 2008). This has potential to be used to set the clock to the optimal time when components involved in IRI is suppressed if the hypothesis proves true. Arginine vasopressin is another circadian messenger and will be analysed for its documented role as a circadian messenger and in renal function.

1.5.4.3 Arginine Vasopressin

Arginine vasopressin has previously been analysed using the live kidney slice model (Kennedy-Lydon *et al.*, 2013). Knowing that arginine vasopressin is a known vasoconstrictor, with the knowledge that it displays a similar circadian rhythm as melatonin, this messenger could be used more as a positive control for the experiment.

1.5.4.4 Corticosterone

Cortisol was initially selected due to prior information implicating cortisol to have potential therapeutic properties against IRI in humans. An issue of using cortisol for this experiment is cortisol is not the main stress hormone in mouse models; thus, corticosterone is set to be used in place of cortisol. Corticosterone is released early in the morning during the sleep-wake transition. Therefore, analysing corticosterone with melatonin will give insight to pericyte vasoactivity at opposite ends of the circadian rhythm, observing the pericyte response at key points of day and night within the cycle.

1.5.4.5 Melatonin

The therapeutic benefits of melatonin as an antioxidant scavenging molecule led to the speculation that the circadian clock can influence the pathogenesis of IRI. As melatonin has established effects to alleviate renal IRI, melatonin is suggested as an ideal candidate to be a therapeutic agent to reduce ischaemia-reperfusion related damage. Melatonin has been found to have various effects on the immune system including immunosuppressive effects. Supporting evidence has found that melatonin has very low toxicity to animal and human studies; supporting that melatonin is an ideal candidate for therapeutic use (Malhotra, Sawhney and Pandhi, 2004). This anti-inflammatory property of melatonin has led to the focus of melatonin in the ability to improve the donor pool and be able to protect the graft from rejection. For some end-stage organ diseases, the only potentially curative treatment option is organ transplantation (Stiegler *et al.*, 2018). In end-stage renal failure for example, a kidney transplant is considered a better treatment option than haemodialysis, as it is more cost effective and improves the quality of life.

1.5.4.6 Experiment design

The initial line of work is to investigate the vascular activity via pericytes after exposure to circadian hormones, to observe the mechanobiological response to these circadian regulators. This will elucidate if these hormones contribute to immune cell entry by pericyte-mediated activity altering renal blood flow. GABA in these experiments shall be utilised to analyse at first whether GABA induces pericyte-mediated constriction or dilation and if this activity is reversible. This would ensure that GABA could be used to entrain the clock without impeding renal and pericyte function. The experiment will prioritise the action of corticosterone and melatonin to attempt to reveal pericyte activity during the day and night respectively. These experiments will be subdivided between experiments that are initially pre-treated with GABA and those treated solely with Physiological saline solution (PSS). This could indicate whether GABA induces pericyte-mediated activity while it is used to reset the peripheral clock and whether it

influences activity induced by corticosterone or melatonin. The null hypothesis of this experiment is that the administration of corticosterone and melatonin will have no effect on pericyte activity.

The aim of this thesis is to understand the influence if any that melatonin imposes pericyte activity. As pericytes influences immune cell entry, it is the aim to uncover whether melatonin, as the primary hormone under focus, influences this aspect of pericyte function. Melatonin can successfully ameliorate immune cell entry as part of its immune regulatory function by its interaction with pericytes, melatonin would be proven to be beneficial to preparing organ transplants for Ischaemia and reperfusion. A secondary aim of the thesis is to identify if GABA could be used to reset peripheral clocks as well as the SCN. The initial experimental design involved using light to induce phase shifts in the central and peripheral clocks, but GABA was utilised in its place when light could not be used. It was the preconception that GABA as a secondary messenger could influence peripheral clocks and exert similar phase resetting properties as observed in the SCN. If proven to have some accuracy it would provide a method to reset the renal clock directly using the *in situ* live kidney slice model.

The animal model to be used for this thesis will be the C57BL/6J murine model. It is essential to consider the limitations of the model as this model produces little to no quantities of melatonin (Zhang *et al.*, 2018; Kennaway, 2019; Korf and von Gall, 2024). However, as discussed in section 1.1.4.8, C57BL/6J mice still possess functional components of melatonin signalling and the model has still proven to be useful in exogenous melatonin studies, which this research will be utilising. Melatonin has been shown to have protective effects in the kidney as explained in section 1.3.3.4. Melatonin have also been shown to still exhibit protective effects in C57BL/6J mice specifically in studies investigating gemcitabine and cisplatin induced kidney injury (Wang *et al.*, 2023) and in folic acid induced AKI induce renal IRI (Zhu et al., 2017). This evidence and the availability of this model lead to this model being chosen, on the assumption that melatonin influence identified in this work would be similar to melatonin proficient strains of mice.

Chapter 2

Methodology

Chapter 2: Methodology

The use of the murine live kidney slice model will be used in order to investigate the vasoactivity of renal pericytes.

2.1 Tissue preparation and slicing

Male C57BL/6J mice (Charles River UK Ltd, Margate, UK) aged 63-70 days were used to utilise the live kidney slice model. These experiments were conducted in accordance with the United Kingdom Animal Scientific Procedures Act of 1986. Animals upon arrival are maintained in laboratory room temperature conditions (22^{o}C) until euthanasia by cervical dislocation via the schedule 1 (SK1) process within the above act. The kidneys were isolated, with the renal capsule being removed. Kidneys were immediately placed in ice cold physiological saline solution (PSS), oxygenated with 95% O_2/ 5% CO_2 prior to preparation for slicing.

PSS was prepared on the day of experiments, prior to SK1, in the following concentrations; NaCl (100 mM), KCl (5 mM), NaH_2PO_4 (0.24 mM), Na_2HPO_4 (0.96 mM), Na acetate (10 mM), $CaCl_2$ (1 mM), $MgSO_4$ (1.2 mM), glucose (5 mM), $NaHCO_3$ (25 mM) and Na pyruvate (5 mM) (Sigma-Aldrich Ltd, Dorset, UK). The PSS pH was determined using a Hanna bench pH209 meter (Hanna Instruments, Bedfordshire, UK) and adjusted to pH 7.4 using either O_2, HCl 1M or NaOH 1M. Kidneys were secured on an ice-cold slicing block and sliced using a Leica VT 1200 S vibrating microtome (Leica, Germany) and submerged in a buffer tray full of ice-cold PSS bubbled with 95% O_2/5% CO_2. To expose the renal medulla, the outer cortical dome region of the mouse kidney was removed (approximately 1200µm removed from the exposed surface). After which, 200µm thick slices were cut in a series (consisting of both medulla and cortex) until the remaining kidney does not possess an adequate amount of medulla. Approximately 3 to 6 slices were obtained per kidney dependent the size and condition of the kidneys and each slice. Post slicing, kidney slices were maintained for up to 4 hours in a holding chamber containing oxygenated PSS with 95% O_2/ 5% CO_2 at room temperature (25^{o}C) prior to experimentation (Crawford et al., 2012).

2.2 Functional Experiments and Analysis

A single C57BL/6J kidney slice was secured in a 1.25ml bath chamber on the stage of an upright Olympus microscope (model BX51WI, Olympus microscopy, Essex, UK) using a purpose-built platinum slice anchor. The slice was continuously perfused at a rate of approximately 2.5ml min^{-1} with PSS oxygenated with 95% O_2/5% CO_2 and maintained at laboratory room temperature (~25°C). Pericytes on vasa recta capillaries were identified using the previously established 'bump on the log' morphology (Crawford et al., 2012). DIC images were captured through an Olympus 60x water immersion objective (0.9 NA). Real-time video images of changes in vasa recta diameter were collected every second by an attached Rolera XR CCD camera and recorded using Image Pro Software (Media Cybernetics Inc., Marlow, Bucks, UK). Live kidney slices were superfused with PSS alone for 120 seconds to establish a baseline diameter at both a pericyte and non-pericyte site and then subjected to one of the following vasoactive compounds for 500s: γ-Aminobutyric acid (GABA, 50 µM), arginine vasopressin (AVP, 300 nM), corticosterone (Corticosterone, 50 µM) and melatonin (Melatonin, 50 µM). After exposure to the stimuli; slices are subsequently subjected to a PSS wash until the experiment end. All vasoactive compounds were purchased from Sigma-Aldrich Ltd, and working solutions were prepared in oxygenated PSS.

Doses were approximated to induce pericyte response at the concentration for a set exposure timeframe of 500s. This timing was chosen to allow for a large enough window of exposure for a response to be elicited, and to provide a washout period of at least 600s to assess reversibility to said exposure timeframe. The initial total timeframe of 1200s was chosen as it is the standard in previous experiments of this department(Lilley, 2020; Lilley *et al.*, 2023; Wildman *et al.*, 2023). The mediators were chosen that are regulators for the circadian rhythm and known to influence kidney function. Experiments that undergo GABA pre-treatment were introduced to GABA for 30 minutes in oxygenated PSS not under the microscope and then allowed to rest for a further 30 minutes to allow pericyte activity, if any, to reverse.

Live tissue differential interference contrast (DIC) images for the 1200s and 1800s experiments were captured through a 63x water immersion objective (0.9 NA; Olympus, Southend-on-Sea, UK). Real-time video images of changes in vasa recta diameter were collected every second via an attached Rolera XR Charged Coupled Device (CCD) camera, and recorded using Image Pro Software (Media Cybernetics Inc., Marlow, UK). Time-Series analysis of live kidney slice experiments was conducted using ImageJ software as previously described (Crawford et al., 2012). In brief, measurements of the vessel diameter were taken from an identified pericyte and non-pericyte site from single vasa recta every 5s for the duration of the experiment. Each capillary acted as its own control. The first 5 measurements were used to determine the

baseline diameter, and represented the resting value (D_b), expressed as 100%. All subsequent measurements are expressed as a percentage of the corresponding baseline resting value for both pericyte and non-pericyte sites (Crawford et al., 2012). Pericyte response was classified as a change to the vessel diameter that is greater than 5% at the pericyte site. From that point, returning to within 5% of the baseline was considered return to rest. Later experiments were refined to be 1800 seconds long. This was to extend the PSS wash timeframe and then add Angiotensin II (300 nM) for the final 300s. This is to act as a positive control to slices that appear to not respond to identify if the vessel is still active. This is to determine if the vessel unresponsiveness is due to the drug or if the slice is no longer viable.

$$\%\,\Delta Vessel\ diameter = \left(\frac{\text{Measured diameter (D)}}{\text{Mean baseline diameter } (D_b)}\right) * 100$$

$$D_b - \%\,\Delta vesseldiameter = \%\ constriction\ or\ dilation$$

Equation 1- Calculation of the percentage of changes in the capillary vessel diameter. An average was calculated from the first 5 5 measurements was used to represent the baseline vessel diameter. All subsequent measurements are expressed as a percentage of the corresponding baseline resting value for both pericyte and non-pericyte sites. Adapted from (Crawford et al., 2012).

2.3 Statistics

Values are presented as the mean $\pm$ the standard error of the mean (SEM). The *n* numbers of each of the experiments are presented as the number of animal's that the functional slices were obtained from then the total number of kidney slices used from these animals. A Shapiro-Wilk test was performed for each data set to identify if the results follow normal distribution. Statistical significance of the data was calculated using a paired two-tailed student's t-test. A 2-way ANOVA and Tukey's Honestly Significant Difference (HSD) test were performed for comparing the means generated from slices that were treated with corticosterone and melatonin only and those that were preincubated with GABA before introducing these drugs. The Tukey's test was chosen to perform comparison of the multiple means and maintain confidence intervals. It also assumes that all variances of the groups are equal. Values were calculated using GraphPad PRISM 8.0 software. Any value of $p<0.05$ were considered statistically significant.

Chapter 3

Results

Chapter 3: Results

3.1 GABA at 50 µM did not induce a significant change in pericyte diameter.

We first observed GABA vasoactivity in the live kidney slice model. The effects of GABA as a regulator of vasoactivity in the descending vasa recta has been studied previously, however, GABAs exact circadian influence is still being uncovered. Using GABA in an attempt to reset the renal clock is a relatively new field that is being explored and was devised in an attempt to replace light as the entraining signal for these experiments. Using GABA as a circadian synchroniser was based on experiments by Liu and Reppert, achieving phase shifts and synchronisation of SCN cell culture to an individual level (Liu and Reppert, 2000). To extend this notion, the theory was GABA will achieve a similar effect with the kidney as with the SCN cells.

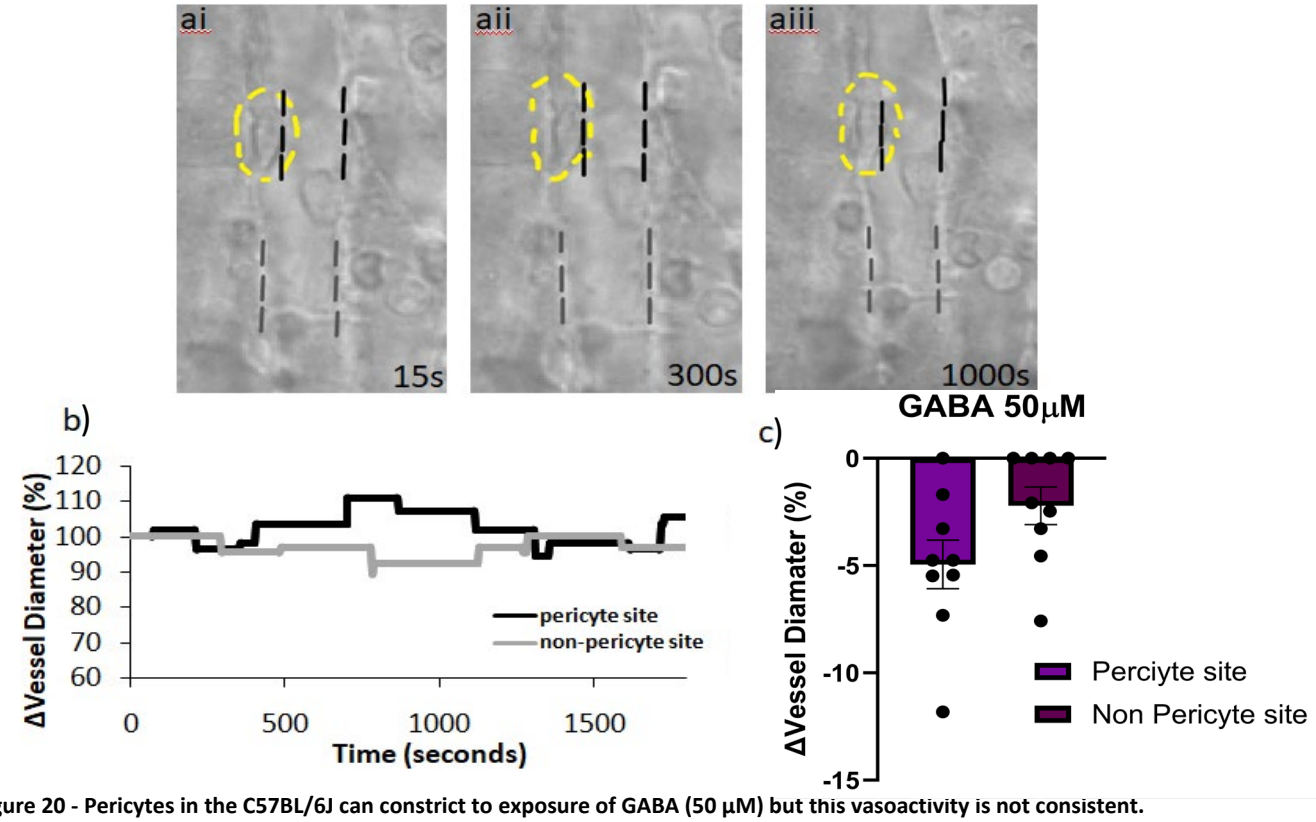

Figure 20 - Pericytes in the C57BL/6J can constrict to exposure of GABA (50 µM) but this vasoactivity is not consistent.

DIC imaging analysis of vasoactive compounds on vasa recta diameter. *In situ* vasa recta were analysed over a time series experiment shown in **(ai-iii)**. **(ai-iii)** show the capillary upon exposure to Gamma aminobutyric acid **(GABA)** at: 15s **(ai)**; prior to GABA exposure, 300s; during exposure **(aii)**; and 1000s, during a "wash out" period **(aiii)**. Pericyte cell bodies are indicated by a yellow dashed circle, while black dashed lines and dark grey dashed lines are used to represent pericyte sites and non-pericyte sites, respectively. **(b)** In the responsive vessel, measurements were obtained from a pericyte site and corresponding non-pericyte site every 5s for the first 100s to establish the baseline diameter, then all subsequent measurements were expressed as the percentage change of that width. The experiments were conducted for 1800s, and exposure to GABA (50 µM) was implemented within the 100s and 600s time period. PSS is used before and after the exposure to GABA. The average change in vessel diameter upon exposure to GABA is presented in **(c)** at pericyte (Light purple bar) and non-pericyte (Dark purple bar) sites (n= 6 animals, n= 9 slices). Values presented are mean ± SEM.

vThe p-values obtained from the Shapiro-Wilk test were p= 0.6241 at the pericyte site and p= 0.0658 at the non-pericyte site, thus proving the data follows normal distribution. Superfusion of live kidney slices with GABA (50 µM) resulted in non-significant constriction of pericytes of vasa recta capillaries (4.951% ± 1.951%, Fig. 20) than non-pericyte sites (2.218% ± 1.515%; Mouse number n = 6; slice number n = 9; P= 0.764 Fig. 20). These results do not correspond with previous findings; therefore, this procedure will need to be repeated to ensure its validity. What has been proven, however, with these findings is the reversibility of vasoconstriction induced by GABA. This was observed by superfusing the same slices with Angiotensin II (300 nM) after washing the slices with PSS to allow time for the slices to reset. This would mean that if GABA superfusion was needed to achieve phase shifts and resetting of the circadian clock, it would not interfere with renal function or in terms of our experiments, using other mediators to ameliorate immune cell entry, if sufficient time is given for the pericytes to reset. GABA's reversibility shall be addressed further in sections 3.5 and 3.6.

3.2 Arginine Vasopressin induces Pericyte-mediated constriction in the C57BL/6J mice.

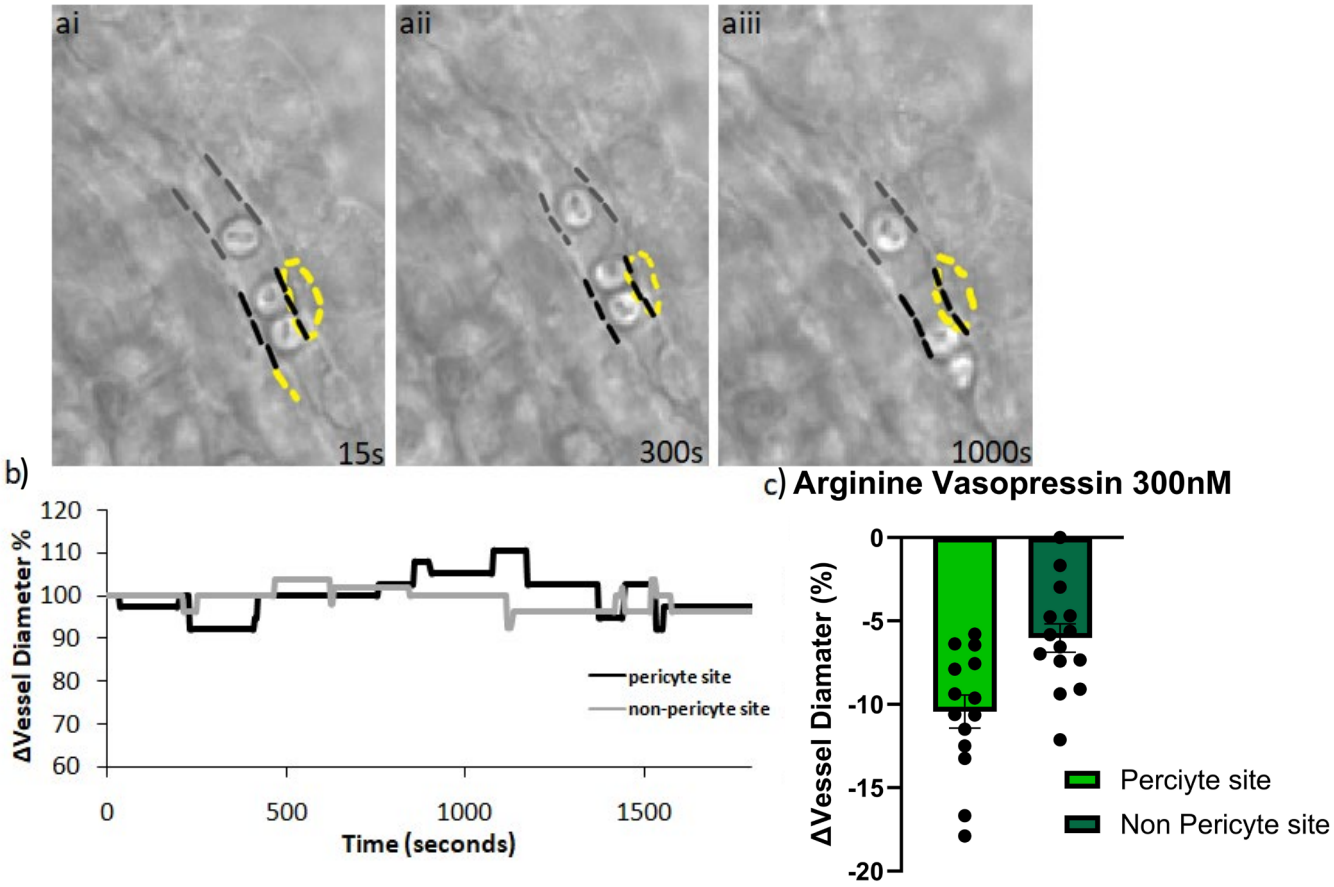

Figure 21 - Exposure to Arginine Vasopressin (300 nM) induces Pericyte-mediated constriction in the C57BL/6J mice.

DIC imaging analysis of vasoactive compounds on vasa recta diameter. *In situ* vasa recta were analysed for a time series experiment shown in **(ai-iii)**. **(ai-iii)** show the capillary upon exposure to Arginine Vasopressin **(AVP)** at: 15s **(ai)**; prior to AVP exposure, 300s; during exposure **(aii)**; and 1000s, during a "wash out" period **(aiii)**. Pericyte cell bodies are indicated by a yellow dashed circle, while black dashed lines and dark grey dashed lines are used to represent pericyte sites and non-pericyte sites, respectively. **(b)** In the responsive vessel, measurements were obtained from a pericyte site and corresponding non-pericyte site every 5s for the first 100s to establish the baseline diameter, then all subsequent measurements were expressed as the percentage change of that width. The experiments were conducted for 1800s, and exposure to AVP (300 nM) was implemented between the 100s and 600s time period. PSS is used before and after the exposure to AVP. The average change in vessel diameter upon exposure to AVP is presented in **(c)** at pericyte (Light green bar) and non-pericyte (Dark green bar) sites (n= 9 animals, 14 slices). Values presented are mean ± SEM.

In search of finding a mediator that is influenced by the circadian clock, Arginine vasopressin or anti diuretic hormone was considered as an ideal candidate, as its expression is coordinated by the SCN and acts as a circadian messenger. As detailed earlier, the primary function of arginine vasopressin is to mediate blood pressure control and vasoactivity in vascular smooth muscle and vascular endothelial cells. Arginine vasopressin has already been established to be a potent vasoconstrictor, reducing renal blood flow to induce water retention. The Shapiro-Wilk test gave p-values of p= 0.3278 at the pericyte site and

p= 0.9944 at the non-pericyte site, showing that the data follows normal distribution at both the pericyte site and at the non-pericyte site. Consistent with previous work, superfusion of live kidney slices with Arginine vasopressin (300 nM) induced a pericyte-mediated constriction of Vasa recta capillaries that was significantly greater at pericyte sites (10.437% ± 2.137%) than non-pericyte sites (6.031% ± 1.831%, Mouse number n=9; Slice number n=14 P= 0.0013).

3.3 Vasoactivity of Corticosterone without GABA pretreatment

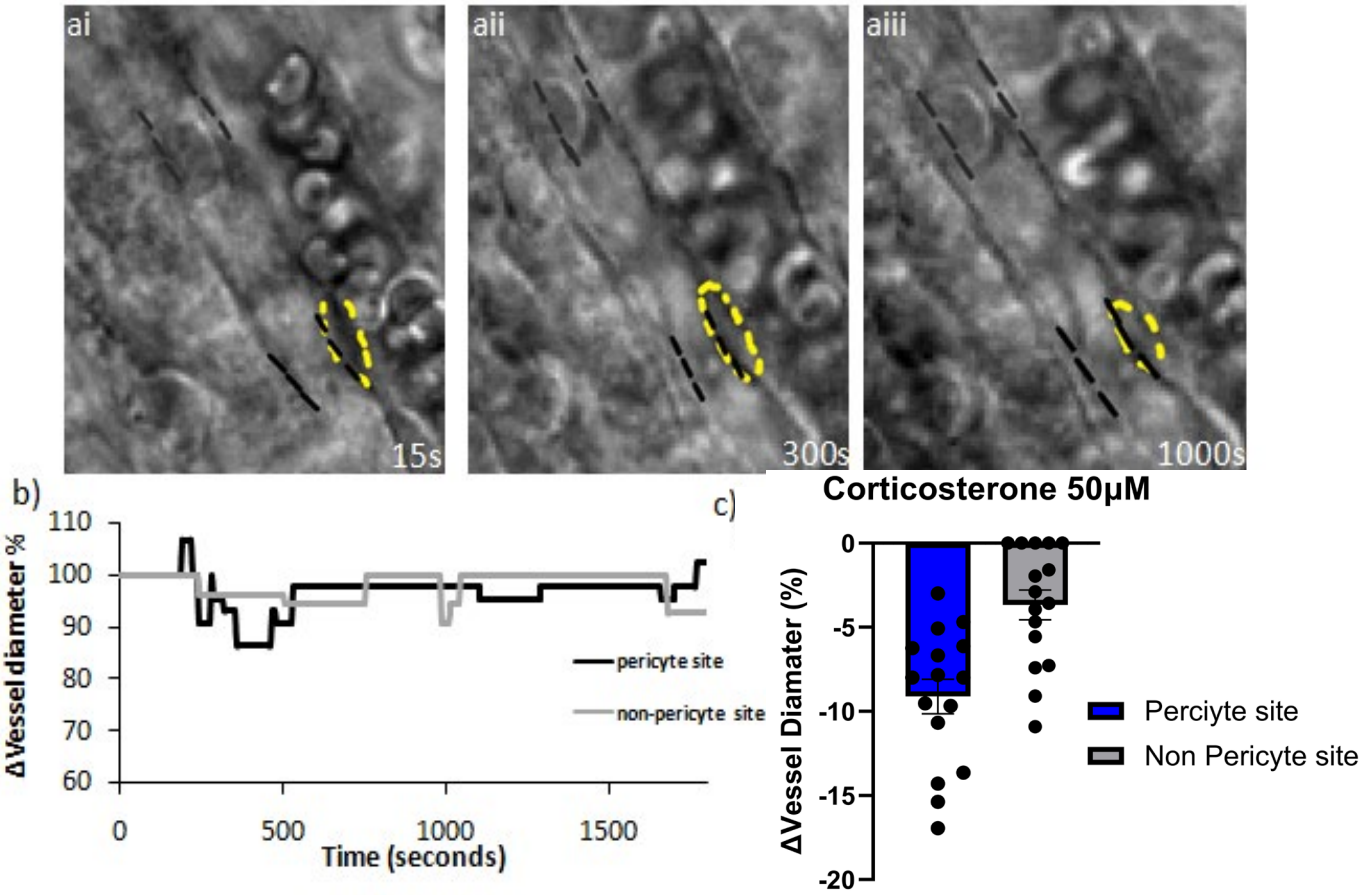

Figure 22 - Introduction of Corticosterone (50 µM) to Pericytes induces vasoconstriction.

DIC imaging analysis of vasoactive compounds on vasa recta diameter. *In situ* vasa recta were analysed for a time series experiment shown in **(ai-iii)**. **(ai-iii)** show the capillary upon exposure to Corticosterone **(Cort)** at: 15s **(ai)**; prior to Cort exposure, 300s; during exposure **(aii)**; and 1000s, during a "wash out" period **(aiii)**. Pericyte cell bodies are indicated by a yellow dashed circle, while black dashed lines and dark grey dashed lines are used to represent pericyte sites and non-pericyte sites, respectively. **(b)** In the responsive vessel, measurements were obtained from a pericyte site and corresponding non-pericyte site every 5s for the first 100s to establish the baseline diameter, then all subsequent measurements were expressed as the percentage change of that width. The experiments were conducted for 1800s, with exposure to Cort (50 µM) being implemented between the 100s and 600s time period. PSS is used before and after the exposure to Cort. The average change in vessel diameter upon exposure to AVP is presented in **(c)** at pericyte (Blue bar) and non-pericyte (Grey bar) sites (n= 13 animals, 16 slices). Values presented are mean ± SEM.

As described previously, corticosterone was utilized in this experimental design because of its rhythmic expression displaying a peak associated with waking and its association with the stress response. Analyzing both corticosterone and melatonin will provide a morning vs night perspective of how circadian messengers act on renal physiology. The Shapiro-Wilk test revealed that the data follows normal distribution at both the pericyte and non-pericyte site with p-values of p= 0.3846 and p= 0.812 respectively. Introducing corticosterone to the kidney slice caused capillary vessel constriction at the pericyte site by 9.111% ± 2.364% compared to 3.680% ± 2.047% constriction at non-pericyte sites (Mouse number n=13, slice number= 16 p= 0.00035).

3.4 Vasoactivity of Melatonin without GABA pretreatment

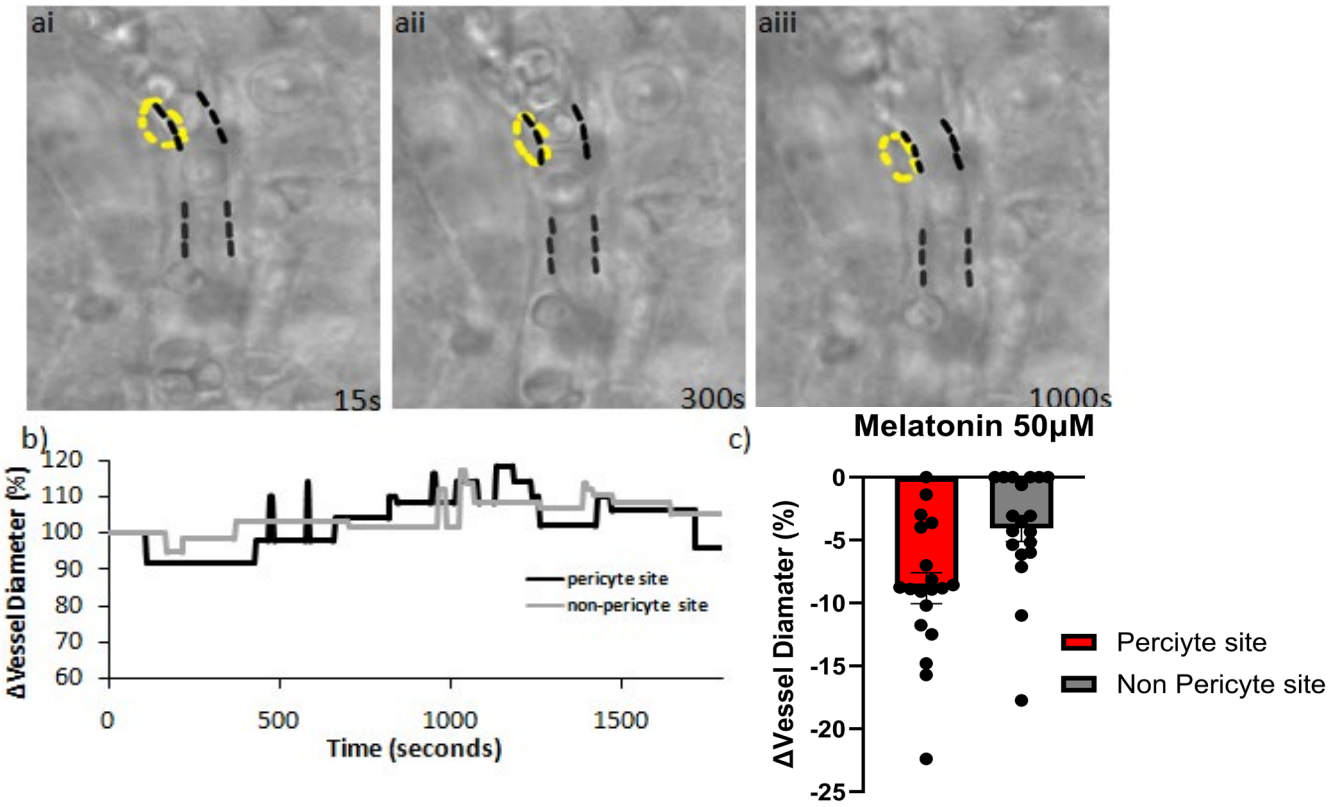

Figure 23 - Melatonin exposure (50 μM) causes a significant vasoconstriction to pericytes in C57BL/6J mice.

DIC imaging analysis of vasoactive compounds on vasa recta diameter. *In situ* vasa recta were analysed for a time series experiment shown in **(ai-iii)**. **(ai-iii)** show the capillary upon exposure to Melatonin **(MT)** at: 15s **(ai)**; prior to MT exposure, 300s; during exposure **(aii)**; and 1000s, during a "wash out" period **(aiii)**. Pericyte cell bodies are indicated by a yellow dashed circle, while black dashed lines and dark grey dashed lines are used to represent pericyte sites and non-pericyte sites, respectively. **(b)** In the responsive vessel, measurements were obtained from a pericyte site and corresponding non-pericyte site every 5s for the first 100s to establish the baseline diameter, then all subsequent measurements were expressed as the percentage change of that width. The experiments were conducted for 1800s, with exposure to Melatonin (50 μM) being implemented between the 100s and 600s time period. PSS is used before and after the exposure to MT. The average change in vessel diameter upon exposure to AVP is presented in **(c)** at pericyte (Red bar) and non-pericyte (Grey bar) sites (n= 15 animals, 19 slices). Values presented are mean ± SEM.

Of the numerous effects that melatonin has been observed to have on the kidney, the effect on the capillary vascular diameter has not been particularly looked at. As a counterpart to corticosterone, melatonin was investigated during these experiments to understand the action of circadian messengers during the evening and night. It was intended to use melatonin to induce a phase shift to the renal vasculature to understand if capillary microvasculature, specifically renal pericytes are affected at all. The p-values from the Shapiro-Wilk test are $p= 0.3538$ and $p= 0.0913$ at the pericyte site and non-pericyte site respectively, proving normal distribution. As stated earlier, melatonin rhythms inversely correlate with ambient blood pressure implying melatonin may regulate blood pressure. Inducing vascular activity in the kidney, specifically the vasa recta is currently unknown. The vessels when exposed to melatonin decreased in vessel diameter by 8.823% ± 3.081% at the pericyte sites and at non pericyte sites of 3.764% ± 2.652% (Mouse number n=15, slice number= 19 $p<0.001$).

3.5 Vasoactivity of Corticosterone after GABA pretreatment

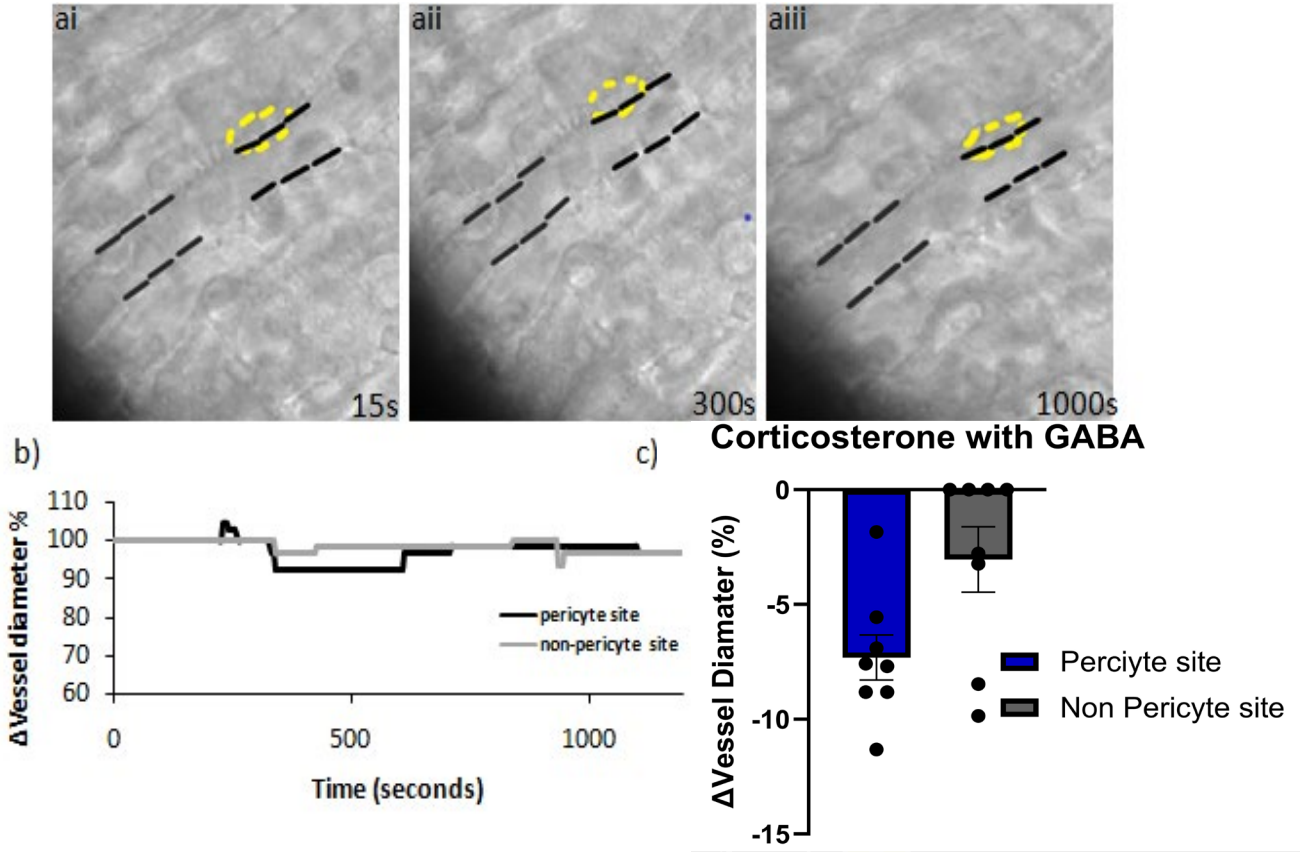

Figure 24 - Preincubation of Kidney slices to GABA did not prevent Pericytes vasoconstriction by Corticosterone in the C57BL/6J mice.

DIC imaging analysis of vasoactive compounds on vasa recta diameter. *In situ* vasa recta were analysed for a time series experiment shown in **(ai-iii)**. Prior to the experiment, kidney slices were perfused with GABA (50 µM) within oxygenated PSS for 15 minutes and then washed with normal PSS for another 10minutes. **(ai-iii)** show the capillary upon exposure to Corticosterone **(Cort)** at: 15s **(ai)**; prior to Cort exposure, 300s; during exposure **(aii)**; and 1000s, during a "wash out" period **(aiii)**. Pericyte cell bodies are indicated by a yellow dashed circle, while black dashed lines and dark grey dashed lines are used to represent pericyte sites and non-pericyte sites, respectively. **(b)** In the responsive vessel, measurements were obtained from a pericyte site and its corresponding non-pericyte site every 5s for the first 100s to establish the baseline diameter, then all subsequent measurements were expressed as the percentage change of that width. The experiments were conducted for 1800s, with Corticosterone exposure (50 µM) being implemented between the 100s and 600s time period. PSS is used before and after the exposure to Cort. The average change in vessel diameter upon exposure to AVP is presented in **(c)** at pericyte (Dark blue bar) and non-pericyte (Grey bar) sites (n= 8 animals, 8 slices). Values presented are mean ± SEM.

Sections 3.5-3.6 address using GABA superfusion on the renal slices to reset the renal clock. The preliminary work of section 3.1 investigated GABA on pericytes of the descending vasa recta to identify if at the concentration of 50 µM would induce pericyte mediated vasoconstriction. Introduction of corticosterone and melatonin after GABA superfusion was implemented because these drugs oscillate at different phases, having a peak in the morning and at night for corticosterone and melatonin, respectively. This would reveal if a reset of the clock would influence the action of what are considered a morning drug

and an evening drug, or if it made the renal clock more malleable to a phase shift to the morning and evening. Another point to consider is if GABA preincubation would affect the corticosterone function. Our experimental procedure analysed the live renal slices over 1200s, analysing changes of vessel diameter during superfusion of corticosterone (100-600s). The Shapiro-Wilk test generated p-values of p= 0.5740 at pericyte sites and p= 0.0747 at the non-pericyte sites, showing that the results follow normal distribution. Our results show, even with GABA preincubation, corticosterone was shown to induce vessel vasoconstriction. The vessel diameter decreased by 7.645% ± 1.046% at the pericyte site compared to the 3.128% ± 1.920% at the non-pericyte site (Mouse number n=8, slice number= 8 p= 0.0098). This means at initial observations reveal GABA pre-treatment did not affect corticosterone activity upon pericytes.

3.6 Vasoactivity of Melatonin after GABA pretreatment

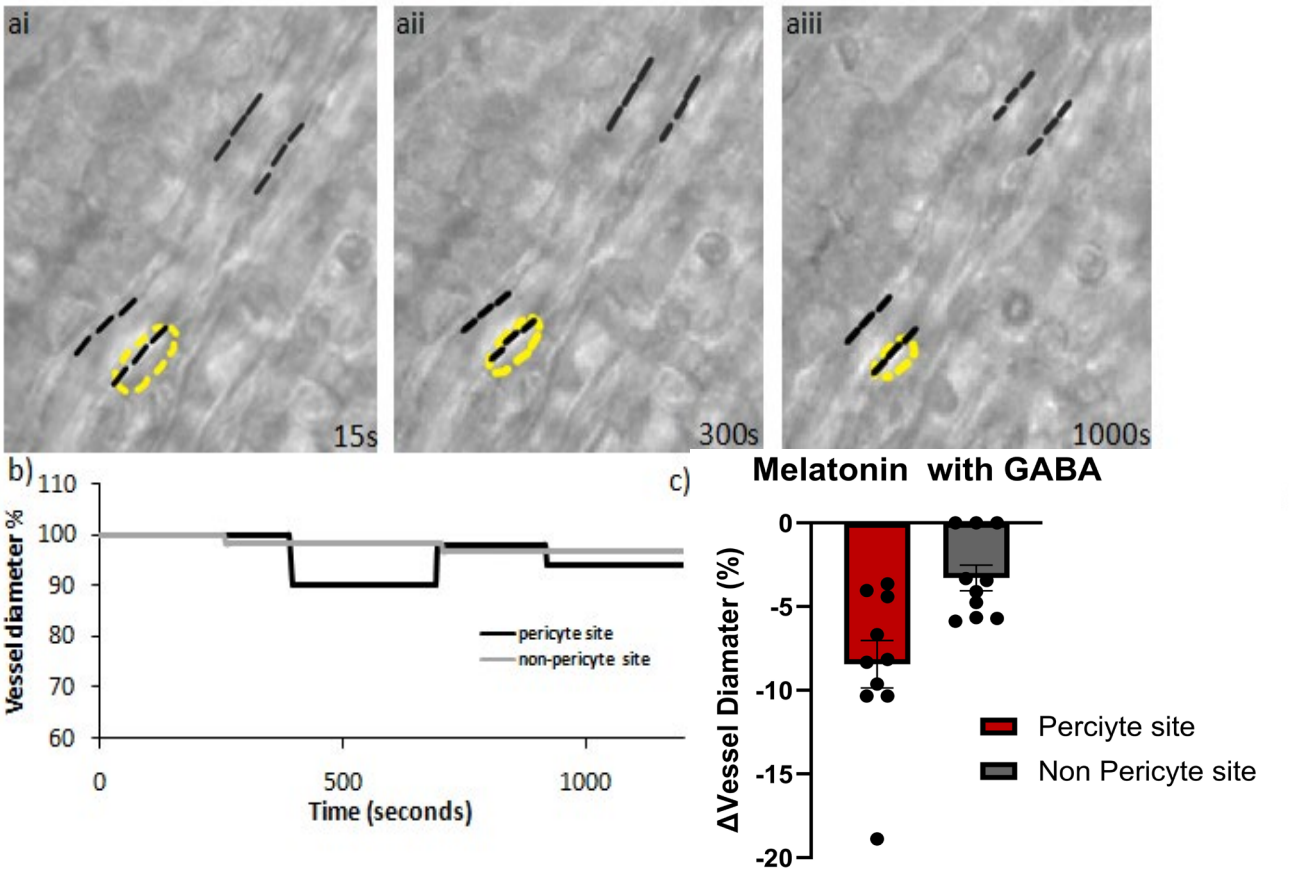

Figure 15 - **Preincubation of Kidney slices to GABA did not prevent Pericytes vasoconstriction by Melatonin in the C57BL/6J mice.**

DIC imaging analysis of vasoactive compounds on vasa recta diameter. *In situ* vasa recta were analysed for a time series experiment shown in **(ai-iii)**. Prior to the experiment, kidney slices were perfused with GABA (50 µM) within oxygenated PSS for 15 minutes and then washed with normal PSS for another 10minutes. **(ai-iii)** show the capillary upon exposure to Melatonin **(MT)** at: 15s **(ai)**; prior to MT exposure, 300s; during exposure **(aii)**; and 1000s, during a "wash out" period **(aiii)**. Pericyte cell bodies are indicated by a yellow dashed circle, while black dashed lines and dark grey dashed lines are used to represent pericyte sites and non-pericyte sites, respectively. **(b)** In the responsive vessel, measurements were obtained from a pericyte site and its corresponding non-pericyte site every 5s for the first 100s to establish the baseline diameter, then all subsequent measurements were expressed as the percentage change of that width. The experiments were conducted for 1800s, with Melatonin exposure (50 µM) within the 100s and 600s time period. PSS is used before and after the exposure to MT. The average change in vessel diameter upon exposure to AVP is presented in **(c)** at pericyte (Dark red bar) and non-pericyte (Grey bar) sites (n= 8 animals, 10 slices). Values presented are mean ± SEM.

Melatonin was assessed after GABA pre-incubation similarly to corticosterone to indicate if the GABA pre-treated slices would alter the vasoactive response. GABA and melatonin are both circadian messengers so could interact with one another. Therefore, this interplay between GABA and melatonin might mean that GABA superfusion might alter melatonin's activity on pericyte mediated vessel activity. The Shapiro-Wilk test gave p-values of p= 0.0866 at pericyte sites and p=0.0515 at non-pericyte sites which reveal that data collected at both pericyte and non-pericyte sites follow normal distribution. These slices showed a

similar response to slices without GABA pretreatment. Vessel diameter was seen to decrease at the pericyte site by 8.443% ± 2.576% whereas the non-pericyte site is reduced by 3.290% ± 1.408%. A paired t test was performed and obtained a p value of p= 0.021 (Mouse number n=8, slice number= 10). This indicates the initial analysis shows GABA pretreatment does not affect the pericyte mediated constriction due to the elevated melatonin concentration.

3.7 Cross comparison of Corticosterone and Melatonin results

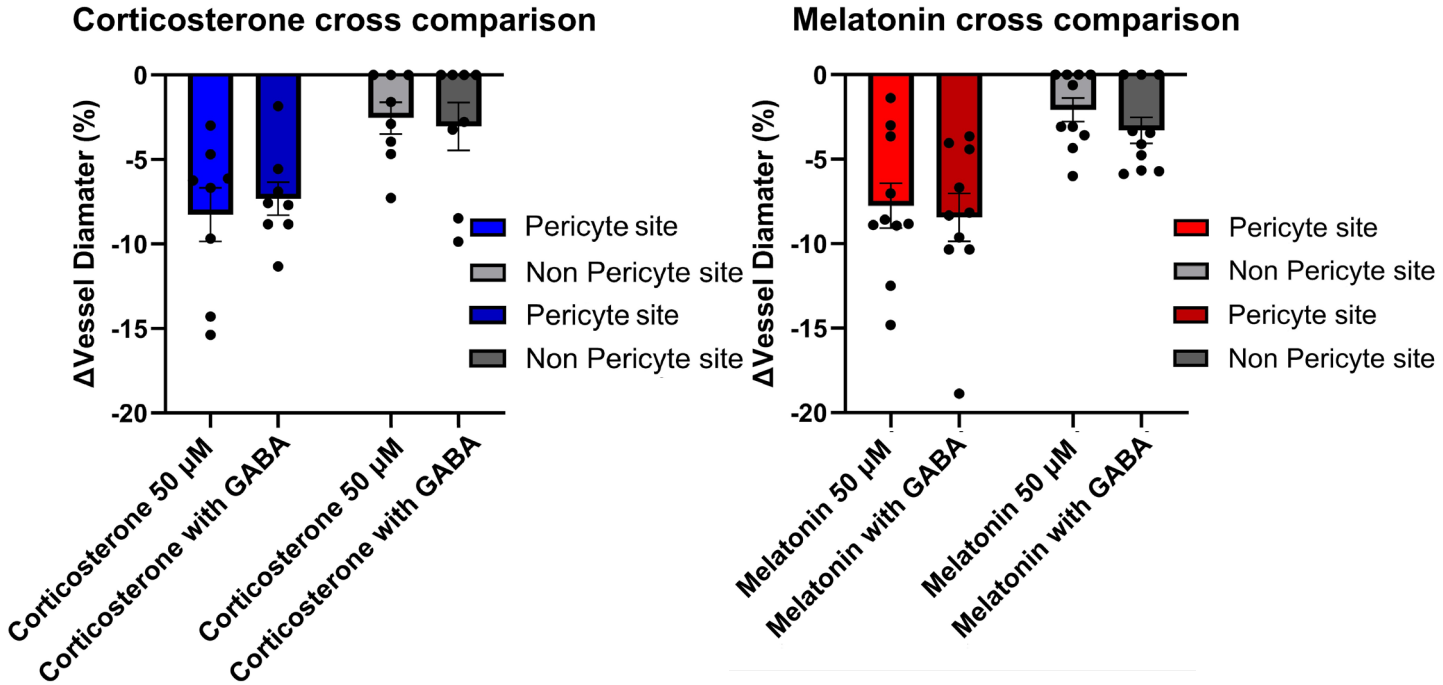

Figure 26 - GABA preincubation did not alter the pericyte vasoconstriction induced by either Corticosterone or Melatonin.

The results obtained for the change in vessel diameter at pericyte and non-pericyte sites were compared for slices that were and were not pre-treated with GABA (50 µM). Averages for corticosterone and melatonin at pericyte sites (blue for corticosterone and red for melatonin) and at non pericyte sites (light grey) were compared to averages obtained in slices preincubated with GABA before analysed (Darker blue and red for pericyte sites for corticosterone and melatonin respectively, darker grey at non pericyte sites). Experiments analysed and compared were slices that came from the same animals (n= 8 animals, 8 slices for Corticosterone, n= 8 animals, 10 slices for Melatonin). In addition to graphs above, a 2-way ANOVA and Tukey's multiple comparison tests were performed for the cross comparison of Corticosterone and Melatonin to determine if there is any significance between the site of analysis (pericyte/non-pericyte sites) and whether GABA was used.

Direct comparison was conducted to inquire whether using GABA preincubation would affect the vasoactivity of corticosterone and melatonin on pericytes. This would indicate whether GABA can be utilized as a SCN synchronizer for these experiments in the kidney in replacement of light. To investigate this, experimental analysis of renal slices using the same animal model were cross analyzed to identify if the preincubation of GABA resulted in any significant changes to vasoconstriction (Mouse number n=8

slice number n=8 for corticosterone, mouse number n= 8, slice number n= 10 for melatonin). A 2-way ANOVA test and Tukey's Honestly Significant Difference (HSD) test were performed, comparing data obtained at the pericyte sites and non-pericyte sites, respectively. For corticosterone, the p-value for comparing vasoactivity with or without GABA pre-incubation is p= 0.8610 which reveals that GABA does not have a significant influence on the pericyte-mediated constriction induced by corticosterone at the pericyte and non-pericyte sites. The p-value addressing the variation caused by diameter is measured at the pericyte site compared to the non-pericyte site is p= 0.0005, thus revealing that addition of corticosterone induces significant difference of constriction at the pericyte site and the non-pericyte site as proven previously by t-test analysis. The interaction p-value also showed non-significance with a value of p=0.5740. The HSD test revealed no significant differences between the means obtained at the pericyte and non-pericyte sites between slices that were introduced to GABA prior and those that were not (p-value between pericyte regions was 0.9518 and p-value between non-pericyte regions was 0.9924). The adjusted p-values remained significant between pericyte and non-pericyte sites without GABA (p= 0.0169) and with GABA pretreatment (p=0.0325).

For melatonin slices, the p-value for comparing vasoactivity with or without GABA is p= 0.3907 which reveals that GABA does not have a significant influence on the pericyte-mediated constriction induced by corticosterone at both the pericyte and non-pericyte sites. The p-value addressing the variation caused by diameter is measured at the pericyte site compared to the non-pericyte site is p< 0.0001, revealing melatonin induces significant pericyte-mediated vascular constriction similar to the t-test analysis. The interaction p-value was p=0.8097, which was non-significant. The adjusted p-value obtained from the comparison of the vasoconstriction at the pericyte sites was p= 0.9706 and the p-value at the non-pericyte was p= 0.8603 which both show that GABA does not significantly alter the effect of melatonin at these respective regions. The HSD test also showed the adjusted p-values between the pericyte and non-pericyte sites without GABA to be p= 0.0043 and after GABA as p= 0.0107, showing that melatonin still induced pericyte-mediated constriction. The combination of corticosterone and melatonin analysis shows that both are not altered by the GABA preincubation. This means GABA has the potential to be used to synchronize the circadian clock and make them more susceptible to phase shifts.

Chapter 4

Discussion

Chapter 4: Discussion

4.1 General discussion

The work here was conducted to analyse if and how circadian messengers influence renal pericytes. The model used for this experiment is a simplified version of assessing the kidneys at different circadian times, using circadian messengers like GABA, corticosterone and melatonin to analyse their influence upon the renal microvasculature. The methodology describes 3-6 slices would be extracted from each kidney. Renal slices from each animal were distributed between each experiment so results can be compared as the same animal. Some slices however were found to be non-viable because these slices did not respond to either the drug but more importantly were not responsive to angiotensin II. As angiotensin II a well-known vasoconstrictor, if no response is found after both the drug and angiotensin then it would be due to the vessel being unresponsive. A possible reason is as the slices reach the end of their viability the capillaries collapse or become occluded by red blood cells. These non-responsive slices were omitted from the results which lowered the number of slices from each animal and thus the ratio to number of slices to the number of animals used.

GABA was utilised in this experiment to attempt to reset the circadian clock. However, the analysis of this thesis was to initially investigate whether preincubation of GABA, similar to the superfusion required to induce a peripheral clock reset, would influence the effect of corticosterone and melatonin on renal pericytes. Corticosterone and melatonin were used to uncover the morning and evening hormone response on renal vasculature respectively. In addition, corticosterone and melatonin have additional roles in stress response and as an antioxidant molecule respectively, which provides insight of pericyte response to stress as found in IRI. Recently, inflammatory mediators TNF-α, IL-18, IL-33, and C5a were found to elicit vasoconstriction on renal pericytes in the vasa recta (Lilley *et al.*, 2023). This decreases pericyte density on the renal vascular network, most probably from pericyte-fibroblasts maturation to facilitate inflammation (Gökçinar-Yagci, Uçkan-Çetinkaya and Çelebi-Saltik, 2015). As myocardial injury was found to be reduced later in the day combined with melatonin's antioxidant properties, the study focuses on melatonin on how it could shift the clock to alleviate renal IRI and relieve ROS accumulation.

4.2 The use of GABA, could synchronise peripheral clocks for *in situ* models?

As discussed in the experimental design, GABA was investigated to determine whether GABA could reset the peripheral clock like the SCN without impeding renal function. The preconceived idea was to treat GABA administration as mediating signals from the central to peripheral clock. To determine if GABA was

a suitable mediator that can be used to reset the circadian clock within renal tissue, live tissue DIC was used to examine any alterations to the capillary vessel diameter. It was documented that GABA can induce pericyte mediated vasoconstriction, therefore affecting medullary blood flow (Wildman *et al.*, 2014). GABAa receptors have been shown to possess an inhibitory and an excitatory role. This would make sense as GABAa receptors have been identified to emerge earlier than GABAb and GABAc receptors and facilitates most GABAergic synaptic transmission as it is distributed more widely. This implicates GABAa involvement in the developing CNS. As discussed earlier, GABA's excitatory response was due to NKCC1 and KCC2 channels and could reveal how GABA induces vasoconstriction therefore implicating NKCC1 with vasoconstriction. This is supported by Everington *et al* as they noted that NKCC1 and NKCC2 are the predominant Cl^- ion transporters within the mouse kidney (Everington *et al.*, 2018).

As Tonic GABAa receptors are involved in pericyte mediated constriction, one potential flaw of GABA to shift/reset the clock is that it can impede pericyte function. To reset the renal clock without impacting pericyte function, it would be implied that we need to induce GABAa Phasic activity rather than GABAa Tonic activity. The association of a γ2 subunit with α1, α2 or α3 subunits were found to predominantly mediate phasic synaptic inhibition whereas receptors with α4, α5 or α6 subunits are more likely to mediate tonic inhibition because they are predominantly or exclusively extra synaptic (Farrant and Nusser, 2005). Of the subunits already identified in the kidney, α1, α2 have been identified in the kidney so this hints at the presence of phasic GABA receptors. However, DeWoskin *et al* suggested that Phasic GABAa activity might not have a significant influence on molecular timekeeping (DeWoskin *et al.*, 2015). Tonic GABA signalling on the other hand affects SCN synchrony and shift molecular rhythms. To reduce activation of tonic GABA receptors the idea was to use the pharmacokinetics of GABA receptors. As previously stated, GABAa Tonic receptors respond to concentrations of 0.5-1 µM, with experiments in our department using slightly higher concentrations of 3 µM to match the concentration observed in urine, which induced pericyte-mediated vasoconstriction in the vasa recta (Wildman *et al.*, 2014, 2023). However, higher concentrations were used to synchronise the clock, so a higher concentration of 50 µM was used. This concentration exceeds the range that activates GABAA tonic receptors, so it is theorised not to trigger Vasa Recta vasoconstriction. GABA was tested using the same protocol as depicted by Wildman et al to identify any capillary vasoactivity (Wildman *et al.*, 2014, 2023). It is the hope that using a higher concentration would not induce a pericyte response. However, it was found out later that concentrations of 100 µM have been used in experiments to induce phase resetting of the SCN so the concentration we used may not be sufficient to induce a reset (Liu and Reppert, 2000; Ehlen *et al.*, 2008). Nevertheless, the results can still indicate whether a higher concentration of GABA would still evoke

pericyte mediated activity. GABA at 50 µM did not significantly induce vasoconstriction which could imply that at concentrations exceeding values stated by Albers *et al.,* GABAa receptors in the kidney would not respond, possibly because they are overwhelmed. This provides potential that GABA could still induce phase shifts/resets, but further experiments are required to confirm this.

In addition, the kidney live slices subjected to GABA pre-treatment showed no significant difference in comparison to slices treated without GABA pre-treatment, thus revealing if there is vascular activity of GABA on pericytes, at this concentration it is reversible. Our GABA analysis reveals GABA's potential to reset the circadian clock, but the information is inconclusive. Further work is required to confirm that GABA would be able to reset the clock outside the SCN. To analyse if GABA can influence the renal clock, a combination of DNA extraction, PCR and gel electrophoresis can be used to analyse expression of clock-controlled genes and compare it to known levels at set points of the day. Comparing genetic expression between slices subjected to GABA pre-treatment and control slices from the same kidney can determine if GABA induces a phase shift. The work of Liu and Reppert used a concentration of 100 µM that was applied to the culture of coronal sections (Liu and Reppert, 2000; Ehlen *et al.*, 2008). It is uncertain that the 100 µM concentration would be sufficient as the solution needs to penetrate the 200µm slice instead of cell lines within culture.

There is one significant issue that was revealed about using GABA to shift the circadian clock. It was revealed after the fact that the amount of time the live kidney slices was incubated to reset the clock may not have been sufficient. Previous incubations of GABA in SCN cultures were used for 3 hours to achieve a circadian reset (Liu and Reppert, 2000; Ehlen *et al.*, 2008). This presents a problem for these types of experiments as the live slice kidney model is viable for approximately 3-4 hours after slicing due to deterioration (Crawford, Kennedy-Lydon, Sprott, Desai, Sawbridge, Munday, Unwin, S. S.P. Wildman, *et al.*, 2012). This leaves a narrow window in which slices could be analysed, with possibility that slices will have degraded. This could mean that using GABA to shift the clock for this model is impractical. The initial need to use GABA to shift the clock arose when designing the experiment. The initial idea was to use housing units so mice could be held and use light as a synchroniser of the circadian clock. Unfortunately, this was not achievable so finding an alternative to set the clock was necessary. There could be possible methods to extend the slice viability model by using cell culture solution rather than PSS, but this has not been documented on the renal slice model so more experiments are required to analyse tissue viability (Saitta *et al.*, 2019). Alternatively, using a kidney preservation solution such as the Wisconsin solution could also increase the viability of slices, but further work is required to investigate. At this stage, the use

of GABA to reset the clock in replacement with the live kidney slice model has proven flawed and therefore requires a lot of work to identify the validity of the work.

4.3 The influence of Arginine Vasopressin on pericytes

Arginine vasopressin induces a significant vasoconstriction at pericyte sites in comparison to non-pericyte sites. This not new information as this is the key function of arginine vasopressin and it was previously identified by Mavani *et al* that V1 receptors were identified on the vessel surface of the vasa recta, the area investigated in the outer medulla (Mavani, DeVita and Michelis, 2015). Because of this, it was intended that arginine vasopressin would represent the positive control of the experiment. The hope of this thesis is to search for a drug/molecule that can be used as a therapeutic agent to reduce the onset and severity of IRI. The only concern of using arginine vasopressin is it is known to influence renal function such as affecting internal water homeostasis and blood osmolarity, thus meaning would not be ideal for this experiment type because of its influence on the renal clock (Sparapani et al., 2021). As vasopressin acts as a circadian messenger and reduces renal blood flow, it could be a potential candidate. Vasopressin increases blood pressure and can apply unnecessary pressure on the cardiovascular system. As a short term, treatment there could be potential to help for kidney IRI, but long-term vasopressin administration could induce pathological conditions by prolonged high blood pressure. As high blood pressure is a condition associated with individuals with advanced age, which many require medication to reduce blood pressure, this method of trying to reduce IRI would be inappropriate. Also, if used to alleviate non renal IRI, there would be the side effect of vasopressin inducing the RAAS system and inducing high blood pressure. Another point to consider explores oxygen distribution after vasopressin arteriolar vasoconstriction (Friesenecker *et al.*, 2004). The paper identifies that AVP can lead to ischaemia in the skin and limbs, and increases oxygen consumption in the microvasculature, the opposite to the desired effect. These reasons would make AVP not an ideal target to help alleviate the onset of IRI in the kidney. Melatonin would be a more ideal target when in comparison to AVP. Although AVP has been observed to have some immunomodulatory effects, melatonin reduces ROS accumulation which is a major contributor for IRI. Also, melatonin ameliorates AKI so melatonin would be a more ideal target.

4.4 Corticosterone and pericyte activity

Kidney slices subjected to corticosterone without GABA treatment reveal that corticosterone induces pericyte-mediated vasoconstriction in the vasa recta, supporting research on glucocorticoid action on the Kidney. This coincides with research that describes corticosteroids to induce adrenergic induced

vasoconstriction (Greaves, 1976) and corticosterone is highlighted as a potentiator of angiotensin II vasoconstriction (Van Acker *et al.*, 2002). As stated previously, there is high glucocorticoid activity in the descending Vasa Recta with high expression of 11β-HSD1 and 11β-HSD2 in the renal outer medulla (Usa *et al.*, 2007). Glucocorticoids were previously found to regulate vascular activity through endothelial cells and vascular muscle cells, but our results highlight pericytes are also affected (Yang and Zhang, 2005). It is assumed that cortisol shares a similar effect in humans as the primary glucocorticoid, but other work is required to confirm. The rhythm of corticosterone and cortisol aligns prior to waking in mice and humans as they enter their active phase (Gomez-Sanchez and Gomez-Sanchez, 2014; Mohd Azmi *et al.*, 2021). The initial study from Montaigne *et al* identified that ischaemic injury after perioperative cardiac surgery had greater injury in the morning than later in the day (Montaigne *et al.*, 2018). Knowing corticosterone and cortisol peaks at a similar time, it hints that this increase in injury severity could be due to corticosterone/cortisol. Multiple studies describe glucocorticoid receptor sensitivity was reduced during chronic stress and resisted anti-inflammatory actions of cortisol (Lange *et al.*, 2022). Bruder *et al* commented that acute hypoxia increases corticosterone in the blood, probably to regulate the immune response to hypoxia (Bruder *et al.*, 2008). Additionally, using mineralocorticoid receptor antagonists have been observed to reduce oxidative stress, inflammation and apoptosis in cardiac IRI, thus reducing infarct size (Dragasevic *et al.*, 2020). MR antagonists exert similar effects in renal tissue, suppressing pro-inflammatory cytokines, chemoattractants and prooxidants and promoting anti-inflammatory cytokine production (Luan *et al.*, 2022). In renal IRI, these antagonists improve renal function by reducing oxidative stress, inflammation and proteinuria, and improving glomerular filtration and renal blood flow. This was utilised as both preconditioning and postconditioning for the tissue, reducing post ischaemic remodelling. In addition to inducing vasoconstriction, glucocorticoids also suppress the production of vasodilators such as nitric oxide (Yang and Zhang, 2005). This effect of corticosterone/cortisol is another key element observed in IRI, increasing severity of injury by increased irreversible vasoconstriction.

The information presented provides insight to why ischaemic damage is greater in earlier stages of daytime. It was found that the blood count of stress associated leukocytes such as neutrophils, natural killer cells, and highly differentiated cytotoxic T cells presents a rhythm with a daytime peak making them more readily available to infiltrate tissue (Lange *et al.*, 2022). Cortisol was shown to promote CXCR4-driven leukocyte traffic, promoting migration of eosinophils and less differentiated T cells into tissues, most notably bone marrow during the daytime. Although leukocyte movement into the blood is primarily driven by adrenaline, cortisol also promotes neutrophil egress from the bone marrow and prevents traffic via CXCR4 cells to other peripheral tissues, inflamed tissues in particular. This synergistic relationship of

cortisol and adrenaline makes immune cell traffic that could be involved in the daytime damage. The study from Lange *et al* also notes sleep suppresses cortisol and adrenaline levels, which in turn modulates the immune rhythm (Lange *et al.*, 2022). In the circadian rhythm, cortisol is known to entrain *Bmal1, Per1* and *Per2* in the SCN (Woodruff *et al.*, 2016). Cortisol and corticosterone rhythm co aligns with the rhythm of REV-ERBα. In intestinal epithelial cells, high REV-ERBα activity resulted in enhanced corticosterone production (Druzd, De Juan and Scheiermann, 2014). Supporting this, both *Rev-Erbα* and glucocorticoid receptors influence similar metabolic and inflammatory processes and REV-ERBα influences the stability and nuclear localization of GR which alters GR target gene expression such as IκBα and alcohol dehydrogenase 1 (Okabe *et al.*, 2016).

REV-ERBα was previously speculated to regulate blood pressure and inflammation via the circadian clock, therefore may be an ideal target for this study (Ramakrishnan and Muscat, 2006). Tracking molecular clock components in mice and representing the stages of molecular events in circadian time (CT), revealed that *Per*, *Rev-erbα* and *Cry* mRNA begin to rise from CT0, which is mid evening transitioning into late night (Dunlap, Loros and DeCoursey, 2004). At CT6, *Rev-Erbα* is translated and translocated to the nucleus which leads to suppression of BMAL1 expression. This activity occurs in the human brain under the same time frames. REV-ERBα is a known transcriptional repressor in many tissues and is implicated in regulating inflammation and metabolism (Griffin *et al.*, 2020). The REV-ERB subgroup is present in peripheral tissues including skeletal muscle, brain, liver and kidney, displaying high energy demand in these tissues (Ramakrishnan and Muscat, 2006). Inflammatory cytokines play crucial roles in endothelial and smooth muscle cell response in vasculature associated diseases such as IRI. Studies in skeletal and vascular smooth muscle suggest that REV-ERBα regulates inflammation by regulating IκBα/NF-κB dependent gene expression. Vascular smooth muscle cells that over-express REV-ERBα induced NF-κB mediated p65 trans-activation and translocation and displayed increased expression of pro-inflammatory cytokines IL-6 and COX-2 (Ramakrishnan and Muscat, 2006). This also occurred in macrophages, where utilising synthetic REV-ERBα ligands and REV-ERBα knockdown cells modulated IL-6 production (Gibbs *et al.*, 2012). REV-ERBα may not directly regulate cytokine response but may be an indirect response by REV-ERBα repressing a repressor.

There is conflicting evidence whether REV-ERBα invokes detrimental or protective effects in response to ischaemia reperfusion injury. Rev-Erbα ablation sensitises mice to hepatic IRI by exacerbating Nlrp3 inflammasome activity (Lin *et al.*, 2020). Both the SR9009 pharmaceutical Rev-Erbα agonist and surgery time corresponding to high Rev-Erbα expression were found to reduce the magnitude of injury, thus

implying a protective effect. Similarly, SR9009 reduces the severity of myocardial ischaemic injury by reducing ferritinophagy/ferroptosis signalling (Huang *et al.*, 2022). In contrast, REV-ERBα ablation or antagonism ameliorated ischaemia-reperfusion injury by promoting CDKN1a/p21 (Montaigne *et al.*, 2018). Wang *et al* noted other factors influence IRI such as calcium overload, oxidative/nitrosative stress and the inflammatory response (Wang *et al.*, 2020). Overall, it is unknown whether REV-ERBα is involved in protective effects in relation to IRI but could have an indirect effect by regulating cortisol or corticosterone. Acute stress via glucocorticoids acts as an effective pre-conditioning mechanism to protect tissues and reduce the severity of IRI (Filep, 2014; Imani *et al.*, 2019; Filaretova *et al.*, 2021). However, chronic/dysregulated corticosterone and cortisol is associated with greater injury by ischaemia and reperfusion (Filaretova *et al.*, 2021; Lange *et al.*, 2022). This is associated with MR activity, where chronic or dysregulated cortisol/corticosterone increases inappropriate MR activation, leading to a rise in ROS and inflammation, therefore increasing renal disease (Gomez-Sanchez and Gomez-Sanchez, 2014).

4.5 The use of melatonin as a therapeutic agent for Ischaemia reperfusion Injury

4.5.1 Melatonin and pericyte activity

The work from this thesis assessed melatonin on pericytes to identify an effect of melatonin, if any, upon pericyte constriction/dilation. It was revealed that melatonin induces vasoconstriction via pericyte activity. For most nocturnal mice, normal nocturnal behaviour describes that melatonin signalling correlates with a decline in mid-nighttime activity and increased mid-night sleep episodes as endogenous melatonin reduces the threshold from sleep-wake transition (Kim et al., 2024). In terms of renal and vascular biology, the promotion of sleep onset would result in reduced renal blood flow, filtration and therefore urine production. However, as mentioned earlier in section 1.5.4.6, C57BL/6J mice are melatonin deficient, therefore exhibit different nocturnal behaviour to melatonin proficient mice. Specifically, melatonin proficient mice display locomotor activity in the first half of the dark phase, and declines in the second half, coinciding with the night peak of melatonin (Pfeffer et al., 2022). C57BL/6J mice on the other hand, experience locomotor activity throughout the entire dark phase. Timed exogenous melatonin in C57BL/6J mice was found to exhibit behaviour similar to melatonin proficient mice. Exogenous melatonin also helped re-entrain rhythms in jet lag studies in melatonin-deficient mice (Pfeffer et al., 2022).Zhang et al, also developed melatonin proficient C57BL/6J mice that displayed a similar phase advancement of locomotor activity (Zhang *et al.*, 2018, 2021; Pfeffer *et al.*, 2022). These articles describe as expressed earlier in section 1.5.4.6, that C57BL/6J mice still possess the functional downstream components of melatonin signalling. This would mean that the results obtained from

experiments of this thesis are results of exogenous melatonin but could allude to effects on renal physiology with endogenous melatonin.

For humans, similar effects would occur to prepare to initiate sleep. There is an established circadian variation of renal blood flow and urinary excretion, decreasing flow through the night and urinary excretion showing a peak in the afternoon and evening (Eckerbom *et al.*, 2020). There is a well described rhythmicity in some diuretic and anti-diuretic hormones associated with implementing a circadian rhythm in urine output (Duffy, Scheuermaier and R. Loughlin, 2016). Melatonin secretion and nocturia in the elderly population is inversely proportional, with melatonin deterioration with age seen to increase incidence of sleep disruption and nocturia (Obayashi, Saeki and Kurumatani, 2014). It was discovered that melatonin receptors are present in human vasculature, which implicates melatonin in the local control on vasculature and possibly in cardiovascular disease (H. Zhou *et al.*, 2018). During the pathology of ischaemia reperfusion injury, vasoconstriction leads to loss of microvascular blood supply and pericyte cell death if not reversed. Therefore, on this function of melatonin alone, pre-treatment of patients using melatonin as a prediction, would not appear beneficial. However, as discussed in section 1.4.3, there are underlying factors that contribute to the 'No reflow' phenomenon leading to the irreversible constriction and damage. Persistent pericytes constriction was reversed by suppressing ROS/RNS accumulation and peroxynitrite formation, highlighting these factors in the onset and severity of ischaemic injury. Thus, melatonin still may have therapeutic potential as an immune regulator.

4.5.2 Corticosterone and Melatonin, the day and night perspective

The use of melatonin in this study, in opposition to corticosterone, was to give a nighttime perspective on pericyte activity. Both melatonin and corticosterone act on the immune response, with their production being regulated in response to Pathogen-associated molecular (Cyrino *et al.*, 2022). It is speculated that coupling melatonin and corticosterone could be physiologically beneficial, to allow melatonin to counteract the adverse effects of free radicals associated with stressful conditions (Barriga, Marchena, et al., 2002; Barriga, Martín, et al., 2002). Previous work identified a positive correlation between increased circulating melatonin in the night; the decline of free radicals and melatonin levels ameliorates exaggerated T cell apoptosis induced by high glucocorticoids. In the latter scenario, melatonin activity was observed via an antioxidant mechanism by upregulating the gene *Bcl-2* (Barriga, Marchena, *et al.*, 2002). Melatonin and corticosterone influence the inflammatory response and play essential, complex immunomodulatory roles. In nocturnal animals, corticosterone peaks at light/dark transition at their sleep to wake transition, while melatonin peaks at the middle of the night in both nocturnal and diurnal animals

(Da Silveira Cruz-Machado *et al.*, 2017). Crosstalk between adrenal and pineal glands in response to inflammatory conditions indicate that corticosterone can upregulate nocturnal melatonin synthesis by reducing NF-κB activity (Da Silveira Cruz-Machado *et al.*, 2017).

4.5.3 The relationship between melatonin and arginine vasopressin

Our work demonstrates that melatonin induces pericyte-mediated vasoconstriction similar to arginine vasopressin. These molecules exhibit similar circadian regulation with peaks during the nighttime hours. This similar action implies an underlying relationship between melatonin and arginine vasopressin. Cell culture studies document melatonin inhibits AVP release in the SCN, facilitated via MT2 receptors (Isobe, Fujioi and Nishino, 2001). In the hypothalamus, there are conflicting evidence that melatonin alters AVP secretion, *in vitro* studies showing melatonin stimulating AVP release and suppressed basal AVP levels yet reporting no effect on AVP levels in the hypothalamus in response to long-term melatonin administration (Mogulkoc and Baltaci, 2010). Stimulated and non-stimulated AVP release via L-thyroxine was also shown to be inhibited by melatonin. Mogulkoc and Baltaci added that the response of AVP to hypertonic and hypovolemic conditions are not affected by both L-thyroxine and melatonin. This suggests in physiological conditions, these molecules alter arginine vasopressin only when it does not interfere with blood pressure regulation (Mogulkoc and Baltaci, 2010). Melatonin also reduces AVP levels during physical exercise (Chiodera *et al.*, 1998). In addition, melatonin was shown to influence the synthesis and release of AVP and oxytocin *In vivo* and *in vitro*, thus altering the activity of hypothalamic supraoptic nuclei (SON) and PVN (Roszczyk and Juszczak, 2014). Interestingly, the response of AVP and OT to melatonin was dependent on melatonin concentration, causing inhibition in lower concentrations (10^{-7}-10^{-9}M) but stimulated AVP and OT at higher concentrations of 10^{-3}M. More recently, melatonin alleviated arginine vasopressin induced cardiomyocyte apoptosis (Chen *et al.*, 2022). Patients that suffered heart failure have elevated AVP levels, which induce peripheral vasoconstriction, cell apoptosis and cardiac hypertrophy by increased oxidative stress. Melatonin administration reduced these effects, partially via activation of the Nrf2 Pathway (Chen *et al.*, 2022). In addition, other studies illustrate melatonin reduced oxidative stress and apoptosis by activating Nrf2 after ischaemia-reperfusion in H9c2 cells (Zhang Yan *et al.*, 2018), Similarly, the Nrf2/HO-1 pathway was induced by melatonin in smooth muscle cells in response to oxidative stress/apoptosis (Tang *et al.*, 2019). It was previously revealed melatonin regulates cell death in response to inflammation by increasing protein expression of Bcl-2, which blocks the proapoptotic ability of Bax via utilising the SIRT1/ NF-κB axis (Tarocco *et al.*, 2019). This in turn leads to significant inhibition of Cyt C release, the decline of caspase 3 activation and forms apoptosomes.

4.5.4 The therapeutic potential of melatonin on Ischaemia-Reperfusion Injury

Overall, this study reveals growing evidence supporting the therapeutic potential of melatonin to alleviate ischaemia-reperfusion injury, due to its multifaceted influence on different stages of IRI including: mitochondrial dysfunction, ROS production and promoting anti-apoptotic effects. The role of melatonin in renal ischemia-reperfusion injury through different mechanisms has been summarised using figure 27 (Ahammed et al., 2022).

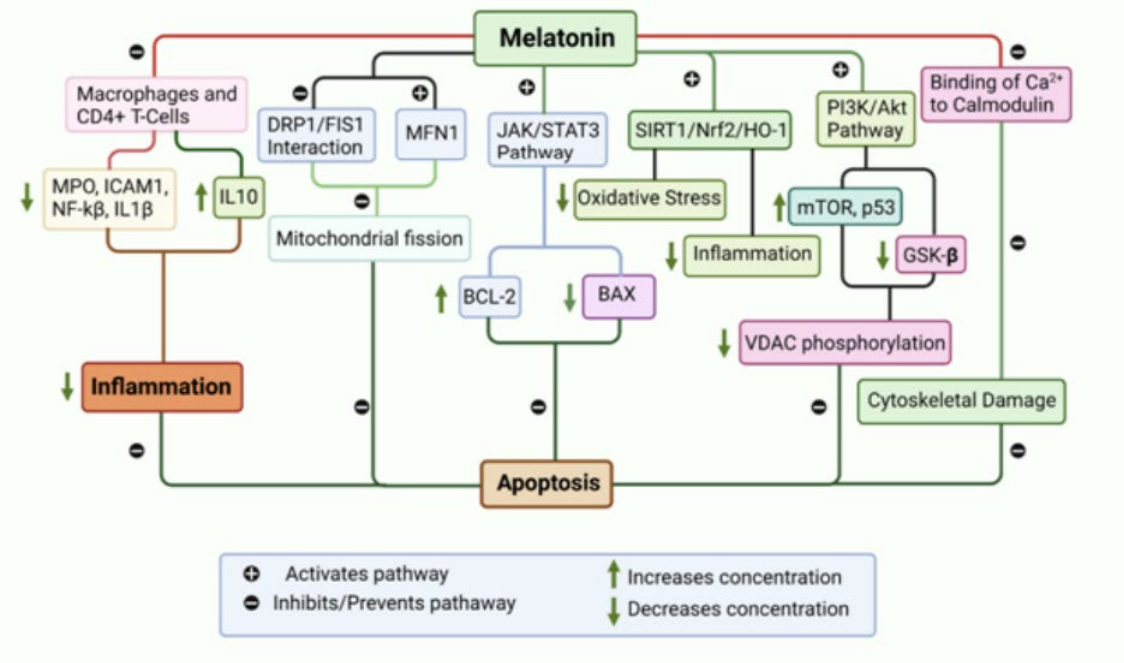

Figure 177 - Summary of Melatonin's effect on preventing renal ischaemia-Reperfusion Injury, taken from Ahammed *et al.,* (Ahammed *et al.*, 2022).

Malfunctioning mitochondria contributes to a multitude of disorders including IRI (Reiter, Ma and Sharma, 2020). Melatonin governs the conversion of pyruvate to acetyl-CoA in the mitochondria and was found to reprogram glucose metabolism in cancer cells to a normal physiological phenotype, thus indicating melatonin influences mitochondria physiology IRI (Reiter, Ma and Sharma, 2020). In addition, melatonin acts at multiple levels of the electron transport chain by stimulating NADH-coenzyme Q reductase (Complex I) and cytochrome c oxidase (Complex IV) enzyme activity, which leads to reduced electron leakage and production of free radicals (Ahammed et al., 2022). In addition, melatonin attenuates endothelial cell pyroptosis induced by hyperglycaemia. Damaged endothelial cells release proinflammatory and procoagulant cytokines causing; adhesion and infiltration of mononuclear macrophages, platelet aggregation, thrombosis and further release of inflammatory factors (Wang Xuebin

et al., 2023). Melatonin alleviates these symptoms by reducing reactive oxygen species and activating the NLRP3 inflammasome pathway via the Nrf2 pathway. Nuclear factor erythroid 2-related factor 2 (Nrf2) activates antioxidant response elements (ARE), binding to Keap1 in the cytoplasm under physiological conditions (Wang Xuebin *et al.*, 2023). When exposed to oxidative stress, both extracellular protein kinase C and phosphatidylinositol 3-kinase signalling pathways activate Nrf2 through phosphorylation. Activated Nrf2 translocate to the nucleus and binds to the cis-acting AREs. This leads to transcription of heme oxygenase-1, NADPH Quinone Oxidoreductase-1, superoxide dismutase and glutathione peroxidase, which helps maintain stability of the intracellular environment (Wang Xuebin *et al.*, 2023).

Melatonin is already known as a potent free radical scavenger, able to scavenge up to 10 reactive oxygen species (Ahammed et al., 2022). Melatonin prevents a rise in Nitric oxide, which could explain the vasoconstriction observed in our work, as it reduces the availability of vasodilatory molecules. Nitric oxide is typically seen as beneficial as NO induces vasodilation. However, NO is postulated to be a primary cause of renal injury and aggravate ischaemic insult in IRI, as NO interacts with superoxide to produce peroxynitrite. This RNS/ROS is a powerful oxidant, producing hydroxyl radicals and causing damage, including lipid peroxidation (Ahammed et al., 2022). Melatonin chelates transition metals which reduce toxic hydroxyl radicals forming, further contributing to reduce oxidative damage (Reiter *et al.*, 2016). Melatonin also inhibits Ca2+ binding to binding proteins like calmodulin and calretinin thus preventing cytoskeleton damage (Ahammed et al., 2022). Melatonin activates the PI3K/Akt/mTOR pathway and exerts renoprotective properties following ischemia-reperfusion injury (Ahammed *et al.,* 2022). Melatonin also enhances autophagy through the TLR4/MyD88/MEK/ERK/mTORC1 signalling pathway in ischemia-reperfusion injury (Ahammed et al., 2022). Finally, melatonin acts on the Akt pathway to prevent decline of phosphorylated Akt and Bad levels after hypoxia-ischaemia injury (Tarocco *et al.*, 2019). The indirect antioxidant effect of melatonin is mediated by upregulating enzyme synthesis of glutathione synthase, superoxide dismutase and catalase, which catalyse the conversion of free radicals and downregulates pro-oxidant enzymes (Ahammed et al., 2022).

Melatonin has been speculated to alleviate resistant hypertension. Hypertension is influenced by endothelial dysfunction, increased oxidative stress, neurohumoral imbalance and alterations upon renal function (Simko, Reiter and Paulis, 2019). Melatonin has been speculated to alleviate resistant hypertension by its antioxidant capabilities, limiting oxidative stress via intracellular and extracellular mechanisms such as direct radical scavenging, improving mitochondrial electron transport, stimulating antioxidant enzyme expression and downregulating pro-oxidant enzymes, supporting synthesis and

recycling of glutathione and protecting other antioxidants (Simko, Reiter and Paulis, 2019). Using the two-kidney one-clip model, Nishi *et al*. identified that ischaemic effects induced by resistant hypertension was partially reversed by renal denervation but also melatonin treatment (Nishi *et al.*, 2018, 2019). The latter reduces the mean arterial pressure by 45mmHg, attenuates sympathetic excitation in the ischaemic kidney and normalises the baroreflex and splanchnic activity. It is speculated melatonin could affect sympathetic activity by GABAergic inhibition from the SCN to PVN, by nitric oxide. Supporting this notion, melatonin suppressed sympathetic activity via increased GABAa receptors in the hypothalamus PVN (Yu *et al.*, 2023). This provides evidence that melatonin could counteract excessive sympathetic stimulation (Simko, Reiter and Paulis, 2019; Yu *et al.*, 2023). In addition, melatonin secretion is associated with Renin-Angiotensin-System activity. Impaired melatonin signalling was associated with increased night-time renal-RAS activation, and melatonin improved the structure and function in animal models of renal damage (Simko, Reiter and Paulis, 2019).

4.5.5 Current studies of melatonin action on transplantation

Melatonin has already shown to protect against ischaemia-reperfusion injury in the lung, liver, kidney and other organs (Wang *et al.*, 2018). Melatonin reduced lung IRI by inhibiting oxidative stress, inflammation and apoptosis. Histological analysis showed that lung parenchymal damage was ameliorated in the groups pre-treated with melatonin compared to the IR group, with melatonin pre-treatment groups exhibiting greater expression of superoxide dismutase, glutathione peroxidase and glutathione reductase (Wang *et al.*, 2018; Hu *et al.*, 2021). In addition, melatonin displayed a protective role against lipid peroxidation and thus cell damage by diminishing malondialdehyde production. Meurisse *et al*. analysed a combined drug approach to reduce the severity of ischaemia reperfusion injury on liver transplants (Meurisse *et al.*, 2023). The study used a combination of drugs including melatonin following cold static preservation but prior to anaesthesia and found no significant difference in the post-transplant peak in serum aspartate transaminase, frequency of early allograft dysfunction, surgical complications, acute kidney injury and the IRI score (Meurisse *et al.*, 2023). This lack of response is surprising as melatonin has been shown to have a protective role in hepatic IRI when assessing melatonin with warm and cold IRI previously (Hu *et al.*, 2021). Within hepatic tissue, melatonin protected against IRI by increasing respiration and ATP synthesis, relieving mitochondrial swelling and reducing lipid peroxidation. This discrepancy between IR models between Hu *et al*. and Meurisse *et al* alludes to higher complexity in alleviating IRI and translating results from IR models to clinical practice. One possibility is combined drug treatment just before transplantation is insufficient. Longer pre-treatment could better prime the donor organ for transplantation. Meurisse *et*

al suggested this as the "proof of concept" study they described combined drug therapy *in situ* to the donor during the flush prior to static cold storage as an upstream strategy, whereas their approach was used as a downstream strategy so this could explain the unexpected result (Meurisse *et al.*, 2023). Hu *et al.* stated that melatonin has the capacity to preserve liver function after cold preservation IRI and promotes regeneration by enhancing inflammatory Ly6C+ F4/80+ monocytes and promoting IL-6, IL-10 and TNF-α release (Hu *et al.*, 2021). Similarly, cryopreserved ovarian tissue exhibited protective effects to apoptosis and oxidative stress. Cold IRI similarly alleviated by melatonin via activating the Nrf2/HO-1 signalling pathway (Sun *et al.*, 2020). Hu *et al* commented on a trial where a drug cocktail including melatonin provided protection against IRI in steatotic liver grafts by reducing leukocyte infiltration, vacuolization and cell death (Hu *et al.*, 2021).

Clinical studies have proven inconclusive in myocardial IRI, with some studies showing melatonin promoting cardioprotective effects and others not (Bermudez-Gonzalez *et al.*, 2022). Melatonin's activation of the Nrf2 pathway was found to induce cardioprotective effects in myocardial IRI (Xu *et al.*, 2021). In this study, melatonin significantly improved post-ischaemic cardiac function, decreased oxidative stress and reduced infarct size. Numerous animal experiments demonstrate melatonin is effective in reducing the severity of myocardial IRI, limiting post ischaemic arrhythmias and improving cardiac recovery. These studies as well as Zhou *et al,* highlight the cardioprotective effects of melatonin may be due to its antioxidant properties (H. Zhou et al., 2018). There is a strong association with taking oral melatonin before bed 1 month prior to surgery with a significant rise in plasma melatonin and Nrf2 levels which can be vital in enhancing antioxidant defence (Bermudez-Gonzalez *et al.*, 2022). Singhanat *et al* also highlighted melatonin as a therapy for cardiac IRI, as melatonin alleviated cardiac IRI at all time points administered. In H9C2 cell lines, melatonin increased cell viability by reducing Bax protein expression and promoting mitochondrial dynamic balance (Singhanat *et al.*, 2021). The cardioprotective effects of melatonin were found to be dependent on MT2 receptors, highlighting a future target for study.

In terms of renal IR injury, a study that used combined therapy of erythropoietin (EPO) and melatonin exhibited beneficial alleviating of renal IRI (Ahmadiasl *et al.*, 2014). The underlying mechanism to this protective effect was due elevated antioxidant capacity, with EPO complimenting melatonin as a scavenger by acting as an enhancer of antioxidant enzymes (H. Zhou *et al.*, 2018). Similarly, melatonin was used with vitamin D3, which regulated and reduced reactive nitrogen species such as NO_2 and $ONOO^-$, promoting angiogenesis and endothelial stability in IRI (H. Zhou *et al.*, 2018). Oral melatonin was tested

to alleviate renal IRI like the previous study on myocardial IRI (Panah et al., 2019). Patients were randomly assigned to take a placebo or 3mg of melatonin a day, from 24hr prior to transplant until time of discharge. This was a lower concentration than Bermudez-Gonzalez *et al* yet still found that serum levels of MDA, CP, 8-OHdG (markers of lipid peroxidation, protein oxidation and DNA damage), TNF-α markers, neutrophil gelatinase-associated lipocalin, a renal functional marker, all significantly decreased (Panah *et al.*, 2019; Bermudez-Gonzalez *et al.*, 2022). The combined work provides evidence that melatonin exhibits therapeutic effects for transplants with a long-term pretreatment, potentially explaining the result from Meurisse *et al*. Chronic melatonin treatment reduces renal damage by restricting NO production and lipid oxidation in streptozotocin-induced diabetic rats that leads to renal ischemia-reperfusion injury (Ahammed *et al.*, 2022). Melatonin also downregulates caspase 3, caspase 9, caspase 12, iNOS, p65 and NF-κB (Zang *et al.*, 2020; Ahammed *et al.*, 2022). Furthermore, melatonin previously was found to be beneficial in kidney transplant patients as it protected the graft from ischemia reperfusion injury (Ahammed *et al.*, 2022). This further supported Li *et al.,* who explained melatonin protected kidney donor grafts through anti-oxidative, anti-apoptotic and NF-κB activity (Li *et al.*, 2009). A summary on the actions of melatonin on various forms of IRI is illustrated in figure 28, (H. Zhou *et al.*, 2018).

Culminating evidence suggests melatonin administration before IRI promotes preservation of renal function by decreasing pro-inflammatory cytokines, oxidative stress, and neutrophil/macrophage infiltration (Ahammed *et al.*, 2022). Despite melatonin showing promise, Costello *et al*. found mice share similar pharmacological profiles to humans, but these results may not translate exactly (Costello *et al.,* 2022). Our work shows potential, but the safety profile, dosage and duration of melatonin therapy still require investigation, paving the way for further study to understand melatonin's full therapeutic potential (Ahammed *et al.*, 2022). Currently, melatonin exhibits protective effects when used as long-term therapy. Melatonin intraperitoneal injection prior to renal IRI, with and without ischaemic preconditioning, alleviated renal IRI, with combined therapy being the most effective (Abdel-Razek *et al.,* 2023). Recent studies utilize melatonin in combined drug therapy to help relieve ischaemic insult and damage in IRI. Melatonin provided better results in long term pre-treatment, meaning patients could benefit from melatonin supplementation by taking weeks before surgery to post surgery or sometime after. To assess melatonin's beneficial effects via pericytes, immunostaining could be useful to assess melatonin's influence on pericyte-endothelial communication during IRI. Immunostaining can analyse immune cell infiltration by investigating adhesion molecule expression on renal microvasculature. In conclusion, current work implies melatonin could be a therapeutic agent to alleviate renal damage by

ischaemia-reperfusion injury. Future work should refine melatonin therapy, with oral supplementation or photoentrainment being targets to reduce injury.

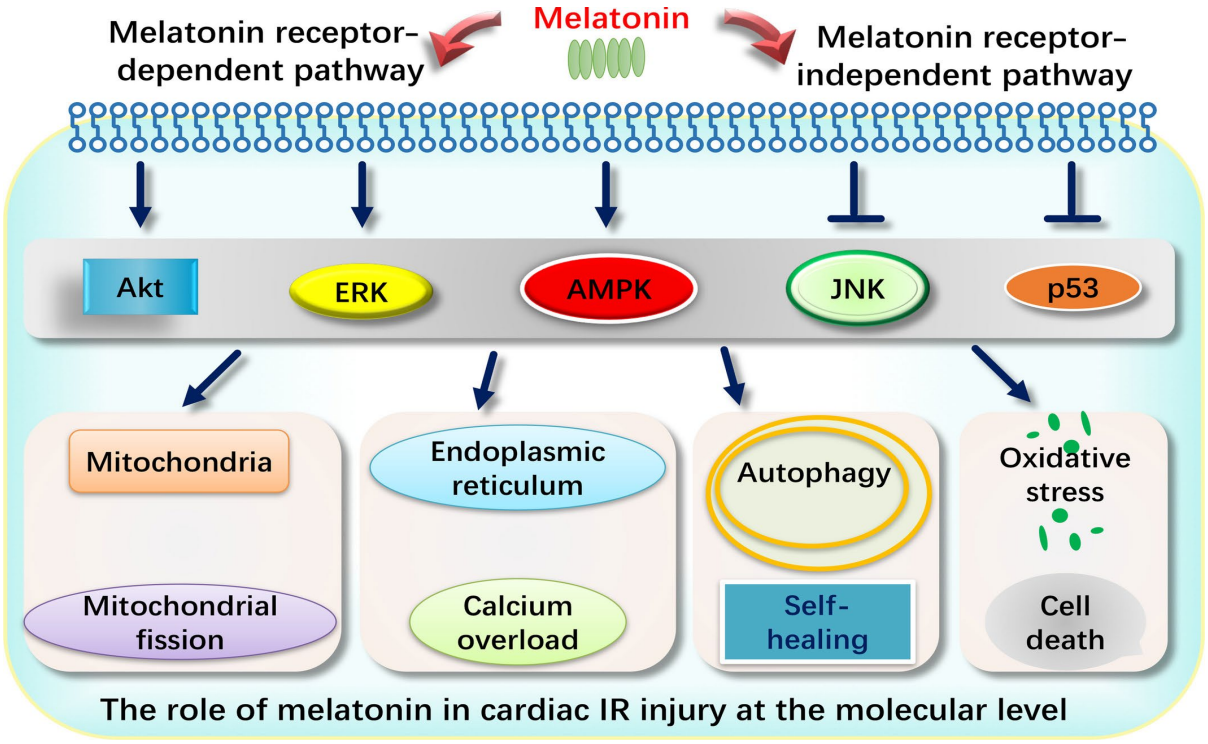

Figure 28 - A summary of the action of melatonin on the onset and severity on cardiac IRI and other variations of IRI at a molecular level, taken from Zhou *et al.,* (H. Zhou et al., 2018).

The protective effect of melatonin is divided into the receptor-dependent and receptor-independent pathways. The receptor-dependent pathway specifies the action of melatonin action on MT1, MT2 and ROR receptors. Receptor dependent action on the Akt, ERK and AMPK pathways exerts protective effects to IRI by alleviating mitochondrial fission and calcium overload in the mitochondria and ER as well as promoting self-healing via autophagy. The receptor independent pathway involved the inhibitory action upon JNK and p53 which reduces oxidative stress and apoptotic signalling that results in cell death.

4.6 Strengths and limitations of the study

The research conducted within this thesis has revolved around the therapeutic potential of melatonin in ameliorating ischaemia reperfusion injury with recent studies starting to use melatonin as combined therapy for organ preservation. This has provided merit to understand the full therapeutic potential melatonin and the work conducted and reviewed in the thesis is to address an additional function of melatonin and how this can influence IRI. Most research focuses on the antioxidant properties of melatonin so analysing melatonin on pericyte activity and immune cell entry provides a new perspective on how melatonin can alleviate IRI. Analysis specifically in the *in situ* kidney live slice model to

corticosterone and melatonin provides real time analysis on how circadian messengers influence pericyte biology.

Despite this, the thesis line of study has faced limitations. A major question from this work is will the findings of exogenous melatonin in C57BL/6J mice translate into melatonin proficient animals and human studies. Although presence of functional receptors in these melatonin deficient mice, future work with melatonin proficient strains of mice can reinforce these findings. Another limitation evokes from trying to design on generating circadian resets from the live slice model. The length of time to induce a circadian reset and the narrow window of renal slice viability means that another model might be able to better analyse GABA inducing a reset in peripheral clocks than the *in situ* model. Finally, the concentrations of GABA, corticosterone and melatonin used in this study were estimations to elicit a response. Refining this work by performing dose response curves for each messenger would help find a concentration that matches endogenous levels or levels that can achieve a circadian reset as with GABA.

Conclusion

Collated research identifies circadian influence on components of the immune system. This study focussed on renal ischaemia-reperfusion injury, an inevitable consequence in transplantation as blood supply is severed during the transfer from donor to recipient. GABA, arginine vasopressin, corticosterone and melatonin demonstrated pericyte mediated vasoconstriction in the DVR. The main focus analyses melatonin on pericytes due to melatonin previously ameliorating kidney injury and Ischaemia-reperfusion injury. Melatonin induces pericyte mediated vasoconstriction, but prolonged constriction would not relieve IRI pathogenesis. Therefore, future experiments should focus on melatonin as an antioxidant scavenger and influence on immune cell entry. The secondary aim addresses whether GABA could be used to reset the renal peripheral clock in the kidney live slice model. This line of research was proven more complex due to the narrow window of slice viability and the length of time required to induce a phase clock reset. Despite this setback, the thesis has proven that the influence of GABA on these slices is reversible so will not impact future studies on vascular activity if phase shifts or clock resets can be induced. Future work could explore if the viability of the live slice model could be extended to improve the window that these experiments can be conducted.

5 References

Van Acker, S.A.B.E. *et al.* (2002) 'Centrally Regulated Blood Pressure Response to Vasoactive Peptides is Modulated by Corticosterone', *Journal of Neuroendocrinology*, 14(1), pp. 56–63. Available at: https://doi.org/10.1046/J.1365-2826.2002.00740.X.

Ahammed, M.R. *et al.* (2022) 'Role of melatonin on renal ischemia-reperfusion injury', *Discoveries Reports*, 5(1), p. 29. Available at: https://doi.org/10.15190/drep.2022.3.

Ahmadiasl, N. *et al.* (2014) 'Effect of a combined treatment with erythropoietin and melatonin on renal ischemia reperfusion injury in male rats', *Clinical and Experimental Nephrology*, 18(6), pp. 855–864. Available at: https://doi.org/10.1007/S10157-014-0937-6/FIGURES/2.

Albers, H.E. *et al.* (2017) 'The dynamics of GABA signaling: Revelations from the circadian pacemaker in the suprachiasmatic nucleus', *Frontiers in Neuroendocrinology*, 44, pp. 35–82. Available at: https://doi.org/10.1016/J.YFRNE.2016.11.003.

Albus, H. *et al.* (2005) 'A GABAergic mechanism is necessary for coupling dissociable ventral and dorsal regional oscillators within the circadian clock', *Current Biology*, 15(10), pp. 886–893. Available at: https://doi.org/10.1016/j.cub.2005.03.051.

Amaral, F.G. Do and Cipolla-Neto, J. (2018) 'A brief review about melatonin, a pineal hormone', *Archives of Endocrinology and Metabolism*, 62(4), pp. 472–479. Available at: https://doi.org/10.20945/2359-3997000000066.

Aschoff, J. (1979) 'Circadian Rhythms: Influences of Internal and External Factors on the Period Measured in Constant Conditions', *Zeitschrift für Tierpsychologie*, 49(3), pp. 225–249. Available at: https://doi.org/10.1111/j.1439-0310.1979.tb00290.x.

De Assis, L.V.M. and Oster, H. (2021) 'The circadian clock and metabolic homeostasis: entangled networks', *Cellular and Molecular Life Sciences 2021 78:10*, 78(10), pp. 4563–4587. Available at: https://doi.org/10.1007/S00018-021-03800-2.

Aton, S.J. *et al.* (2006) 'GABA and Gi/o differentially control circadian rhythms and synchrony in clock neurons', *Proceedings of the National Academy of Sciences of the United States of America*, 103(50), pp. 19188–19193. Available at: https://doi.org/10.1073/PNAS.0607466103/SUPPL_FILE/IMAGE536.GIF.

Attwell, D. *et al.* (2016) 'What is a pericyte?', *Journal of Cerebral Blood Flow and Metabolism*, 36(2), pp. 451–455. Available at: https://doi.org/10.1177/0271678X15610340/ASSET/DA388224-4D0B-459B-9ABB-31414409EC5B/ASSETS/IMAGES/LARGE/10.1177_0271678X15610340-FIG2.JPG.

Banaei, S., Ahmadiasl, N. and Alihemmati, A. (2016) 'Comparison of the Protective Effects of Erythropoietin and Melatonin on Renal Ischemia-Reperfusion Injury', *Trauma monthly*, 21(3). Available at: https://doi.org/10.5812/TRAUMAMON.23005.

Banaei, S., Rezagholizadeh, L. and Azimian, E. (2019) 'The role of hormones in renal disease and ischemia-reperfusion injury', *Iranian Journal of Basic Medical Sciences*, 22(5), pp. 469–476. Available at: https://doi.org/10.22038/IJBMS.2019.34037.8095.

Barclay, J.L., Tsang, A.H. and Oster, H. (2012) 'Interaction of central and peripheral clocks in physiological regulation', *Progress in Brain Research*, 199, pp. 163–181. Available at: https://doi.org/10.1016/B978-0-444-59427-3.00030-7.

Barrera-Chimal, J. and Jaisser, F. (2020) 'Vascular and inflammatory mineralocorticoid receptors in kidney disease', *Acta Physiologica*, 228(2), p. e13390. Available at: https://doi.org/10.1111/APHA.13390.

Barriga, C. *et al.* (2001) 'Circadian rhythm of melatonin, corticosterone and phagocytosis: effect of stress', *Journal of Pineal Research*, 30(3), pp. 180–187. Available at: https://doi.org/10.1034/J.1600-079X.2001.300307.X.

Barriga, C., Marchena, J.M., *et al.* (2002) *Effect of stress and dexamethasone treatment on circadian rhythms of melatonin and corticosterone in ring dove (Streptopelia risoria)*, *Molecular and Cellular Biochemistry*.

Barriga, C., Martín, M.I., *et al.* (2002) 'Physiological concentrations of melatonin and corticosterone in stress and their relationship with phagocytic activity', *Journal of Neuroendocrinology*, 14(9), pp. 691–695. Available at: https://doi.org/10.1046/j.1365-2826.2002.00823.x.

Bellini, M.I. *et al.* (2019) 'Cold Pulsatile Machine Perfusion versus Static Cold Storage in Kidney Transplantation: A Single Centre Experience', *BioMed Research International*, 2019(1), p. 7435248. Available at: https://doi.org/10.1155/2019/7435248.

Ben-Ari, Y. *et al.* (2007) 'GABA: A pioneer transmitter that excites immature neurons and generates primitive oscillations', *Physiological Reviews*, 87(4), pp. 1215–1284. Available at: https://doi.org/10.1152/PHYSREV.00017.2006/ASSET/IMAGES/LARGE/Z9J0040724500011.JPEG.

Bermudez-Gonzalez, J.L. *et al.* (2022) 'Role of the Antioxidant Activity of Melatonin in Myocardial Ischemia-Reperfusion Injury', *Antioxidants*. MDPI. Available at: https://doi.org/10.3390/antiox11040627.

Bhandage, A.K. (2016) 'Glutamate and GABA signalling components in the human brain and in immune cells.', in. Uppsala: Acta Universitatis Upsaliensis, pp. 1–81. Available at: http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-282422.

Bhandage, A.K. *et al.* (2018) 'GABA Regulates Release of Inflammatory Cytokines From Peripheral Blood Mononuclear Cells and CD4+ T Cells and Is Immunosuppressive in Type 1 Diabetes', *EBioMedicine*, 30, pp. 283–294. Available at: https://doi.org/10.1016/J.EBIOM.2018.03.019/ATTACHMENT/1FBE71F0-2D4D-4AC8-8173-DF3936F81F58/MMC1.DOCX.

Bhat, R. *et al.* (2010) 'Inhibitory role for GABA in autoimmune inflammation', *Proceedings of the National Academy of Sciences of the United States of America*, 107(6), pp. 2580–2585. Available at: https://doi.org/10.1073/PNAS.0915139107.

Birbrair, A. *et al.* (2015) 'Pericytes at the intersection between tissue regeneration and pathology', *Clinical Science*, 128(2), pp. 81–93. Available at: https://doi.org/10.1042/CS20140278.

Birbrair, A. (2018) *Pericyte Biology - Novel Concepts*. Edited by A. Birbrair. Cham: Springer International Publishing (Advances in Experimental Medicine and Biology). Available at: https://doi.org/10.1007/978-3-030-02601-1.

Birder, L.A. and Van Kerrebroeck, P.E.V. (2019) 'Pathophysiological Mechanisms of Nocturia and Nocturnal Polyuria: The Contribution of Cellular Function, the Urinary Bladder Urothelium, and Circadian Rhythm', *Urology*, 133, pp. 14–23. Available at: https://doi.org/10.1016/J.UROLOGY.2019.07.020/ASSET/3717C932-48E1-4D27-B397-8484A1798F34/MAIN.ASSETS/GR5.JPG.

Boutin, J.A. and Jockers, R. (2021) 'Melatonin controversies, an update', *Journal of Pineal Research*, 70(2), p. e12702. Available at: https://doi.org/10.1111/JPI.12702.

Boyd, J.H. *et al.* (2008) 'Vasopressin decreases sepsis-induced pulmonary inflammation through the V2R', *Resuscitation*, 79(2), pp. 325–331. Available at: https://doi.org/10.1016/j.resuscitation.2008.07.006.

Brainard, G.C. *et al.* (2008) 'Sensitivity of the human circadian system to short-wavelength (420-nm) light', *Journal of biological rhythms*, 23(5), pp. 379–386. Available at: https://doi.org/10.1177/0748730408323089.

Brown, T.M. and Piggins, H.D. (2007) 'Electrophysiology of the suprachiasmatic circadian clock', *Progress in Neurobiology*, pp. 229–255. Available at: https://doi.org/10.1016/j.pneurobio.2007.05.002.

Bruder, E.D. *et al.* (2008) 'Development of the ACTH and corticosterone response to acute hypoxia in the neonatal rat', *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, 295(4), pp. 1195–1203. Available at: https://doi.org/10.1152/AJPREGU.90400.2008/ASSET/IMAGES/LARGE/ZH60100865060004.JPEG.

Burek, M. *et al.* (2020) 'Kidney Ischemia/Reperfusion Injury Induces Changes in the Drug Transporter Expression at the Blood–Brain Barrier in vivo and in vitro', *Frontiers in Physiology*, 11, p. 569881. Available at: https://doi.org/10.3389/FPHYS.2020.569881/BIBTEX.

Burke, M. *et al.* (2014) 'Molecular Mechanisms of Renal Blood Flow Autoregulation', *Current Vascular Pharmacology*, 12(6), pp. 845–858. Available at: https://doi.org/10.2174/15701611113116660149.

Carney, E.F. (2016) 'Functional roles of the renal tubular circadian clock', *Nature Reviews Nephrology 2016 12:6*, 12(6), pp. 316–316. Available at: https://doi.org/10.1038/nrneph.2016.60.

Carrillo-Vico, A. *et al.* (2005) 'A review of the multiple actions of melatonin on the immune system', *Endocrine*, 27(2), pp. 189–200. Available at: https://doi.org/10.1385/ENDO:27:2:189/METRICS.

Chalmers, J. and Macdonald, J. (2018) 'Characteristics of special circulations', *Anaesthesia & Intensive Care Medicine*, 19(9), pp. 502–506. Available at: https://doi.org/10.1016/J.MPAIC.2018.06.012.

Chen, C. *et al.* (2019) 'The Roles of GABA in Ischemia-Reperfusion Injury in the Central Nervous System and Peripheral Organs', *Oxidative Medicine and Cellular Longevity*, 2019(1), p. 4028394. Available at: https://doi.org/10.1155/2019/4028394.

Chen, S. *et al.* (2022) 'Melatonin alleviates arginine vasopressin-induced cardiomyocyte apoptosis via increasing Mst1-Nrf2 pathway activity to reduce oxidative stress', *Biochemical Pharmacology*, 206, p. 115265. Available at: https://doi.org/10.1016/J.BCP.2022.115265.

Chen, Y. *et al.* (2019) 'Preservation Solutions for Kidney Transplantation: History, Advances and Mechanisms', *Cell Transplantation*, 28(12), pp. 1472–1489. Available at: https://doi.org/10.1177/0963689719872699/ASSET/4F0BF1AC-5796-4953-9C44-4D18275FBE68/ASSETS/IMAGES/LARGE/10.1177_0963689719872699-FIG1.JPG.

Chiodera, P. *et al.* (1998) 'Effect of melatonin on arginine vasopressin secretion stimulated by physical exercise or angiotensin II in normal men', *Neuropeptides*, 32(2), pp. 125–129. Available at: https://doi.org/10.1016/S0143-4179(98)90027-0.

Choi, H.J. *et al.* (2008) 'Excitatory Actions of GABA in the Suprachiasmatic Nucleus', *Journal of Neuroscience*, 28(21), pp. 5450–5459. Available at: https://doi.org/10.1523/JNEUROSCI.5750-07.2008.

Chojnacki, C. *et al.* (2013) 'Evaluation of enterochromaffin cells and melatonin secretion exponents in ulcerative colitis', *World Journal of Gastroenterology*, 19(23), pp. 3602–3607. Available at: https://doi.org/10.3748/wjg.v19.i23.3602.

Cipolla-Neto, J. and Do Amaral, F.G. (2018) 'Melatonin as a Hormone: New Physiological and Clinical Insights', *Endocrine Reviews*, 39(6), pp. 990–1028. Available at: https://doi.org/10.1210/ER.2018-00084.

Coe, F. (2017) *Glomerular Filtration | Kidney Stone Program*, *The university of Chicago- Kidney stone program*. Available at: https://kidneystones.uchicago.edu/2017/08/29/glomerular-filtration/ (Accessed: 20 March 2025).

Costello, H.M. *et al.* (2022) 'CIRCADIAN CLOCKS OF THE KIDNEY: FUNCTION, MECHANISM, AND REGULATION', *Physiological Reviews*, 102(4), pp. 1669–1701. Available at: https://doi.org/10.1152/PHYSREV.00045.2021/ASSET/IMAGES/LARGE/PHYSREV.00045.2021_F009.JPEG.

Costello, R.B. *et al.* (2014) 'The effectiveness of melatonin for promoting healthy sleep: A rapid evidence assessment of the literature', *Nutrition Journal*, 13(1), pp. 1–17. Available at: https://doi.org/10.1186/1475-2891-13-106/TABLES/5.

Coutinho, A.E. and Chapman, K.E. (2011) 'The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights', *Molecular and Cellular Endocrinology*, 335(1), pp. 2–13. Available at: https://doi.org/10.1016/J.MCE.2010.04.005.

Crawford, C., Kennedy-Lydon, T., Sprott, C., Desai, T., Sawbridge, L., Munday, J., Unwin, R.J., Wildman, S. S.P., *et al.* (2012) 'An Intact Kidney Slice Model to Investigate Vasa Recta Properties and Function in situ', *Nephron Physiology*, 120(3), pp. p17–p31. Available at: https://doi.org/10.1159/000339110.

Crawford, C., Kennedy-Lydon, T., Sprott, C., Desai, T., Sawbridge, L., Munday, J., Unwin, R.J., Wildman, S. S P, *et al.* (2012) 'An intact kidney slice model to investigate vasa recta properties and function in situ', *Nephron - Physiology*, 120(3). Available at: https://doi.org/10.1159/000339110.

Crepeau, L.J. *et al.* (2006) *LIGHTING AS A CIRCADIAN RHYTHM-ENTRAINING AND ALERTNESS-ENHANCING STIMULUS IN THE SUBMARINE ENVIRONMENT*. Available at: https://doi.org/https://dx.doi.org/10.2139/ssrn.3075632.

Cuffe, J.S.M. *et al.* (2016) 'Maternal corticosterone exposure in the mouse programs sex-specific renal adaptations in the renin–angiotensin–aldosterone system in 6-month offspring', *Physiological Reports*, 4(8), p. e12754. Available at: https://doi.org/10.14814/PHY2.12754.

Curtis, A.M. *et al.* (2014) 'Circadian Clock Proteins and Immunity', *Immunity*, 40(2), pp. 178–186. Available at: https://doi.org/10.1016/J.IMMUNI.2014.02.002/ASSET/DE733450-A373-445D-9485-996A5632AB05/MAIN.ASSETS/GR3.JPG.

Custom MemPro™ 11-beta Hydroxysteroid Dehydrogenases (HSD-11β) - Creative Biostructure (2020) *Creative Biostructure*. Available at: https://www.creative-biostructure.com/custom-mempro%E2%84%A2-11-beta-hydroxysteroid-dehydrogenases-hsd-11%CE%B2-84.htm (Accessed: 20 June 2021).

Cyrino, J.C. *et al.* (2022) 'Day Versus Night Melatonin and Corticosterone Modulation by LPS in Distinct Tissues of Toads (Rhinella Icterica)', *Integrative and Comparative Biology*, 62(6), pp. 1606–1617. Available at: https://doi.org/10.1093/ICB/ICAC028.

Dessalles, C.A. *et al.* (2021) 'Pericyte mechanics and mechanobiology', *Journal of Cell Science*, 134(6). Available at: https://doi.org/10.1242/JCS.240226/237881.

DeWoskin, D. *et al.* (2015) 'Distinct roles for GABA across multiple timescales in mammalian circadian timekeeping', *Proceedings of the National Academy of Sciences of the United States of America*, 112(29), pp. E3911–E3919. Available at: https://doi.org/10.1073/PNAS.1420753112/SUPPL_FILE/PNAS.1420753112.SM02.MP4.

Dragasevic, N. *et al.* (2020) 'The role of aldosterone inhibitors in cardiac ischemia–reperfusion injury1', *https://doi.org/10.1139/cjpp-2020-0276*, 99(1), pp. 18–29. Available at: https://doi.org/10.1139/CJPP-2020-0276.

Druzd, D., De Juan, A. and Scheiermann, C. (2014) 'Circadian rhythms in leukocyte trafficking', *Seminars in Immunopathology*, 36(2), pp. 149–162. Available at: https://doi.org/10.1007/S00281-013-0414-4/TABLES/4.

Dryer, S.E. (2015) 'Glutamate receptors in the kidney', *Nephrology Dialysis Transplantation*, 30(10), pp. 1630–1638. Available at: https://doi.org/10.1093/NDT/GFV028.

Duffy, J.F., Scheuermaier, K. and R. Loughlin, K. (2016) 'Age-Related Sleep Disruption and Reduction in the Circadian Rhythm of Urine Output: Contribution to Nocturia?', *Current aging science*, 9(1), p. 34. Available at: https://doi.org/10.2174/1874609809666151130220343.

Dunlap, J.C., Loros, J.J., and DeCoursey, P.J. (2004) *Chronobiology: Biological Timekeeping*. Sinauer Associates. Sunderland.

Ebihara, S. *et al.* (1986) 'Genetic Control of Melatonin Synthesis in the Pineal Gland of the Mouse', *Science*, 231(4737), pp. 491–493. Available at: https://doi.org/10.1126/science.3941912.

Eckerbom, P. *et al.* (2020) 'Circadian variation in renal blood flow and kidney function in healthy volunteers monitored with noninvasive magnetic resonance imaging', *American Journal of Physiology - Renal Physiology*, 319(6), pp. F966–F978. Available at: https://doi.org/10.1152/AJPRENAL.00311.2020/ASSET/IMAGES/LARGE/AJ-AFLU200030F013.JPEG.

Edvinsson, L. and Krause, D.N. (1979) 'Pharmacological characterization of GABA receptors mediating vasodilation of cerebral arteries in vitro', *Brain Research*, 173(1), pp. 89–97. Available at: https://doi.org/10.1016/0006-8993(79)91098-9.

Ehlen, J.C. *et al.* (2008) 'Interactions of GABAA Receptor Activation and Light on Period mRNA Expression in the Suprachiasmatic Nucleus', *Journal of Biological Rhythms*, 23(1), pp. 16–25. Available at: https://doi.org/10.1177/0748730407310785.

Erdö, S.L. and Wolff, J.R. (1990) 'γ-Aminobutyric Acid Outside the Mammalian Brain', *Journal of Neurochemistry*, 54(2), pp. 363–372. Available at: https://doi.org/10.1111/J.1471-4159.1990.TB01882.X.

Everington, E.A. *et al.* (2018) 'Molecular characterization of GABA-A receptor subunit diversity within major peripheral organs and their plasticity in response to early life psychosocial stress', *Frontiers in Molecular Neuroscience*, 11. Available at: https://doi.org/10.3389/fnmol.2018.00018.

Farrant, M. and Nusser, Z. (2005) 'Variations on an inhibitory theme: phasic and tonic activation of GABAA receptors', *Nature Reviews Neuroscience 2005 6:3*, 6(3), pp. 215–229. Available at: https://doi.org/10.1038/nrn1625.

Fernández, A.R. *et al.* (2020) 'Review: Ischemia reperfusion injury—a translational perspective in organ transplantation', *International Journal of Molecular Sciences*. MDPI AG, pp. 1–21. Available at: https://doi.org/10.3390/ijms21228549.

Filaretova, L. *et al.* (2021) 'Non-Invasive Remote Ischemic Preconditioning May Protect the Gastric Mucosa Against Ischemia-Reperfusion-Induced Injury Through Involvement of Glucocorticoids', *Frontiers in Pharmacology*, 12, p. 682643. Available at: https://doi.org/10.3389/FPHAR.2021.682643/BIBTEX.

Filep, J.G. (2014) 'Glucocorticoid protection against myocardial ischemia-reperfusion injury: Central Role for the PGD2-Nrf2 Pathway', *Hypertension*, 63(1), pp. 22–23. Available at: https://doi.org/10.1161/HYPERTENSIONAHA.113.01832/ASSET/C8864C52-FDF6-49F4-87D1-FE493723A038/ASSETS/GRAPHIC/22FIG01.JPEG.

Firsov, D. and Bonny, O. (2018) 'Circadian rhythms and the kidney', *Nature Reviews Nephrology 2018 14:10*, 14(10), pp. 626–635. Available at: https://doi.org/10.1038/s41581-018-0048-9.

Flaherty, R.L. *et al.* (2017) 'Glucocorticoids induce production of reactive oxygen species/reactive nitrogen species and DNA damage through an iNOS mediated pathway in breast cancer', *Breast Cancer Research*, 19(1), pp. 1–13. Available at: https://doi.org/10.1186/S13058-017-0823-8/FIGURES/6.

Fligny, C. and Duffield, J.S. (2013) 'Activation of pericytes: Recent insights into kidney fibrosis and microvascular rarefaction', *Current Opinion in Rheumatology*, 25(1), pp. 78–86. Available at: https://doi.org/10.1097/BOR.0B013E32835B656B.

Freitas, F. and Attwell, D. (2021) 'Pericyte-mediated constriction of renal capillaries evokes no-reflow and kidney injury following ischemia', *bioRxiv*, p. 2021.09.24.461675. Available at: https://doi.org/10.1101/2021.09.24.461675.

Friesenecker, B. *et al.* (2004) 'Oxygen distribution in microcirculation after arginine vasopressin-induced arteriolar vasoconstriction', *American Journal of Physiology - Heart and Circulatory Physiology*, 287(4 56-4). Available at: https://doi.org/10.1152/AJPHEART.00283.2004/ASSET/IMAGES/LARGE/ZH40100433820004.JPEG.

Garrahy, A. and Thompson, C.J. (2019) 'Vasopressin', *Encyclopedia of Endocrine Diseases*, pp. 29–35. Available at: https://doi.org/10.1016/B978-0-12-801238-3.65216-9.

George, C.P.L. *et al.* (1975) 'Diurnal Variation of Plasma Vasopressin in Man', *The Journal of Clinical Endocrinology & Metabolism*, 41(2), pp. 332–338. Available at: https://doi.org/10.1210/JCEM-41-2-332.

Gibbs, J.E. *et al.* (2012) 'The nuclear receptor REV-ERBα mediates circadian regulation of innate immunity through selective regulation of inflammatory cytokines', *Proceedings of the National Academy of Sciences of the United States of America*, 109(2), pp. 582–587. Available at: https://doi.org/10.1073/PNAS.1106750109/SUPPL_FILE/PNAS.201106750SI.PDF.

Ginty, D.D. *et al.* (1993) 'Regulation of CREB Phosphorylation in the Suprachiasmatic Nucleus by Light and a Circadian Clock', *Science*, 260(5105), pp. 238–241. Available at: https://doi.org/10.1126/SCIENCE.8097062.

Gobbo, V.D. *et al.* (1989) *SHORT COMMUNICATION PINEALECTOMY INHIBITS INTERLEUKIN-2 PRODUCTION AND NATURAL KILLER ACTIVITY IN MICE*, *Int. Z Immunopharmac*.

Gökçinar-Yagci, B., Uçkan-Çetinkaya, D. and Çelebi-Saltik, B. (2015) 'Pericytes: Properties, functions and applications in tissue engineering', *Stem Cell Reviews and Reports*, 11(4), pp. 549–559. Available at: https://doi.org/10.1007/S12015-015-9590-Z/TABLES/1.

Goltsev, A. V. *et al.* (2022) 'Generation and Disruption of Circadian Rhythms in the Suprachiasmatic Nucleus: A Core-Shell Model', *Journal of Biological Rhythms*, 37(5), pp. 545–561. Available at: https://doi.org/10.1177/07487304221107834/ASSET/33494FE4-CE9F-4DF5-BFDE-C9B4D1C0B6A3/ASSETS/IMAGES/LARGE/10.1177_07487304221107834-FIG4.JPG.

Gomez-Sanchez, E. and Gomez-Sanchez, C.E. (2014) 'The Multifaceted Mineralocorticoid Receptor', *Comprehensive Physiology*, 4(3), pp. 965–994. Available at: https://doi.org/10.1002/CPHY.C130044.

Greaves, M.W. (1976) 'Anti-inflammatory action of corticosteroids', *Postgraduate Medical Journal*, 52(612), pp. 631–633. Available at: https://doi.org/10.1136/PGMJ.52.612.631.

Gribkoff, V.K., Pieschl, R.L. and Dudek, F.E. (2003) 'GABA receptor-mediated inhibition of neuronal activity in rat SCN in vitro: Pharmacology and influence of circadian phase', *Journal of Neurophysiology*, 90(3), pp. 1438–1448. Available at: https://doi.org/10.1152/JN.01082.2002/ASSET/IMAGES/LARGE/9K0933339008.JPEG.

Griffin, P. *et al.* (2020) 'Rev-erbα mediates complement expression and diurnal regulation of microglial synaptic phagocytosis', *eLife*, 9, pp. 1–17. Available at: https://doi.org/10.7554/ELIFE.58765.

Gumz, M.L. *et al.* (2009) 'The circadian clock protein Period 1 regulates expression of the renal epithelial sodium channel in mice', *The Journal of Clinical Investigation*, 119(8), pp. 2423–2434. Available at: https://doi.org/10.1172/JCI36908.

Hamilton, N.B., Attwell, D. and Hall, C.N. (2010) 'Pericyte-mediated regulation of capillary diameter: a component of neurovascular coupling in health and disease', *Frontiers in Neuroenergetics*, 2, p. 1453. Available at: https://doi.org/10.3389/FNENE.2010.00005/PDF.

Hara, M. *et al.* (2017) 'Robust circadian clock oscillation and osmotic rhythms in inner medulla reflecting cortico-medullary osmotic gradient rhythm in rodent kidney', *Scientific Reports 2017 7:1*, 7(1), pp. 1–9. Available at: https://doi.org/10.1038/s41598-017-07767-8.

Hardeland, R. *et al.* (2012) 'Melatonin, the circadian multioscillator system and health: the need for detailed analyses of peripheral melatonin signaling', *Journal of Pineal Research*, 52(2), pp. 139–166. Available at: https://doi.org/10.1111/J.1600-079X.2011.00934.X.

Hastings, M.H., Maywood, E.S. and Brancaccio, M. (2019) 'The mammalian circadian timing system and the suprachiasmatic nucleus as its pacemaker', *Biology*. MDPI AG. Available at: https://doi.org/10.3390/biology8010013.

He, B. *et al.* (2016) 'The nuclear melatonin receptor RORα is a novel endogenous defender against myocardial ischemia/reperfusion injury', *Journal of Pineal Research*, 60(3), pp. 313–326. Available at: https://doi.org/10.1111/JPI.12312.

Holmes, C.L., Landry, D.W. and Granton, J.T. (2003) 'Science review: Vasopressin and the cardiovascular system part 1 - Receptor physiology', *Critical Care*, 7(6), pp. 427–434. Available at: https://doi.org/10.1186/CC2337/FIGURES/2.

Hu, C. *et al.* (2021) 'Melatonin and its protective role in attenuating warm or cold hepatic ischaemia/reperfusion injury', *Cell Proliferation*, 54(4), p. e13021. Available at: https://doi.org/10.1111/CPR.13021.

Huang, Q. *et al.* (2022) 'Rev-erbs agonist SR9009 alleviates ischemia-reperfusion injury by heightening endogenous cardioprotection at onset of type-2 diabetes in rats: Down-regulating ferritinophagy/ferroptosis signaling', *Biomedicine & Pharmacotherapy*, 154, p. 113595. Available at: https://doi.org/10.1016/J.BIOPHA.2022.113595.

Husse, J., Eichele, G. and Oster, H. (2015) 'Synchronization of the mammalian circadian timing system: Light can control peripheral clocks independently of the SCN clock', *BioEssays*, 37(10), pp. 1119–1128. Available at: https://doi.org/10.1002/BIES.201500026.

Hyodo, S. (2016) 'Vasopressin', *Handbook of Hormones: Comparative Endocrinology for Basic and Clinical Research*, pp. 41-e6A-3. Available at: https://doi.org/10.1016/B978-0-12-801028-0.00113-6.

Imani, A. *et al.* (2019) 'Acute Physical Stress Preconditions the Heart Against Ischemia/Reperfusion Injury Through Activation of Sympathetic Nervous System', *Arquivos Brasileiros de Cardiologia*, 113(3), pp. 401–408. Available at: https://doi.org/10.5935/ABC.20190189.

Isobe, Y., Fujioi, J. and Nishino, H. (2001) 'Circadian rhythm of melatonin release in pineal gland culture: arg-vasopressin inhibits melatonin release', *Brain Research*, 918(1–2), pp. 67–73. Available at: https://doi.org/10.1016/S0006-8993(01)02936-5.

Itri, J. *et al.* (2004) 'Circadian rhythm in inhibitory synaptic transmission in the mouse suprachiasmatic nucleus', *Journal of Neurophysiology*, 92(1), pp. 311–319. Available at: https://doi.org/10.1152/JN.01078.2003/ASSET/IMAGES/LARGE/Z9K0070439070006.JPEG.

Kalra, S., Agrawal, S. and Sahay, M. (2012) 'The reno-pineal axis: A novel role for melatonin', *Indian Journal of Endocrinology and Metabolism*, 16(2), p. 192. Available at: https://doi.org/10.4103/2230-8210.93735.

Karasawa, K. *et al.* (2015) 'Vascular-resident CD169-positive monocytes and macrophages control neutrophil accumulation in the kidney with ischemia-reperfusion injury', *Journal of the American Society of Nephrology*, 26(4), pp. 896–906. Available at: https://doi.org/10.1681/ASN.2014020195.

Karatsoreos, I.N. and Silver, R. (2017) 'Body Clocks in Health and Disease', *Conn's Translational Neuroscience*, pp. 599–615. Available at: https://doi.org/10.1016/B978-0-12-802381-5.00043-9.

Katsu, Y. and Iguchi, T. (2015) 'Corticosterone', in *Handbook of Hormones: Comparative Endocrinology for Basic and Clinical Research*. Elsevier, pp. 527,e95A-1-528,e95A–3. Available at: https://doi.org/10.1016/B978-0-12-801028-0.00228-2.

Kennaway, D.J. *et al.* (2002) 'Melatonin in mice: rhythms, response to light, adrenergic stimulation, and metabolism'. Available at: https://doi.org/10.1152/ajpregu.00360.2001.-There.

Kennaway, D.J. (2019) 'Melatonin research in mice: a review', *Chronobiology International*. Taylor and Francis Ltd, pp. 1167–1183. Available at: https://doi.org/10.1080/07420528.2019.1624373.

Kennedy-Lydon, T.M. *et al.* (2013) 'Renal pericytes: regulators of medullary blood flow', *Acta Physiologica*, 207(2), pp. 212–225. Available at: https://doi.org/10.1111/APHA.12026.

Khennouf, L. *et al.* (2018) 'Active role of capillary pericytes during stimulation-induced activity and spreading depolarization', *Brain*, 141(7), pp. 2032–2046. Available at: https://doi.org/10.1093/BRAIN/AWY143.

Kim, P. *et al.* (2024) 'Melatonin's role in the timing of sleep onset is conserved in nocturnal mice', *npj Biological Timing and Sleep*, 1(1). Available at: https://doi.org/10.1038/s44323-024-00013-1.

Kobuchi, S. *et al.* (2009) 'Renoprotective effects of γ-aminobutyric acid on ischemia/reperfusion-induced renal injury in rats', *European Journal of Pharmacology*, 623(1–3), pp. 113–118. Available at: https://doi.org/10.1016/J.EJPHAR.2009.09.023.

Koo, D.D.H. *et al.* (1998) 'Ischemia/reperfusion injury in human kidney transplantation: An immunohistochemical analysis of changes after reperfusion', *American Journal of Pathology*, 153(2), pp. 557–566. Available at: https://doi.org/10.1016/S0002-9440(10)65598-8/ASSET/7A2AE735-0809-451B-86F2-970F5B15A0EC/MAIN.ASSETS/GR4.JPG.

Korf, H.W. and von Gall, C. (2024) 'Mouse Models in Circadian Rhythm and Melatonin Research', *Journal of Pineal Research*. John Wiley and Sons Inc. Available at: https://doi.org/10.1111/jpi.12986.

Lall, G.S. and Biello, S.M. (2002) 'Attenuation of phase shifts to light by activity or neuropeptide Y: a time course study', *Brain Research*, 957(1), pp. 109–116. Available at: https://doi.org/10.1016/S0006-8993(02)03610-7.

Lall, G.S. and Biello, S.M. (2003) 'Neuropeptide Y, GABA and circadian phase shifts to photic stimuli', *Neuroscience*, 120(4), pp. 915–921. Available at: https://doi.org/10.1016/S0306-4522(03)00396-8.

Lange, T. *et al.* (2022) 'The contribution of sleep to the neuroendocrine regulation of rhythms in human leukocyte traffic', *Seminars in Immunopathology 2021 44:2*, 44(2), pp. 239–254. Available at: https://doi.org/10.1007/S00281-021-00904-6.

Lattin, C.R. and Romero, L.M. (2014) 'Chronic stress alters concentrations of corticosterone receptors in a tissue-specific manner in wild house sparrows (Passer domesticus)', *Journal of Experimental Biology*, 217(14), pp. 2601–2608. Available at: https://doi.org/10.1242/JEB.103788/257795/AM/CHRONIC-STRESS-ALTERS-CONCENTRATIONS-OF.

Leatherby, R.J., Theodorou, C. and Dhanda, R. (2021) 'Renal physiology: blood flow, glomerular filtration and plasma clearance', *Anaesthesia and Intensive Care Medicine*, 22(7), pp. 439–442. Available at: https://doi.org/10.1016/j.mpaic.2021.05.003.

Lee, B. *et al.* (2007) 'Protein kinase C modulates the phase-delaying effects of light in the mammalian circadian clock', *European Journal of Neuroscience*, 26(2), pp. 451–462. Available at: https://doi.org/10.1111/J.1460-9568.2007.05664.X.

Lee, B. *et al.* (2010) 'CREB influences timing and entrainment of the SCN circadian clock', *Journal of Biological Rhythms*, 25(6), pp. 410–420. Available at: https://doi.org/10.1177/0748730410381229.

Legrand, M. *et al.* (2008) 'Renal Hypoxia and Dysoxia After Reperfusion of the Ischemic Kidney', *Molecular Medicine 2008 14:7*, 14(7), pp. 502–516. Available at: https://doi.org/10.2119/2008-00006.LEGRAND.

Li, K. and Xu, E. (2008) 'The role and the mechanism of γ-aminobutyric acid during central nervous system development', *Neuroscience Bulletin*, 24(3), pp. 195–200. Available at: https://doi.org/10.1007/S12264-008-0109-3/METRICS.

Li, Z. *et al.* (2009) 'Melatonin protects kidney grafts from ischemia/reperfusion injury through inhibition of NF-kB and apoptosis after experimental kidney transplantation', *Journal of Pineal Research*, 46(4), pp. 365–372. Available at: https://doi.org/10.1111/J.1600-079X.2009.00672.X.

Liang, D. *et al.* (2020) 'SIRT1/PGC-1 pathway activation triggers autophagy/mitophagy and attenuates oxidative damage in intestinal epithelial cells', *Biochimie*, 170, pp. 10–20. Available at: https://doi.org/10.1016/J.BIOCHI.2019.12.001.

Liang, R. *et al.* (2009) 'Melatonin protects from hepatic reperfusion injury through inhibition of IKK and JNK pathways and modification of cell proliferation', *Journal of Pineal Research*, 46(1), pp. 8–14. Available at: https://doi.org/10.1111/J.1600-079X.2008.00596.X.

Lilley, R. (2020) 'An Investigation into the Multifunctional Nature of Renal Pericytes'.

Lilley, R.J. *et al.* (2023) 'Inflammatory mediators act at renal pericytes to elicit contraction of vasa recta and reduce pericyte density along the kidney medullary vascular network', *Frontiers in Physiology*, 14, p. 1194803. Available at: https://doi.org/10.3389/FPHYS.2023.1194803/BIBTEX.

Lin, Y. *et al.* (2020) 'Rev-erbα regulates hepatic ischemia-reperfusion injury in mice', *Biochemical and Biophysical Research Communications*, 529(4), pp. 916–921. Available at: https://doi.org/10.1016/J.BBRC.2020.06.152.

Linz, D. *et al.* (2018) 'Modulation of renal sympathetic innervation: recent insights beyond blood pressure control', *Clinical Autonomic Research*, 28(4), pp. 375–384. Available at: https://doi.org/10.1007/S10286-018-0508-0/FIGURES/5.

Liu, C. and Reppert, S.M. (2000) 'GABA synchronizes clock cells within the suprachiasmatic circadian clock', *Neuron*, 25(1), pp. 123–128. Available at: https://doi.org/10.1016/S0896-6273(00)80876-4.

Luan, Z.L. *et al.* (2022) 'Nuclear receptors in renal health and disease', *eBioMedicine*, 76, p. 103855. Available at: https://doi.org/10.1016/J.EBIOM.2022.103855/ASSET/E8CCDC54-0937-4839-9662-679AF0FE857C/MAIN.ASSETS/FX1B.JPG.

Maggio, N. and Segal, M. (2019) 'Stress, Corticosterone, and Hippocampal Plasticity', *Stress: Physiology, Biochemistry, and Pathology Handbook of Stress Series, Volume 3*, pp. 93–104. Available at: https://doi.org/10.1016/B978-0-12-813146-6.00008-4.

Majerova, P. *et al.* (2019) 'Trafficking of immune cells across the blood-brain barrier is modulated by neurofibrillary pathology in tauopathies', *PLOS ONE*, 14(5), p. e0217216. Available at: https://doi.org/10.1371/JOURNAL.PONE.0217216.

Malek, M. and Nematbakhsh, M. (2015) 'Renal ischemia/reperfusion injury; from pathophysiology to treatment.', *Journal of renal injury prevention*, 4(2), pp. 20–7. Available at: https://doi.org/10.12861/jrip.2015.06.

Malhotra, S., Sawhney, G. and Pandhi, P. (2004) 'The Therapeutic Potential of Melatonin: A Review of the Science', *Medscape General Medicine*, 6(2), p. 46. Available at: https://pmc.ncbi.nlm.nih.gov/articles/PMC1395802/ (Accessed: 23 March 2025).

Mastrullo, V. *et al.* (2021) 'The vascular clock: a new insight into endothelial cells and pericytes crosstalk', *European Heart Journal*, 42(Supplement_1). Available at: https://doi.org/10.1093/EURHEARTJ/EHAB724.3382.

Mastrullo, V. *et al.* (2022) 'Pericytes' Circadian Clock Affects Endothelial Cells' Synchronization and Angiogenesis in a 3D Tissue Engineered Scaffold', *Frontiers in Pharmacology*, 13, p. 867070. Available at: https://doi.org/10.3389/FPHAR.2022.867070/BIBTEX.

Mateus, V. *et al.* (2017) 'Anti-Inflammatory Effect of Erythropoietin in the TNBS-induced Colitis', *Basic & Clinical Pharmacology & Toxicology*, 120(2), pp. 138–145. Available at: https://doi.org/10.1111/BCPT.12663.

De Matteo, R. and May, C.N. (1997) 'Glucocorticoid-induced renal vasodilatation is mediated by a direct renal action involving nitric oxide', *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, 273(6 42-6). Available at: https://doi.org/10.1152/AJPREGU.1997.273.6.R1972/ASSET/IMAGES/LARGE/AREG7121705.JPEG.

Mavani, G.P., DeVita, M. V. and Michelis, M.F. (2015) 'A Review of the Nonpressor and Nonantidiuretic Actions of the Hormone Vasopressin', *Frontiers in Medicine*, 2(MAR), p. 19. Available at: https://doi.org/10.3389/FMED.2015.00019.

Meurisse, N. *et al.* (2023) 'Effect of a Combined Drug Approach on the Severity of Ischemia-Reperfusion Injury During Liver Transplant: A Randomized Clinical Trial', *JAMA Network Open*, 6(2), pp. e230819–e230819. Available at: https://doi.org/10.1001/JAMANETWORKOPEN.2023.0819.

Mieda, M., Okamoto, H. and Sakurai, T. (2016) 'Manipulating the Cellular Circadian Period of Arginine Vasopressin Neurons Alters the Behavioral Circadian Period', *Current Biology*, 26(18), pp. 2535–2542. Available at: https://doi.org/10.1016/j.cub.2016.07.022.

Mirick, D.K. and Davis, S. (2008) 'Melatonin as a Biomarker of Circadian Dysregulation', *Cancer Epidemiology, Biomarkers & Prevention*, 17(12), pp. 3306–3313. Available at: https://doi.org/10.1158/1055-9965.EPI-08-0605.

Mizoguchi, K. *et al.* (2003) 'Chronic stress attenuates glucocorticoid negative feedback: Involvement of the prefrontal cortex and hippocampus', *Neuroscience*, 119(3), pp. 887–897. Available at: https://doi.org/10.1016/S0306-4522(03)00105-2/ASSET/9BA3EEA1-ED7A-4EC1-8092-BCE8146E51E1/MAIN.ASSETS/GR7.GIF.

Mogulkoc, R. and Baltaci, A.K. (2010) 'Effect of melatonin supplementation on plasma vasopressin response to different conditions in rats with hyperthyroidism induced by l-thyroxine', *Regulatory Peptides*, 161(1–3), pp. 38–42. Available at: https://doi.org/10.1016/J.REGPEP.2009.12.018.

Mohd Azmi, N.A.S. *et al.* (2021) 'Cortisol on Circadian Rhythm and Its Effect on Cardiovascular System', *International Journal of Environmental Research and Public Health 2021, Vol. 18, Page 676*, 18(2), p. 676. Available at: https://doi.org/10.3390/IJERPH18020676.

Moldavan, M., Cravetchi, O. and Allen, C.N. (2021) 'Diurnal properties of tonic and synaptic GABAAreceptor-mediated currents in suprachiasmatic nucleus neurons', *Journal of Neurophysiology*, 126(2), pp. 637–652. Available at: https://doi.org/10.1152/JN.00556.2020/ASSET/IMAGES/LARGE/JN.00556.2020_F007.JPEG.

Montaigne, D. *et al.* (2018) 'Daytime variation of perioperative myocardial injury in cardiac surgery and its prevention by Rev-Erbα antagonism: a single-centre propensity-matched cohort study and a randomised study', *The Lancet*, 391(10115), pp. 59–69. Available at: https://doi.org/10.1016/S0140-6736(17)32132-3.

Mou, Y. *et al.* (2019) 'Ferroptosis, a new form of cell death: opportunities and challenges in cancer', *Journal of Hematology & Oncology 2019 12:1*, 12(1), pp. 1–16. Available at: https://doi.org/10.1186/S13045-019-0720-Y.

Myung, J. *et al.* (2015) 'GABA-mediated repulsive coupling between circadian clock neurons in the SCN encodes seasonal time', *Proceedings of the National Academy of Sciences of the United States of America*, 112(29), pp. E3920–E3929. Available at: https://doi.org/10.1073/PNAS.1421200112/SUPPL_FILE/PNAS.1421200112.SAPP.PDF.

Nava, M. *et al.* (2000) 'Melatonin attenuates acute renal failure and oxidative stress induced by mercuric chloride in rats', *American Journal of Physiology - Renal Physiology*, 279(5 48-5). Available at: https://doi.org/10.1152/AJPRENAL.2000.279.5.F910/ASSET/IMAGES/LARGE/H21100129011.JPEG.

Navarro, R. *et al.* (2016) 'Immune Regulation by Pericytes: Modulating Innate and Adaptive Immunity', *Frontiers in Immunology*, 7(NOV), p. 480. Available at: https://doi.org/10.3389/FIMMU.2016.00480.

Nikolaeva, S. *et al.* (2016) 'Nephron-specific deletion of circadian clock gene BMAL1 alters the plasma and renal metabolome and impairs drug disposition', *Journal of the American Society of Nephrology*, 27(10), pp. 2997–3004. Available at: https://doi.org/10.1681/ASN.2015091055.

Nishi, E.E. *et al.* (2018) 'Renal denervation reduces sympathetic overactivation, brain oxidative stress, and renal injury in rats with renovascular hypertension independent of its effects on reducing blood pressure', *Hypertension Research 2018 42:5*, 42(5), pp. 628–640. Available at: https://doi.org/10.1038/s41440-018-0171-9.

Nishi, E.E. *et al.* (2019) 'Melatonin attenuates renal sympathetic overactivity and reactive oxygen species in the brain in neurogenic hypertension', *Hypertension Research 2019 42:11*, 42(11), pp. 1683–1691. Available at: https://doi.org/10.1038/s41440-019-0301-z.

Nishi, E.E., Bergamaschi, C.T. and Campos, R.R. (2015) 'The crosstalk between the kidney and the central nervous system: the role of renal nerves in blood pressure regulation', *Experimental Physiology*, 100(5), pp. 479–484. Available at: https://doi.org/10.1113/EXPPHYSIOL.2014.079889.

Obayashi, K., Saeki, K. and Kurumatani, N. (2014) 'Association between Melatonin Secretion and Nocturia in Elderly Individuals: a Cross-Sectional Study of the HEIJO-KYO Cohort', *The Journal of Urology*, 191(6), pp. 1816–1821. Available at: https://doi.org/10.1016/J.JURO.2013.12.043.

Ohashi, N., Ishigaki, S. and Isobe, S. (2019) 'The pivotal role of melatonin in ameliorating chronic kidney disease by suppression of the renin–angiotensin system in the kidney', *Hypertension Research 2018 42:6*, 42(6), pp. 761–768. Available at: https://doi.org/10.1038/s41440-018-0186-2.

Okabe, T. *et al.* (2016) 'REV-ERBα influences the stability and nuclear localization of the glucocorticoid receptor', *Journal of Cell Science*, 129(21), pp. 4143–4154. Available at: https://doi.org/10.1242/JCS.190959/265243/AM/REV-ERB-INFLUENCES-STABILITY-AND-NUCLEAR.

Ono, D. *et al.* (2018) 'Role of GABA in the regulation of the central circadian clock of the suprachiasmatic nucleus', *The Journal of Physiological Sciences*, 68(4), pp. 333–343. Available at: https://doi.org/10.1007/S12576-018-0604-X.

Ono, D. *et al.* (2020) 'The mammalian circadian pacemaker regulates wakefulness via CRF neurons in the paraventricular nucleus of the hypothalamus', *Science Advances*, 6(45). Available at: https://doi.org/10.1126/SCIADV.ABD0384/SUPPL_FILE/ABD0384_SM.PDF.

Orozco-Solis, R. and Aguilar-Arnal, L. (2020) 'Circadian Regulation of Immunity Through Epigenetic Mechanisms', *Frontiers in Cellular and Infection Microbiology*, 10, p. 525590. Available at: https://doi.org/10.3389/FCIMB.2020.00096/PDF.

Palsson, R. and Waikar, S.S. (2018) 'Renal Functional Reserve Revisited', *Advances in Chronic Kidney Disease*, 25(3), pp. e1–e8. Available at: https://doi.org/10.1053/J.ACKD.2018.03.001/ASSET/019F17FF-89F6-4CFF-BD14-2B0986860888/MAIN.ASSETS/GR3.SML.

Panah, F. *et al.* (2019) 'The effect of oral melatonin on renal ischemia–reperfusion injury in transplant patients: A double-blind, randomized controlled trial', *Transplant Immunology*, 57, p. 101241. Available at: https://doi.org/10.1016/J.TRIM.2019.101241.

Parducz, A. *et al.* (1992) 'GABA-immunoreactive structures in rat kidney.', *Journal of Histochemistry & Cytochemistry*, 40(5), pp. 675–680. Available at: https://doi.org/10.1177/40.5.1573248.

Paulus, W. and Rothwell, J.C. (2016) 'Membrane resistance and shunting inhibition: where biophysics meets state-dependent human neurophysiology', *The Journal of Physiology*, 594(10), pp. 2719–2728. Available at: https://doi.org/10.1113/JP271452.

Peng, Q. *et al.* (2012) 'C3a and C5a promote renal ischemia-reperfusion injury', *Journal of the American Society of Nephrology*, 23(9), pp. 1474–1485. Available at: https://doi.org/10.1681/ASN.2011111072.

Peppiatt, C.M. *et al.* (2006) 'Bidirectional control of CNS capillary diameter by pericytes', *Nature 2006 443:7112*, 443(7112), pp. 700–704. Available at: https://doi.org/10.1038/nature05193.

Peppiatt-Wildman, C.M. (2013) 'The evolving role of renal pericytes', *Current Opinion in Nephrology and Hypertension*, 22(1), pp. 10–16. Available at: https://doi.org/10.1097/MNH.0B013E32835B4E6E.

Perreau-Lenz, S. *et al.* (2005) 'In vivo evidence for a controlled offset of melatonin synthesis at dawn by the suprachiasmatic nucleus in the rat', *Neuroscience*, 130(3), pp. 797–803. Available at: https://doi.org/10.1016/j.neuroscience.2004.10.014.

Pfeffer, M. *et al.* (2022) 'The Role of the Melatoninergic System in Circadian and Seasonal Rhythms—Insights From Different Mouse Strains', *Frontiers in Physiology*. Frontiers Media S.A. Available at: https://doi.org/10.3389/fphys.2022.883637.

Pistono, C. *et al.* (2021) *Title: Major role of MT2 receptors in the beneficial effect of melatonin on long-term recognition memory in C57BL/6J male mice*.

Pocock, G., Richards, C.D. and Richards, D.A. (2017) *Human Physiology*. 5th ed. Oxford: Oxford University Press.

Poluzzi, C. *et al.* (2019) 'Biglycan evokes autophagy in macrophages via a novel CD44/Toll-like receptor 4 signaling axis in ischemia/reperfusion injury', *Kidney International*, 95(3), pp. 540–562. Available at: https://doi.org/10.1016/J.KINT.2018.10.037/ASSET/49AA2229-A73E-40A1-8200-C46EC5D356AB/MAIN.ASSETS/FIGS3_LRG.JPG.

Prayag, A.S. *et al.* (2019) 'Light Modulation of Human Clocks, Wake, and Sleep', *Clocks & Sleep 2019, Vol. 1, Pages 193-208*, 1(1), pp. 193–208. Available at: https://doi.org/10.3390/CLOCKSSLEEP1010017.

Proebstl, D. *et al.* (2012) 'Pericytes support neutrophil subendothelial cell crawling and breaching of venular walls in vivo', *Journal of Experimental Medicine*, 209(6), pp. 1219–1234. Available at: https://doi.org/10.1084/JEM.20111622/VIDEO-6.

Quan, Z.Y., Walser, M. and Hill, G.S. (1992) 'Adrenalectomy ameliorates ablative nephropathy in the rat independently of corticosterone maintenance level', *Kidney international*, 41(2), pp. 326–333. Available at: https://doi.org/10.1038/KI.1992.45.

Rahman, A., Hasan, A.U. and Kobori, H. (2019) 'Melatonin in chronic kidney disease: a promising chronotherapy targeting the intrarenal renin–angiotensin system', *Hypertension Research 2019 42:6*, 42(6), pp. 920–923. Available at: https://doi.org/10.1038/s41440-019-0223-9.

Ramakrishnan, S.N. and Muscat, G.E.O. (2006) 'The Orphan Rev-Erb Nuclear Receptors: A Link between Metabolism, Circadian Rhythm and Inflammation?', *Nuclear Receptor Signaling*, 4(1), p. nrs.04009. Available at: https://doi.org/10.1621/NRS.04009.

Raza, Z. and Naureen, Z. (2020) 'Melatonin ameliorates the drug induced nephrotoxicity: Molecular insights', *Nefrología*, 40(1), pp. 12–25. Available at: https://doi.org/10.1016/J.NEFRO.2019.06.009.

Reiter, R.J. *et al.* (2016) 'Melatonin as an antioxidant: under promises but over delivers', *Journal of Pineal Research*, 61(3), pp. 253–278. Available at: https://doi.org/10.1111/JPI.12360.

Reiter, R.J., Ma, Q. and Sharma, R. (2020) 'Melatonin in mitochondria: Mitigating clear and present dangers', *Physiology*, 35(2), pp. 86–95. Available at: https://doi.org/10.1152/PHYSIOL.00034.2019/ASSET/IMAGES/LARGE/PHY0022005060004.JPEG.

Richards, J. and Gumz, M.L. (2013) 'Mechanism of the circadian clock in physiology', *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, 304(12), pp. 1053–1064. Available at: https://doi.org/10.1152/AJPREGU.00066.2013/ASSET/IMAGES/LARGE/ZH60121382030002.JPEG.

Rodríguez, A.B. *et al.* (2001) 'Physiological concentrations of melatonin and corticosterone affect phagocytosis and oxidative metabolism of ring dove heterophils', *Journal of Pineal Research*, 31(1), pp. 31–38. Available at: https://doi.org/10.1034/J.1600-079X.2001.310105.X.

Rostron, A.J. *et al.* (2007) 'Hemodynamic resuscitation of the brain-dead organ donor and the potential role of vasopressin', *Transplantation Reviews*, 21(1), pp. 34–42. Available at: https://doi.org/10.1016/J.TRRE.2007.01.003.

Roszczyk, M. and Juszczak, M. (2014) 'Forskolin-stimulated vasopressin and oxytocin release from the rat hypothalamo–neurohypophysial system in vitro is inhibited by melatonin', *Endokrynologia Polska*, 65(2), pp. 125–131. Available at: https://doi.org/10.5603/EP.2014.0018.

Rudziak, P., Ellis, C.G. and Kowalewska, P.M. (2019) 'Role and Molecular Mechanisms of Pericytes in Regulation of Leukocyte Diapedesis in Inflamed Tissues', *Mediators of Inflammation*, 2019(1), p. 4123605. Available at: https://doi.org/10.1155/2019/4123605.

Sage, D. *et al.* (2004) 'Influence of the Corticosterone Rhythm on Photic Entrainment of Locomotor Activity in Rats', *Journal of Biological Rhythms*, 19(2), pp. 144–156. Available at: https://doi.org/10.1177/0748730403261894.

Saitta, B. *et al.* (2019) 'Ex vivo kidney slice preparations as a model system to study signaling cascades in kidney epithelial cells', *Methods in Cell Biology*, 153, pp. 185–203. Available at: https://doi.org/10.1016/BS.MCB.2019.04.017.

Sakagami, K. *et al.* (2001) 'Nitric Oxide/cGMP-Induced Inhibition of Calcium and Chloride Currents in Retinal Pericytes', *Microvascular Research*, 62(2), pp. 196–203. Available at: https://doi.org/10.1006/MVRE.2001.2343.

Sakagami, K., Kodama, T. and Puro, D.G. (2001) 'PDGF-induced coupling of function with metabolism in microvascular pericytes of the retina - PubMed', *Investigative ophthalmology & visual science*, 42(8), pp. 1939–1944. Available at: https://pubmed.ncbi.nlm.nih.gov/11431464/ (Accessed: 18 March 2025).

Sarang, S.S. *et al.* (2001) 'Identification of the γ-aminobutyric acid receptor β2 and β3 subunits in rat, rabbit, and human kidneys', *Journal of the American Society of Nephrology*, 12(6), pp. 1107–1113. Available at: https://doi.org/10.1681/ASN.V1261107.

Sasaki, H. *et al.* (2016) 'Forced rather than voluntary exercise entrains peripheral clocks via a corticosterone/noradrenaline increase in PER2::LUC mice', *Scientific Reports 2016 6:1*, 6(1), pp. 1–15. Available at: https://doi.org/10.1038/srep27607.

Sata, Y. *et al.* (2018) 'Role of the sympathetic nervous system and its modulation in renal hypertension', *Frontiers in Medicine*, 5(MAR), p. 349079. Available at: https://doi.org/10.3389/FMED.2018.00082/BIBTEX.

Scheuer, D.A. and Mifflin, S.W. (1997) 'Chronic corticosterone treatment increases myocardial infarct size in rats with ischemia-reperfusion injury', *https://doi.org/10.1152/ajpregu.1997.272.6.R2017*, 272(6 41-6). Available at: https://doi.org/10.1152/AJPREGU.1997.272.6.R2017.

Scott, R.P. and Quaggin, S.E. (2015) 'Review series: The cell biology of renal filtration', *The Journal of cell biology*, 209(2), pp. 199–210. Available at: https://doi.org/10.1083/JCB.201410017.

Shaw, I. *et al.* (2018) 'Pericytes in the renal vasculature: roles in health and disease', *Nature Reviews Nephrology 2018 14:8*, 14(8), pp. 521–534. Available at: https://doi.org/10.1038/s41581-018-0032-4.

Silva, I. *et al.* (2021) 'Potential anti-inflammatory effect of erythropoietin in non-clinical studies in vivo: A systematic review', *Biomedicine & Pharmacotherapy*, 139, p. 111558. Available at: https://doi.org/10.1016/J.BIOPHA.2021.111558.

Da Silveira Cruz-Machado, S. *et al.* (2017) 'Daily corticosterone rhythm modulates pineal function through NFκB-related gene transcriptional program', *Scientific Reports 2017 7:1*, 7(1), pp. 1–11. Available at: https://doi.org/10.1038/s41598-017-02286-y.

Simko, F., Reiter, R.J. and Paulis, L. (2019) 'Melatonin as a rational alternative in the conservative treatment of resistant hypertension', *Hypertension Research 2019 42:11*, 42(11), pp. 1828–1831. Available at: https://doi.org/10.1038/s41440-019-0318-3.

Singhanat, K. *et al.* (2018) 'Roles of melatonin and its receptors in cardiac ischemia–reperfusion injury', *Cellular and Molecular Life Sciences*, 75(22), pp. 4125–4149. Available at: https://doi.org/10.1007/S00018-018-2905-X/TABLES/10.

Singhanat, K. *et al.* (2021) 'Melatonin as a therapy in cardiac ischemia-reperfusion injury: Potential mechanisms by which MT2 activation mediates cardioprotection', *Journal of Advanced Research*, 29, pp. 33–44. Available at: https://doi.org/10.1016/J.JARE.2020.09.007.

Slegtenhorst, B.R. *et al.* (2014) 'Ischemia/Reperfusion Injury and its Consequences on Immunity and Inflammation', *Current Transplantation Reports*, 1(3), pp. 147–154. Available at: https://doi.org/10.1007/S40472-014-0017-6/FIGURES/1.

Soares, R.O.S. *et al.* (2019) 'Ischemia/Reperfusion Injury Revisited: An Overview of the Latest Pharmacological Strategies', *International Journal of Molecular Sciences 2019, Vol. 20, Page 5034*, 20(20), p. 5034. Available at: https://doi.org/10.3390/IJMS20205034.

Solocinski, K. and Gumz, M.L. (2015) 'The Circadian Clock in the Regulation of Renal Rhythms', *Journal of Biological Rhythms*, 30(6), pp. 470–486. Available at: https://doi.org/10.1177/0748730415610879/ASSET/5CA57B30-E159-4399-9A7A-7196D9595A97/ASSETS/IMAGES/LARGE/10.1177_0748730415610879-FIG2.JPG.

Song, Z. *et al.* (2018) 'Exogenous melatonin protects small-for-size liver grafts by promoting monocyte infiltration and releases interleukin-6', *Journal of Pineal Research*, 65(1). Available at: https://doi.org/10.1111/JPI.12486.

Sparks, M.A. *et al.* (2014) 'Classical Renin-Angiotensin System in Kidney Physiology', *Comprehensive Physiology*, 4(3), pp. 1201–1228. Available at: https://doi.org/10.1002/CPHY.C130040.

Spitzer, N.C. (2010) 'How GABA generates depolarization', *The Journal of Physiology*, 588(5), pp. 757–758. Available at: https://doi.org/10.1113/JPHYSIOL.2009.183574.

Stefani, O. *et al.* (2021) 'Changing color and intensity of LED lighting across the day impacts on circadian melatonin rhythms and sleep in healthy men', *Journal of Pineal Research*, 70(3). Available at: https://doi.org/10.1111/jpi.12714.

Stefanska, A. *et al.* (2016) 'Human kidney pericytes produce renin', *Kidney International*, 90(6), pp. 1251–1261. Available at: https://doi.org/10.1016/J.KINT.2016.07.035/ASSET/EA1230B5-E12E-4C6B-B613-4129AABB0245/MAIN.ASSETS/FIGS2.JPG.

Stiegler, P. *et al.* (2018) 'Impact of Melatonin in Solid Organ Transplantation—Is It Time for Clinical Trials? A Comprehensive Review', *International Journal of Molecular Sciences 2018, Vol. 19, Page 3509*, 19(11), p. 3509. Available at: https://doi.org/10.3390/IJMS19113509.

Stokkan, K.A. *et al.* (2001) 'Entrainment of the Circadian Clock in the Liver by Feeding', *Science*, 291(5503), pp. 490–493. Available at: https://doi.org/10.1126/SCIENCE.291.5503.490.

Stow, L.R. and Gumz, M.L. (2011) 'The circadian clock in the kidney', *Journal of the American Society of Nephrology*, 22(4), pp. 598–604. Available at: https://doi.org/10.1681/ASN.2010080803.

Sun, Q. *et al.* (2021) *Mechanism of circadian regulation of the NRF2/ARE pathway in renal ischemia-reperfusion*. Available at: https://doi.org/https://doi.org/10.3892/etm.2021.9622.

Sun, T.C. *et al.* (2020) 'Melatonin Inhibits Oxidative Stress and Apoptosis in Cryopreserved Ovarian Tissues via Nrf2/HO-1 Signaling Pathway', *Frontiers in Molecular Biosciences*, 7, p. 555976. Available at: https://doi.org/10.3389/FMOLB.2020.00163/BIBTEX.

Sweeney, M.D., Ayyadurai, S. and Zlokovic, B. V. (2016) 'Pericytes of the neurovascular unit: key functions and signaling pathways', *Nature Neuroscience 2016 19:6*, 19(6), pp. 771–783. Available at: https://doi.org/10.1038/nn.4288.

Tagawa, T. *et al.* (1993) 'Vasodilatory effect of arginine vasopressin is mediated by nitric oxide in human forearm vessels.', *The Journal of Clinical Investigation*, 92(3), pp. 1483–1490. Available at: https://doi.org/10.1172/JCI116726.

Takano, K. *et al.* (2014) 'Characteristic Expressions of GABA Receptors and GABA Producing/Transporting Molecules in Rat Kidney', *PLOS ONE*, 9(9), p. e105835. Available at: https://doi.org/10.1371/JOURNAL.PONE.0105835.

Talebi, N. *et al.* (2016) 'The Protective Effect of γ-aminobutyric Acid on Kidney Injury Induced by Renal Ischemia-reperfusion in Ovariectomized Estradiol-treated Rats', *International Journal of Preventive Medicine*, 7, p. 6. Available at: https://doi.org/10.4103/2008-7802.173796.

Tang, Z. *et al.* (2019) 'Melatonin Treatment Ameliorates Hyperhomocysteinemia-Induced Impairment of Erectile Function in a Rat Model', *The Journal of Sexual Medicine*, 16(10), pp. 1506–1517. Available at: https://doi.org/10.1016/J.JSXM.2019.07.003.

Tarocco, A. *et al.* (2019) 'Melatonin as a master regulator of cell death and inflammation: molecular mechanisms and clinical implications for newborn care', *Cell Death & Disease 2019 10:4*, 10(4), pp. 1–12. Available at: https://doi.org/10.1038/s41419-019-1556-7.

Tavakoli, M. (2014) 'Kidney protective effects of melatonin', *Journal of Nephropharmacology*, 3(1), p. 7. Available at: https://pmc.ncbi.nlm.nih.gov/articles/PMC5297592/ (Accessed: 19 March 2025).

Tordjman, S. *et al.* (2017) 'Melatonin: Pharmacology, Functions and Therapeutic Benefits', *Current Neuropharmacology*, 15(3), p. 434. Available at: https://doi.org/10.2174/1570159X14666161228122115.

Usa, K. *et al.* (2007) 'Renal interstitial corticosterone and 11-dehydrocorticosterone in conscious rats', *American Journal of Physiology - Renal Physiology*, 293(1), pp. 186–192. Available at: https://doi.org/10.1152/AJPRENAL.00484.2006/ASSET/IMAGES/LARGE/ZH20070747640006.JPEG.

Vairetti, M. *et al.* (2005) 'Exogenous melatonin enhances bile flow and ATP levels after cold storage and reperfusion in rat liver: implications for liver transplantation', *Journal of Pineal Research*, 38(4), pp. 223–230. Available at: https://doi.org/10.1111/J.1600-079X.2004.00193.X.

Vitaterna, M.H., Takahashi, J.S. and Turek, F.W. (2001) 'Overview of Circadian Rhythms', *Alcohol Research & Health*, 25(2), p. 85. Available at: https://pmc.ncbi.nlm.nih.gov/articles/PMC6707128/ (Accessed: 19 March 2025).

Wahl, S. *et al.* (2019) 'The inner clock—Blue light sets the human rhythm', *Journal of Biophotonics*. Wiley-VCH Verlag. Available at: https://doi.org/10.1002/jbio.201900102.

Wang, M-L *et al.* (2018) 'Melatonin attenuates lung ischaemia–reperfusion injury via inhibition of oxidative stress and inflammation', *Interactive CardioVascular and Thoracic Surgery*, 26(5), pp. 761–767. Available at: https://doi.org/10.1093/ICVTS/IVX440.

Wang, S. *et al.* (2020) 'Targeting REV-ERBα for therapeutic purposes: promises and challenges', *Theranostics*, 10(9), pp. 4168–4182. Available at: https://doi.org/10.7150/THNO.43834.

Wang, S.-C. *et al.* (2023) 'Melatonin exhibits partial protective effects against gemcitabine- and cisplatin-induced kidney and reproductive injuries in mice', *Aging*, 15(23), pp. 14372–14383. Available at: https://doi.org/10.18632/aging.205307.

Wang Xuebin *et al.* (2023) *Melatonin attenuates high glucose-induced endothelial cell pyroptosis by activating the Nrf2 pathway to inhibit NLRP3 inflammasome activation*. Available at: https://doi.org/https://doi.org/10.3892/mmr.2023.12958.

Wei, R.M. *et al.* (2025) 'Melatonin attenuates intermittent hypoxia-induced cognitive impairment in aged mice: The role of inflammation and synaptic plasticity', *Psychoneuroendocrinology*, 171, p. 107210. Available at: https://doi.org/10.1016/J.PSYNEUEN.2024.107210.

Wheaton, K.L. *et al.* (2018) 'The Phosphorylation of CREB at Serine 133 Is a Key Event for Circadian Clock Timing and Entrainment in the Suprachiasmatic Nucleus', *Journal of Biological Rhythms*, 33(5), pp. 497–514. Available at: https://doi.org/10.1177/0748730418791713.

Wight, J.P. *et al.* (2003) 'Pulsatile machine perfusion vs. cold storage of kidneys for transplantation: a rapid and systematic review', *Clinical Transplantation*, 17(4), pp. 293–307. Available at: https://doi.org/10.1034/J.1399-0012.2003.00077.X.

Wildman, S.S. *et al.* (2023) 'A novel functional role for the classic CNS neurotransmitters, GABA, glycine, and glutamate, in the kidney: potent and opposing regulators of the renal vasculature', *American Journal of Physiology - Renal Physiology*, 325(1), pp. F38–F49. Available at: https://doi.org/10.1152/AJPRENAL.00425.2021/ASSET/IMAGES/LARGE/AJPRENAL.00425.2021_F006.JPEG.

Wildman, S.S.P. *et al.* (2014) 'Current Perspective on the Location and Function of Gamma-Aminobutyric Acid (GABA) and its Metabolic Partners in the Kidney.', *Journal of Nephrology and Urology Research*, 2(2), pp. 47–57. Available at: https://doi.org/10.12970/2310-984X.2014.02.02.5.

Woodruff, E.R. *et al.* (2016) 'Diurnal Corticosterone Presence and Phase Modulate Clock Gene Expression in the Male Rat Prefrontal Cortex', *Endocrinology*, 157(4), pp. 1522–1534. Available at: https://doi.org/10.1210/EN.2015-1884.

Xiang, Y.Y. *et al.* (2007) 'A GABAergic system in airway epithelium is essential for mucus overproduction in asthma', *Nature Medicine 2007 13:7*, 13(7), pp. 862–867. Available at: https://doi.org/10.1038/nm1604.

Xu, C. *et al.* (2021) 'Cardioprotective effects of melatonin against myocardial ischaemia/reperfusion injury: Activation of AMPK/Nrf2 pathway', *Journal of Cellular and Molecular Medicine*, 25(13), pp. 6455–6459. Available at: https://doi.org/10.1111/JCMM.16691.

Yamaguchi, Y. (2018) 'Arginine vasopressin signaling in the suprachiasmatic nucleus on the resilience of circadian clock to jet lag', *Neuroscience Research*, 129, pp. 57–61. Available at: https://doi.org/10.1016/J.NEURES.2017.10.007.

Yambe, Y. *et al.* (2002) 'Diurnal changes in arginine vasopressin gene transcription in the rat suprachiasmatic nucleus', *Molecular Brain Research*, 104(2), pp. 132–136. Available at: https://doi.org/10.1016/S0169-328X(02)00327-3.

Yang, S. *et al.* (2017) 'Diverse Functions and Mechanisms of Pericytes in Ischemic Stroke', *Current Neuropharmacology*, 15(6), p. 892. Available at: https://doi.org/10.2174/1570159X15666170112170226.

Yang, S. and Zhang, L. (2005) 'Glucocorticoids and Vascular Reactivity', *Current Vascular Pharmacology*, 2(1), pp. 1–12. Available at: https://doi.org/10.2174/1570161043476483.

Ye, P. *et al.* (2022) 'BMAL1 regulates mitochondrial homeostasis in renal ischaemia-reperfusion injury by mediating the SIRT1/PGC-1α axis', *Journal of Cellular and Molecular Medicine*, 26(7), pp. 1994–2009. Available at: https://doi.org/10.1111/JCMM.17223.

Yu, Q. *et al.* (2023) 'Melatonin suppresses sympathetic vasomotor tone through enhancing GABAA receptor activity in the hypothalamus', *Frontiers in Physiology*, 14, p. 1166246. Available at: https://doi.org/10.3389/FPHYS.2023.1166246/BIBTEX.

Yu, Q., Miller, S.C. and Osmond, D.G. (2000) 'Melatonin inhibits apoptosis during early B-cell development in mouse bone marrow', *Journal of Pineal Research*, 29(2), pp. 86–93. Available at: https://doi.org/10.1034/j.1600-079X.2000.290204.x.

Zang, M. *et al.* (2020) 'The circadian nuclear receptor RORα negatively regulates cerebral ischemia–reperfusion injury and mediates the neuroprotective effects of melatonin', *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1866(11), p. 165890. Available at: https://doi.org/10.1016/J.BBADIS.2020.165890.

Zaoualí, M.A. *et al.* (2011) 'Melatonin protects steatotic and nonsteatotic liver grafts against cold ischemia and reperfusion injury', *Journal of Pineal Research*, 50(2), pp. 213–221. Available at: https://doi.org/10.1111/J.1600-079X.2010.00831.X.

Zhang, C. *et al.* (2021) 'Impact of endogenous melatonin on rhythmic behaviors, reproduction, and survival revealed in melatonin-proficient C57BL/6J congenic mice', *Journal of Pineal Research*, 71(2). Available at: https://doi.org/10.1111/jpi.12748.

Zhang, E.E. and Kay, S.A. (2010) 'Clocks not winding down: unravelling circadian networks', *Nature Reviews Molecular Cell Biology 2010 11:11*, 11(11), pp. 764–776. Available at: https://doi.org/10.1038/nrm2995.

Zhang, Y. *et al.* (2022) 'New insight into ischemic stroke: Circadian rhythm in post-stroke angiogenesis', *Frontiers in Pharmacology*, 13, p. 927506. Available at: https://doi.org/10.3389/FPHAR.2022.927506/PDF.

Zhang Yan *et al.* (2018) *Melatonin protects H9c2 cells against ischemia/reperfusion-induced apoptosis and oxidative stress via activation of the Nrf2 signaling pathway*. Available at: https://doi.org/https://doi.org/10.3892/mmr.2018.9315.

Zhang, Z. *et al.* (2018) 'A congenic line of the C57BL/6J mouse strain that is proficient in melatonin synthesis', *Journal of Pineal Research*, 65(3). Available at: https://doi.org/10.1111/jpi.12509.

Zhou, H. *et al.* (2018) 'Protective role of melatonin in cardiac ischemia-reperfusion injury: From pathogenesis to targeted therapy', *Journal of Pineal Research*, 64(3), p. e12471. Available at: https://doi.org/10.1111/JPI.12471.

Zhou, Y. *et al.* (2018) 'SIRT1/PGC-1α Signaling Promotes Mitochondrial Functional Recovery and Reduces Apoptosis after Intracerebral Hemorrhage in Rats', *Frontiers in Molecular Neuroscience*, 10, p. 443. Available at: https://doi.org/10.3389/FNMOL.2017.00443.

Zhu, F. *et al.* (2017) *Melatonin promoted renal regeneration in folic acid-induced acute kidney injury via inhibiting nucleocytoplasmic translocation of HMGB1 in tubular epithelial cells*, *Am J Transl Res*. Available at: www.ajtr.org.