A Mechanistic Investigation of the Neuropharmacological and Metabolic Actions of Lithium

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Abstract

Bipolar disorder is a chronic mood disorder characterised by cycling episodes of mania and depression. These episodes can have devastating impacts on life quality and expectancy in patients. Currently, the ion lithium is the gold standard mood stabiliser in BD – alleviating both manic and depressive episodes. Lithium pharmacotherapy is accompanied with side effects, however, such as weight gain and nephrotoxicity which limit the application of treatment and patient compliance. Given that the mechanisms underlying lithium's mood stabilisation and side effects remain unknown, we embarked on a detailed mechanistic investigation to shed light on lithium's molecular interactions.

Previous research has focused on lithium's inhibition of glycogen synthase kinase-3β (GSK-3β) as a possible explanation for its mood stabilising properties. We explored the hypothesis that lithium inhibits a wide array of kinases beyond GSK-3β and found that lithium is indeed capable of inhibiting 76 kinases in a cell-free assay *in vitro*. A functional consequence of the inhibition/knockout of 50 lithium-sensitive kinases was demonstrated by alterations to the circadian rhythms of immortalised cell lines *in vitro*. Upon administering lithium to mice *in vivo*, we made the surprising discovery that lithium tolerance is greater in female mice than male mice. Using cryptochrome knockout mice, we further established that period change mediated by cryptochrome does not play a role in lithium's modulation of circadian wheel-running activity *in vivo*. Finally, lithium's selective induction of weight gain in obese patients was examined using a diet-induced obesity mouse model. The transcription factor peroxisome proliferator activated receptor γ was identified as a lithium-sensitive target which could be responsible for lithium-induced weight gain and improved insulin and glucose tolerance in obese patients. Thus this DPhil thesis proposes new cellular targets for lithium which explain how it drives widespread alterations of disparate signalling pathways and further explain lithium's promotion of weight gain.

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Abbreviations

ABL2	ABL Proto-Oncogene 2 Non-Receptor Tyrosine Kinase
ACAM	Acyl-CoA dehydrogenase
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AP2/FABP4	Adipocyte protein 2
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
AUC	Area under the curve
BD	Bipolar disorder
BDNF	Brain-derived neurotrophic factor
BMAL	Brain muscle arnt-like
BMI	Body mass index
BRSK	Brain-selective kinase
C/EBP	CCAAT-enhancer-binding protein
CAM	Ca2+/calmodulin-dependent
CAMK	Calcium/calmodulin kinase
CAMKK	Calcium/calmodulin-dependent protein kinase kinase
cAMP	Cyclic adenosine monophosphate
CBP	CREB binding protein
CDP	Cytidine diphosphate
CHIP	Chromatin immunoprecipitation
ChIP	Chromatin immunoprecipitation
CK	Casein kinase
CLOCK	Circadian locomotor output cycles kaput
CNS	Central nervous system
CREB	Cyclic adenosine monophosphate response element binding protein
CRY	Cryptochrome
Cry1KO	Cry1 homozygous knockout mice
Cry2KO	Cry2 homozygous knockout mice
CSK	C-terminal Src kinase
DAG	Diacylglycerol

DALYs	Disability-adjusted life-years
DAPI	4',6-diamidino-2-phenylindole
DBP	D element-binding protein
DD	Constant darkness
DEC	Differentially expressed in chondrocytes protein
DIO	Diet-induced obesity
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DR1	Direct repeat spaced by one nucleotide
DRN	Dorsal raphe nucleus
DSM-V	Fifth edition of the Diagnostic and Statistical Manual of Mental Disorders
Dvl	Dishevelled
eIF2B	Eukaryotic translation initiation factor 2B
EphA4	Ephrin type-A receptor 4
EPM	Elevated plus maze
ERK	Extracellular signal-regulated kinase
ESR	Estrogen receptor
ESRRB	Estrogen Related Receptor β
FBS	Fetal bovine serum
FFAs	Free fatty acids
FST	Forced swim test
Fzd	Frizzled
G-6-P	Glucose-6-phosphate
GLUT	Glucose transporter
GOI	Gene of interest
GSH-EE	Glutathione reduced ethyl ester
GSK	Glycogen synthase kinase
GTP	Guanosine triphosphate
GWAS	Genome-wide association studies
HFD	High-fat diet
HFHS	High-fat, high-sucrose-fed
HPA	Hypothalamic-pituitary-adrenal

IBMX	3-isobutyl-1-methylxanthine
IKK	Inhibitor of nuclear factor kappa-B kinase
IMPase	Inositol monophosphatase
INSR	Insulin receptor
IP_1	Inositol monophosphate
IP_3	Inositol (1,4,5) trisphosphate
IPPase	Inositol polyphosphate phosphatase
ITT	Insulin tolerance test
JNK	c-Jun N-terminal kinase
KD	Knockdown
KEGG	Kyoto Encyclopedia of Genes and Genomes
KSPs	Keystone sleep phosphoproteins
LD	Light–dark
LEF	Lymphoid enhancer factor
LiCl	Lithium chloride
LL	Constant light
LRP	Lipoprotein receptor-related protein coreceptor 5 or 6
LTD	Long-term depression
LTP	Long-term potentiation
LUC	Luciferase
LXR	Liver X receptor
MAP2K1	Dual specificity mitogen-activated protein kinase kinase 1
MAPK	Mitogen-activated protein kinase
MAPKAPK	MAPK-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MARK	Microtubule affinity regulating kinase
MIPS	L-myo-inositol-phosphate synthase
mTOR	Mammalian/mechanistic target of rapamycin
NaCl	Sodium chloride
NBCS	New born calf serum
NF-κB	Nuclear factor kappa-B
NFIL3	Nuclear factor, interleukin-3-regulated protein
NGF	Nerve growth factor

NIFL	Nuclear factor interleukin-3
NMDA	N-methyl-D-aspartate
NTC	Non-targeting control
OFT	Open field test
OGTT	Oral glucose tolerance test
PAK	p21-activated kinase
PBS	Dulbecco's phosphate-buffered saline
PC	Principal component
PCK1	Phosphoenolpyruvate carboxykinase
PCR	Polymerase chain reaction
PDK	Phosphoinositide-dependent protein kinase
PER	Period
PI	Phosphatidylinositol
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidyl(4,5) bisphosphate
PKA	Protein kinase A
PKB/AKT	Protein kinase B
PKC	Protein Kinase C
PKIS	Published kinase inhibitor set
PLC	Phospholipase C
PPAR	Peroxisome proliferator activated receptor
PPMs	Metal-dependent protein phosphatases
PPPs	Phosphoprotein phosphatases
PPRE	Peroxisome proliferator responsive elements
PTPs	Tyrosine phosphatases
REF	Reference housekeeping genes
RIN	RNA integrity number
RORE	Retinoid-related orphan receptor response elements
RORα	Retinoid-related orphan receptor α
RPMI	Roswell Park Memorial Institute media
RSK	Ribosomal S6 kinase
RXRa	Retinoid X receptor a
SCN	Suprachiasmatic nucleus

SCRD	Sleep and circadian rhythm disruption
SEM	Standard error of the mean
SIK	Salt-inducible kinase
SMIT1	Sodium–myo-inositol transporter 1
SNIPPs	Synaptic sleep-need-index phosphoproteins
SNP	Single nucleotide polymorphism
SRC	Sarcoma kinase
STAT	Signal transducer and activator of transcription
SWA	Slow wave activity
SWS	Slow wave sleep
TAF7L	TATA-Box Binding Protein Associated Factor 7 Like
TCA	Tricarboxylic acid
TCF	T-cell factor
TESK	Testis-specific protein kinase
TLK	Tousled-like kinase
TNF	Tumour necrosis factor
TRH	Thyrotropin-releasing hormone
TSH	Thyroid stimulating hormone
TTFL	Transcription/translation feedback loops
TZD	Thiazolidinedione
VPA	Valproic acid
WAT	White adipose tissue
Wnt	Wingless/int-1
WT	Wildtype
ZT	Zeitgeber timepoint

1. GENERAL INTRODUCTION

Lithium is believed to be one of the first three elements created in the big bang (Walker *et al.*, 1991). Since this explosive start, lithium has rather serendipitously become one of the first-line mood stabilisers in the treatment of bipolar disorder (BD) (Goodwin *et al.*, 2016). The mechanisms of action of this primordial medication, however, remain largely unknown. This thesis aims to elucidate lithium's alteration of signalling cascades to better understand its role as a mood stabiliser and the mechanisms underlying its side effects.

1.1 The History of Bipolar Disorder

Scientific investigations into the affective states of mania and depression can be traced back to the ancient Greeks (Angst and Marneros, 2001). In the fifth century BCE, many ancient Greeks sought to understand their world by breaking it down into constituents such as hot, cold, wet and dry, or the more well-known earth, air, fire and water. Alcmaeon of Croton hypothesised that diseases in the brain were caused by an imbalance in these fundamental 'powers'. This idea of a balance between elements responsible for human health grew into the influential Hippocratic constitutive humoral theory expounded in the treatise *Nature of Man.* According to the treatise, blood, phlegm, and yellow and black bile were the four 'humours' responsible for the human body's health and pathology (Jackson, 2016). Indeed, the very word for the subdued despondent state of 'melancholia' is derived from the Greek 'melas' (meaning black) and 'cholé' (meaning bile) (Angst and Marneros, 2001). The origin of the notion of mania is less clear as it has many

meanings. Some early references from the Ancient Greeks refer to manic fits of rage and others refer to a divine state of inspiration (Angst and Marneros, 2001).

In the first century AD, Aretaeus of Cappadocia was the first physician to link episodes of melancholy and mania in one person. He believed that melancholy and mania were the opposite sides of a continuous spectrum and thus mania was simply a worsened form of melancholy (Angst and Marneros, 2001). Physicians' observations of these episodes in patients continued until two theories emerged in Paris in 1854 that linked mania and melancholy in a single disease. In early January, Parisian psychiatrist Jean Pierre Falret wrote:

> […]it has not until now been realized that there is a category of insanity in which the succession of mania and melancholia manifests itself in a nearly regular manner. We have considered this fact to be of sufficient importance to become the basis of a special form of mental disease we call circular madness (*folie circulaire*) because the course of this type of insanity runs in a repetitive circle of pathological states[...] separated only by rational intervals of fairly short duration (Pichot, 1995).

Falret presented a rather coherent picture of an intermittent disorder characterised by episodes of extreme mood (Pichot, 1995).

Not even 31 days later, however, a fellow Parisian psychiatrist named Jules Baillarger presented a lecture that he later formulated into an essay called 'folie à double forme' (insanity with a double form). He gave a description not unlike Aretaeus' of an illness characterised by episodes of depression and excitement where the mania is a reaction to the depression and there are no intervals between the two (Pichot, 1995). This sparked a bitter rivalry between the two colleagues over who had discovered the new mental disease and what exactly the disease consisted of. Both called for the need to establish distinctions between mental

disorders so that a consensus could be reached and proper treatments found (Sedler, 1983).

Somewhat contrary to Falret and Baillarger's calls for exact delineations of the disease, the influential German psychiatrist Emil Kraepelin championed the idea that a symptom is not specific to a particular disease and instead each disease is characterised by a unique *combination* of symptoms. Thus in 1921 he described multiple manic states, depressive states, and mixed states and combined them all under the umbrella term 'manic-depressive insanity'. This manic-depressive insanity included patients with pure mania, pure depression, the circular madness that combined the two, and delirious insanity. Furthermore, Kraeplin recognised that there was a genetic hereditary component to this disease (Mitchell, 2016).

It was Karl Leonhard who coined the term 'bipolar disorder' in the 1950s. He separated Kraelepin's manic-depressive insanity into bipolar disorders (psychoses in which there are cyclical episodes of mania and depression) and unipolar disorders (psychoses that are either only manic or only depressive) – distinctions that we still use to this day (Mitchell, 2016).

Estimates of the worldwide prevalence of BD today vary from 1% to 5% of the population depending on the subtypes of bipolar disorders that are included (Akiskal *et al.*, 2000). A cross-sectional study of 61,392 adults in 11 countries across the Americas, Europe, and Asia found that the lifetime prevalence of bipolar disorders was 2.4% (Merikangas *et al.*, 2011). These estimates are similar to those for the prevalence of cancer which vary from 0.4% to 5.5% (Roser and Ritchie, 2019). In fact, BDs are a bigger cause of loss of disability-adjusted life-years (DALYs)

than cancer because of their earlier onset and chronicity (Merikangas *et al.*, 2011). Partly responsible for the loss of DALYs is suicidal ideation which is prevalent in manic, depressive, and euthymic stages of BDs. 80% of patients with BDs have suicidal ideations and 20% actually attempt suicide (Valtonen *et al.*, 2005). A recent study found that BDs cost the UK economy £6.4 billion in 2018-2019 largely due to productivity loss and healthcare costs (Simon *et al.*, 2021).

1.2 The Symptoms and Characterisation of Bipolar Disorder

BDs are mood disorders characterised by episodes of mania/hypomania, and depression. These episodes can cycle over the course of the disorder, causing swings between extreme moods (World Health Organization, 2019). The most recent (fifth) edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) published by the American Psychiatric Association in 2013 divides bipolar and "related" disorders into seven categories: bipolar I disorder, bipolar II disorder, cyclothymic disorder, substance/medication-induced bipolar and related disorder, bipolar and related disorder due to another medical condition, other specified bipolar and related disorder, and unspecified bipolar and related disorder (American Psychiatric Association, 2013). The manual defines a manic episode as:

> A distinct period of abnormally and persistently elevated, expansive, or irritable mood and abnormally and persistently goal-directed behavior or energy, lasting at least 1 week and present most of the day, nearly every day (or any duration if hospitalization is necessary) (American Psychiatric Association, 2013).

It also defines a hypomanic episode as:

> A distinct period of abnormally and persistently elevated, expansive, or irritable mood and abnormally and persistently increased activity or energy, lasting at least 4 consecutive days and present most of the day, nearly every day (American Psychiatric Association, 2013).

Symptoms of a major depressive episode include "depressed mood most of the day, nearly every day" and "markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day" (American Psychiatric Association, 2013).

The main distinction between bipolar I disorder (BD-I) and bipolar II disorder (BD-II) is that manic episodes are necessary for a BD-I diagnosis, whereas patients with BD-II experience hypomanic episodes. Furthermore, while those with a BD-I diagnosis may experience a depressive episode, depressive episodes are a necessity for a BD-II diagnosis. Cyclothymic disorder is defined as a milder form of BD-II where some symptoms for hypomanic and depressive episodes are present, but the full criteria are not met. As the name suggests, substance/medication-induced bipolar and related disorder is diagnosed when the mood disruptions are linked to a medication. Similarly, bipolar and related disorder due to another medical condition is diagnosed when the mood disruptions are attributable to a different medical condition. Other specified bipolar and related disorder is diagnosed when some of the symptoms of BD are present enough to produce clinically relevant distress, but the patient doesn't meet the full criteria for any of the aforementioned BDs. The unspecified bipolar and related disorder diagnosis is given when the clinician does not wish to specify why the criteria for any of the disorders above are not met and cannot make a specific diagnosis (American Psychiatric Association, 2013). The eleventh edition of the International

Classification of Diseases produced by the World Health Organization largely agrees with the criteria laid out in the DSM-V (World Health Organization, 2019).

1.3 The Aetiology of Bipolar Disorder

The onset of BD usually occurs in late adolescence to early adulthood, with more severe forms having an earlier average onset (18.4 and 20 years old for BD-I and BD-II, respectively) than the less severe forms (21.9 years old), though the exact ages vary between studies (Wittchen, Mühlig and Pezawas, 2003; Merikangas *et al.*, 2011). Given that BD is chronic and prevalent worldwide, a lot of research has focused on potential causes of the mood disorder.

1.3.1 Genes Associated with Bipolar Disorder

Although the exact cause(s) of BD remain unknown, it is clear there is a strong genetic component. A meta-analysis of eight studies that compared the risk of BD-I in first degree relatives of bipolar probands to the risk in first degree relatives of controls found an odds ratio of 7 (Craddock and Sklar, 2013). The risk of BD-II is also increased in first degree relatives of BD-I patients and vice versa. Twin and adoption studies provided further evidence for the heritability of BD (Smoller and Finn, 2003; Craddock and Sklar, 2013). One study found the biological parents of children with BD were significantly more likely to also have BD than adoptive parents (Mendlewicz and Rainer, 1977).

As the DSM-V lays out, BD is multifaceted and complex, making the identification of the genes involved difficult. Early linkage studies desperately tried

to find a single gene responsible for BD, but to no avail (Craddock and Sklar, 2013). Researchers have tried to narrow down the search by focusing on genes that are known to have a neurobiological role in the regulation of emotion and behaviour. Using these methods, genetic markers in *SLC6A4* (a serotonin transporter), d-amino acid oxidase activator, and brain-derived neurotrophic factor (BDNF), among others, have been identified, but do not have enough supporting evidence to be proven as the cause of BD (Rowland and Marwaha, 2018; Gordovez and McMahon, 2020). Polymorphisms in catechol-O-methyl transferase (an enzyme involved in dopamine degradation) have also been observed in BD and it has been noted that dopamine agonists and a dopamine precursor L-DOPA can cause manic episodes (Cousins, Butts and Young, 2009; Rowland and Marwaha, 2018).

Genome-wide association studies (GWAS) have enabled researchers to focus on groups of gene loci that may contribute to the development of BD. A number of polymorphisms have been detected in the nearly 20 GWAS that have been conducted on BD, but many single nucleotide polymorphisms (SNPs) are in noncoding regions or have odd ratios that are too small to be clinically relevant. Two genes, *ANK3* and *CACNA1C*, appear to be of significance (Gordovez and McMahon, 2020).

Studies have identified multiple SNPs in *ANK3* (the gene encoding the scaffolding protein ankyrin-G which is required for the function of the axon initial segment and nodes of Ranvier) as markers for susceptibility to BD-I (Schulze *et al.*, 2009). Region specific knockdown of *ANK3* in mice leads to increased risk-taking behaviour. The SNP rs10994336 has also been associated with increased risk-

taking, and another BD-risk haplotype of *ANK3* (SNP rs1938526) reduces *ANK3* expression in the cerebellum which is known to play a role in behavioural regulation (Rueckert *et al.*, 2013). Ankyrin-G is a negative regulator of the Wnt signalling pathway that regulates neural proliferation and neurogenesis, supporting the hypothesis that neurodevelopmental changes could predispose individuals to BD (Durak *et al.*, 2015).

Another possible genetic culprit, *CACNA1C*, has been implicated in BD susceptibility. *CACNA1C* is a subunit of the L-type voltage-gated calcium channel. *Cacna1c* haploinsufficiency alters behaviour in mice with an anti-depressant-like phenotype (Dao *et al.*, 2010). Calcium plays a critical role in many signalling cascades, especially synaptic transmission, and it can alter gene expression relating to synaptic plasticity, neuronal fate, dendritic development, learning, and memory (Bhat *et al.*, 2012). Given that *ANK3* also regulates voltage-gated sodium channels, one group concluded that ion channelopathies could contribute to BD (Ferreira *et al.*, 2008). Other risk loci have been implicated in various GWAS, but all told, the common gene variants are responsible for 25% of the heritability of BD (Gordovez and McMahon, 2020). All in all, the heritability of BD is estimated at 60-85%, suggesting that environmental factors also play a role in the aetiology of BD (Smoller and Finn, 2003).

1.3.2 Environmental Contributions to the Prognosis of Bipolar Disorder

Childhood abuse and emotional abuse have been linked to the development of BD and worsened symptoms of BD, including increased affective episodes and

mood cycling. In fact, BD patients are four times more likely to have experienced emotional abuse than controls. The mechanisms of these interactions, however, remain unknown (Palmier-Claus *et al.*, 2016). Though there is a lack of consensus, several studies have reported stressful life events to precede the onset of and precipitate further affective episodes in BD (Tsuchiya, Byrne and Mortensen, 2003; Lex, Bäzner and Meyer, 2017). The DSM-V recognises that BD can also be comorbid with substance use and with other medical conditions, such as asthma, obesity, head trauma and multiple sclerosis (American Psychiatric Association, 2013). The connection of socioeconomic status with BD remains unclear with studies coming to opposing conclusions (Tsuchiya, Byrne and Mortensen, 2003; Rowland and Marwaha, 2018).

1.3.3 Sleep and Circadian Disruptions in Bipolar Disorder

Sleep and circadian rhythm disruptions (SCRDs) have been implicated in metabolic syndrome and psychiatric disorders – and BD is no exception (Jagannath *et al.*, 2017). In fact, sleep disturbances are one of the diagnostic criteria for recognising BD, particularly in mania and hypomania during which the DSM-V notes there is a decreased need for sleep (American Psychiatric Association, 2013). Recent work has therefore focused on disruptions in the circadian rhythms of BD patients hoping to uncover potential mechanisms underlying the mood disorder (McCarthy *et al.*, 2019; Sanghani *et al.*, 2021).

1.3.3.1 The Circadian Rhythm

The molecular mechanisms underlying circadian rhythms are found in most lifeforms on Earth. Daily 24-hour (circadian) rhythms are an adaptive response developed by disparate organisms including cyanobacteria, plants, fungi, and animals to cope with their changing environment. Daily light, food availability, and predatory changes made it advantageous for these organisms to sequester various cellular processes to the appropriate time of day. They evolved clocks that contain positive processes whose negative products slow down the original positive elements, creating rhythmic oscillations away from equilibrium. In mammals, these clocks took the form of transcription/translation feedback loops (TTFLs) that regulate daily rhythms throughout the body (Dunlap, 1999).

The core autoregulatory TTFL in mammals is comprised of the transcription factors brain muscle arnt-like 1 (BMAL1) and circadian locomotor output cycles kaput (CLOCK) (the positive arm) and period 1/2/3 (PER1/2/3) and cryptochrome 1/2 (CRY1/2) (the negative arm). The CLOCK-BMAL1 complex heterodimerises with peak activation during the day and binds to E-box promoters that drive the transcription of rhythmic genes including PER1/2/3 and CRY1/2, which accumulate at dusk. Casein Kinase 1 (CK1) phosphorylates PER, forming a brief complex that allows CRY to bind. PER and CRY are then phosphorylated within the CRY-PER-CK1 complex, allowing it to translocate into the nucleus at night where it interacts with CLOCK-BMAL1 to inhibit PER and CRY transcription. As PER and CRY levels fall, the repression on CLOCK-BMAL1 eases, and CLOCK-BMAL1 can build up again to initiate another cycle of transcription in the morning, setting the period of the clock (Fig. 1.1) (Jagannath *et al.*, 2017; Takahashi, 2017).

BMAL1 expression is driven by a secondary TTFL. The nuclear receptors retinoid-related orphan receptor alpha (RORα) and REV-ERBα contain an E-box in their promoters and thus are also activated by the CLOCK-BMAL1 complex. They are transcribed and translated at different rates, however, such that RORα levels peak at dawn and REV-ERBα levels peak at dusk. *Bmal1* transcription is activated by RORα binding ROR response elements (ROREs) in its promoter and inhibited when REV-ERBα binds. This alternation results in rhythmic transcription of *Bmal1* that is antiphase to *Per* and *Cry*, while *Clock* is constitutively expressed (Fig. 1.1B) (Jagannath *et al.*, 2017; Takahashi, 2017).

Two tertiary TTFLs regulate the activity of CLOCK-BMAL1 through different promoters. Differentially expressed in chondrocytes protein 1 and 2 (DEC1 and 2) are rhythmic proteins that reinforce the actions of PER and CRY by binding directly to E-boxes and blocking transcription driven by CLOCK-BMAL1 (Nakashima *et al.*, 2008; Jagannath *et al.*, 2017). Nuclear factor, interleukin-3 regulated protein (NFIL3) suppresses the expression of RORα and other circadian genes at D-box elements. RORα in turn binds to ROREs in the regulatory region of NFIL3 and drives its expression. This loop is repressed in a circadian fashion by REV-ERBα which also rhythmically binds to ROREs in the regulatory region of NFIL3 (Fig. 1.1A) (Takahashi, 2017). These molecular rhythms are present in most cells in the body and must be entrained to the proper time of day and synchronised with each other. The suprachiasmatic nucleus (SCN) in the anteroventral hypothalamus is the master circadian pacemaker that plays just this role.

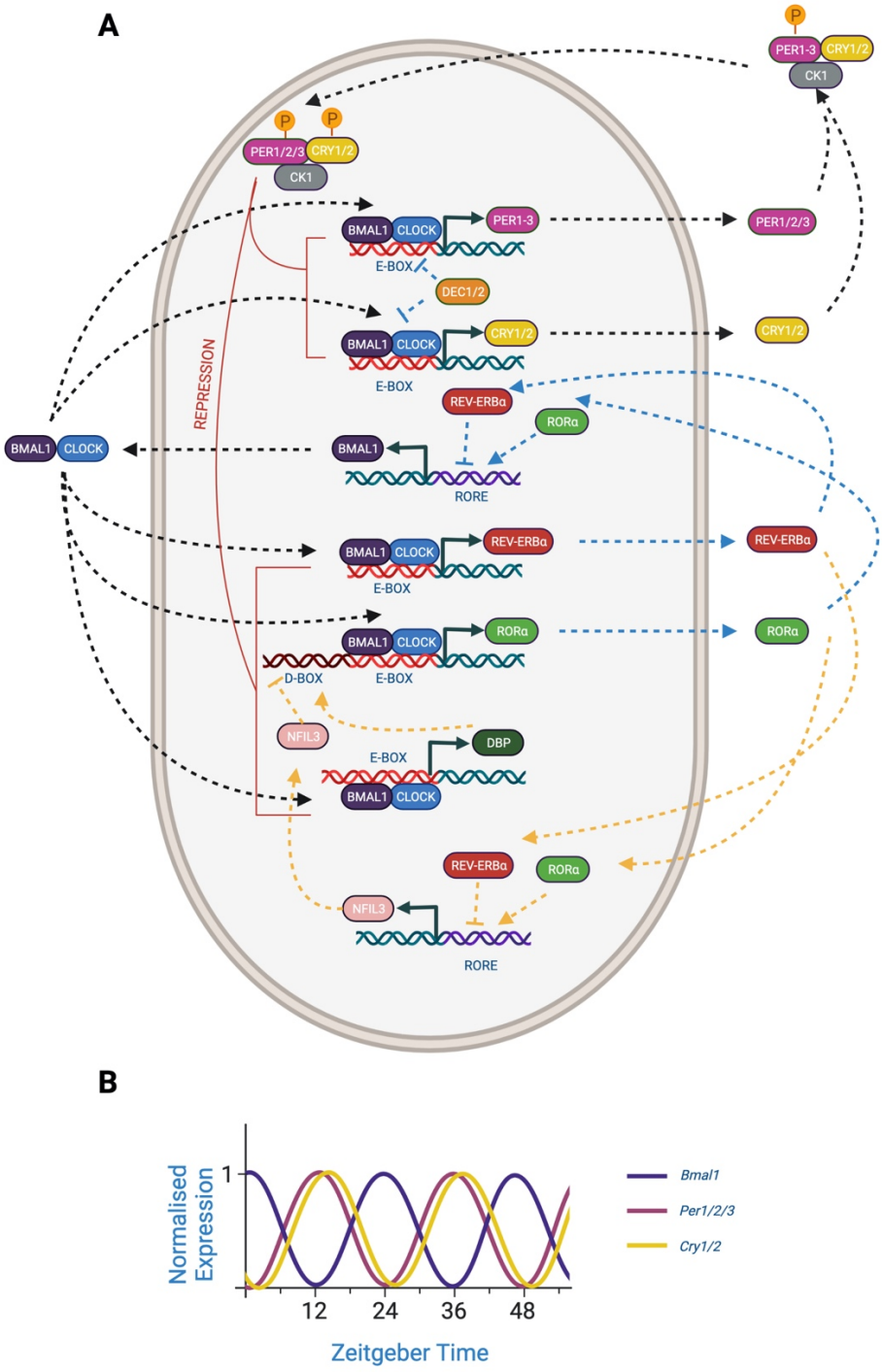

Figure 1.1 The Circadian Clock. A) The primary TTFL of the circadian clock (black arrows and red brackets) is positively driven by BMAL1 and CLOCK, which heterodimerise and translocate into the nucleus where they bind E-boxes in the regulatory region of circadian transcription factors, including PER and CRY. PER and CRY, in turn, translocate into the nucleus and repress CLOCK-BMAL1 activity. A secondary TTFL (blue arrows) reinforces the primary TTFL by regulating BMAL1 expression. REV-ERBα inhibits BMAL1 expression, while RORα promotes it. The activity of these transcription factors occurs at different rates to give BMAL1 rhythmic expression. A tertiary TTFL (yellow arrows) involves DEC1 and 2, which block CLOCK-BMAL1 activity to reinforce PER and CRY. Another tertiary TTFL involves NFIL3 and RORα. RORα activates NFIL3, and NIFL3 represses RORα activity where DBP activates it. B) The transcription of the primary TTFL drivers (*Bmal1*, *Per1/2/3*, and *Cry1/2*) oscillates in a circadian fashion such that BMAL1 accumulates at dawn (ZT0) and PER and CRY accumulate at dusk (~ZT12).

Temporal cues (zeitgebers) change the period (length), amplitude (strength) and phase (advance or delay) of the circadian clock to match the environment. Perhaps the most obvious zeitgeber is light. The SCN receives light cues directly from the retina via photosensitive retinal ganglion cells which project to the SCN through the retinohypothalamic tract. This input reaches the ventral 'core' region of the SCN, where innervation of the core neurons results in depolarisation and an influx of calcium through N-methyl-D-aspartate (NMDA) receptors and voltage-gated ion channels. The intracellular increase in calcium activates a cascade of kinases that leads to the phosphorylation of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) at Ser133 and Ser142. Phosphorylated CREB binds calcium/cAMP response elements (CREs) with its coactivator CREB binding protein (CBP), inducing the transcription of target genes including PER1/2. CREB phosphorylation is induced within minutes of light exposure and only occurs during subjective night, as this is the only time it would be needed to reset the clock (Ginty *et al.*, 1993; Gau *et al.*, 2002; Travnickova-Bendova *et al.*, 2002; Welsh, Takahashi and Kay, 2009). The mechanisms of light-induced shifts to the circadian clock are still being examined, however, as mice with targeted disruption of *Per1* and *Per2* have still been shown to phase shift in response to light (Bae and Weaver, 2003).

Through neuronal connections and hormone release, the SCN send outputs from the dorsal 'shell' region to synchronise peripheral circadian clocks in tissues throughout the body. These outputs combined with zeitgebers (including feeding schedule, sleep/wake rhythms, glucocorticoids and temperature) all set the phase of peripheral clocks. Depending on the peripheral tissue, 2-30% of genes are

expressed with circadian rhythmicity (Welsh, Takahashi and Kay, 2009; Jagannath *et al.*, 2017).

The sleep/wake cycle and circadian rhythms are tightly intertwined. In fact, it has been proposed that sleep consists of two processes – one that is driven by the circadian rhythm (Process C) and another that is driven and increased by sleep pressure (Process S). Together, Process C and Process S combine to determine sleep propensity along with sleep duration (Borbély, 1982). Protein phosphorylation is an important post-translational modification that rather recently has been found to tie Process C to Process S, as sleep deprivation leads to hyperphosphorylation in the brain (Wang *et al.*, 2018). Further work examining the synaptic proteome found that the expression of rhythmic genes is resilient against sleep deprivation, but the translation of these genes is not. Thus Process C anticipates environmental needs, but Process S drives actual protein availability according to present demand (Noya *et al.*, 2019).

1.3.3.2 Circadian Disruptions in Bipolar Disorder

Disruptions in sleep and circadian processes are well documented in bipolar patients. Case studies and interviews found that sleep deprivation alleviates depression and induces mania (Wehr, 1992; Lewis *et al.*, 2017). In the search for state (mood)-independent endophenotypes in BD, researchers identified circadian rhythm instability, particularly in sleep/wake cycles. Disruptions in circadian body temperature and neuroendocrine rhythms were also apparent, but appeared to be state-dependent (Hasler *et al.*, 2006).

Association studies have examined the link between alterations in circadian genes and BD, but the results have been mixed and many genes aren't replicated between studies (Table 1.1). A nongenomic study used patient fibroblasts to examine the peripheral circadian clocks in 12 BD patients and 12 controls and found no change in period, but a reduction of amplitude in the rhythmic expression of BMAL1, REV-ERBα and D element-binding protein (DBP) (Yang *et al.*, 2009).

Many of the mouse models used to study BD and mania are generated through mutations to circadian genes. Mice with a mutation in CLOCK that produces a dominant-negative protein (ClockΔ19 mice) display behaviours characteristic of mania including reduced anxiety and sleep, depression-like behaviour, and greater preference for rewarding stimuli (Roybal *et al.*, 2007). 'After-hours' mice have a cysteine to serine substitution in F-box/LRR-repeat protein 3, which participates in the degradation of CRY. These mice show increased locomotor activity, reduced anxiety, and diminished depression-like behaviours indicative of mania in behavioural tests (Keers *et al.*, 2012). Intriguingly, *Myshkin* mutant mice are a model of mania that involves an inactivating mutation in a non-circadian gene (the neuron-specific Na+,K+-ATPase α3 subunit), but they display circadian and sleep disruptions (Kirshenbaum *et al.*, 2011). Finally, a paradoxical sleep deprivation mouse model utilises the stress of sleep deprivation to induce mania, further supporting the connection between circadian rhythms and mania (Gessa *et al.*, 1995; Benedetti *et al.*, 2008).

Gene	Studies Showing Association
ADCYAP1	(Soria *et al.*, 2010)
ARNTL (Bmal1)	(Mansour *et al.*, 2006, 2009; Nievergelt *et al.*, 2006; Shi *et al.*, 2008; Soria *et al.*, 2010; McCarthy *et al.*, 2012; Gonzalez *et al.*, 2015)
ARNTL2	(Shi *et al.*, 2008; Soria *et al.*, 2010)
CLOCK	(Shi *et al.*, 2008; Kripke *et al.*, 2009; Soria *et al.*, 2010)
CRY1	(Soria *et al.*, 2010)
CRY2	(Mansour *et al.*, 2009)
CSNK1D	(Kripke *et al.*, 2009)
CSNK1E	(Shi *et al.*, 2008; Mansour *et al.*, 2009; Soria *et al.*, 2010; Gonzalez *et al.*, 2015)
DBP	(Shi *et al.*, 2008)
DEC1	(Shi et al., 2008; McCarthy et al., 2012)
DEC2	(Soria *et al.*, 2010)
EGR3	(Mansour *et al.*, 2009)
NPAS2	(Kripke *et al.*, 2009; Mansour *et al.*, 2009; Soria *et al.*, 2010)
NR1D1 (REV-ERBα)	(Kripke *et al.*, 2009; Severino *et al.*, 2009)
PER1	(Kripke *et al.*, 2009)
PER2	(Kripke *et al.*, 2009)
PER3	(Mansour *et al.*, 2006; Nievergelt *et al.*, 2006; Soria *et al.*, 2010)
PPARGC1B	(Kripke *et al.*, 2009)
RORA	(Soria *et al.*, 2010; Etain *et al.*, 2014)
RORB	(Mansour *et al.*, 2009; McGrath *et al.*, 2009; McCarthy *et al.*, 2012)
TIMELESS	(Mansour *et al.*, 2006; Shi *et al.*, 2008; Etain *et al.*, 2014)
VIP	(Soria *et al.*, 2010)
VIPR	(Soria *et al.*, 2010)

Table 1.1 Mutations in Circadian Genes Associated with Bipolar Disorder.

A connection between the circadian rhythm and the neurotransmitters involved in mood regulation has been observed. Serotonin deficiency has been

linked to depression for the past 50 years with selective serotonin reuptake inhibitors being the primary treatment for depression (Sharp and Cowen, 2011). Decreased serotonergic signalling has also been linked to acute mania. PET evidence gathered from manic patients *in* vivo suggests that serotonin-2 receptor binding potential is reduced in multiple brain regions (Yatham *et al.*, 2010). In addition, mania has been found to reduce platelet serotonin uptake and reduce 5-HIAA (a metabolite of serotonin) levels in cerebrospinal fluid (Coppen *et al.*, 1972; Marazziti *et al.*, 1991). Accordingly, it has been shown that lithium stabilises serotonergic signalling by increasing serotonin release and reducing serotonin receptors in the rat hippocampus. It has been suggested that this mechanism may be responsible for lithium's acute antidepressant action (Treiser *et al.*, 1981; Sharp *et al.*, 1991). In manic patients, lithium increases long-term platelet serotonin uptake and baseline 5-HIAA levels in cerebrospinal fluid (Fyrö, Petterson and Sedvall, 1975; Meltzer, Arora and Goodnick, 1983). Thus, enhancing serotonergic signalling could be a method of mood stabilisation. Evidence for this is mixed, however, and more research is needed (Shiah and Yatham, 2000).

A variety of serotonin receptors have been found in the SCN of rats and mice at pre- and postsynaptic terminals suggesting that serotonin plays a significant role in circadian regulation (Anfuso, Lupo and Alberghina, 1999; Jiang *et al.*, 2000; Belenky and Pickard, 2001). In fact, serotonin has a mainly inhibitory effect on the synaptic depolarisation of pre-and postsynaptic terminals in the SCN, though this varies with zeitgeber timepoint (ZT) suggesting circadian regulation (Jiang *et al.*, 2000). Serotonergic signalling can alter circadian processes through photic and nonphotic pathways. Light activates the retinoraphe pathway which innervates the

dorsal raphe nucleus (DRN) where most serotonergic neurons are found. The DRN is indirectly connected to the SCN and can therefore affect serotonin release there. DRN innervation has differential effects on circadian phase depending on the ZT at which it is received (Morin, 1999; Fite, Wu and Bellemer, 2005). Serotonin can also induce phase shift in response to a nonphotic stimulus such as locomotive activity (Morin, 1999). 8-hydroxy-2-(di-n-propylamino) tetralin (a serotonin 1A/7 receptor agonist) also acts as a nonphotic stimulus in the SCN, reducing *Per1* and *Per2* expression in the hamster SCN, but only during the subjective day (Horikawa *et al.*, 2000). In addition, serotonin is a precursor in the synthesis of melatonin (a hormone that facilitates sleep) (Zhao *et al.*, 2019). Thus serotonergic signalling is altered in BD and also plays a role in the circadian system.

Dopaminergic signalling has been implicated in BD since at least the 1970s and it is also involved in circadian signalling. The "dopamine hypothesis" of BD arose from the observation that antidopaminergic drugs had antimanic properties. It was then proposed by extension that depression was caused by a deficit in dopaminergic signalling. Thus faulty dopamine homeostasis has been proposed as a cause of BD (Ashok *et al.*, 2017). The mesolimbic dopamine system, including the ventral tegmental area, plays a role in the brain's response to rewarding stimuli, such as food or drugs of abuse, and is therefore believed to influence mood (Nestler and Carlezon, 2006). A role for dopamine in BD has been supported by animal and patient studies. Mania has been associated with an elevated density of D_2 dopamine receptors in patients using *in vivo* tracers and post mortem brain tissue (Pearlson *et al.*, 1995; Wong *et al.*, 1997; Zhan *et al.*, 2011). The evidence for the involvement of dopamine in bipolar depression is more mixed as both dopaminergic agonists and

dopaminergic antagonists are effective for treating bipolar depression (Ashok *et al.*, 2017).

The disruption of circadian gene expression has a pronounced effect on dopaminergic signalling and behaviour. The promoter of monoamine oxidase, a key enzyme in the metabolism of dopamine, is regulated by both BMAL1 and PER2. Indeed, dopamine levels are elevated in *Per2* mutant mice, which spend less time immobile in the forced swim test (FST) – used as a measure of depression-like behaviour (Hampp *et al.*, 2008). REV-ERBα has also been tied to dopamine regulation. The knockout of REV-ERBα in mice increases the expression of tyrosine hydroxylase (a rate-limiting enzyme in dopamine biosynthesis), resulting in increased midbrain dopaminergic signalling and simultaneously increasing behavioural changes thought to be associated with mania (Chung *et al.*, 2014).

The dopaminergic system, in turn, can alter circadian signalling. The SCN expresses two dopamine receptors (D_1 and D_5), suggesting a role for dopamine in the regulation of circadian rhythms. The D_2 dopamine receptor activates *Per1* expression through the recruitment of CLOCK-BMAL1 (Korshunov, Blakemore and Trombley, 2017). Dopamine has also been found to inhibit melatonin and play an important role in the circadian adaptation to light. Perhaps the most convincing evidence of dopamine's circadian role comes from the fact that Parkinson's Disease, characterised by the loss of dopamine neurons, is associated with circadian dysfunction (Korshunov, Blakemore and Trombley, 2017). Therefore dopamine also illustrates the connection between circadian signalling and neurotransmitters implicated in the pathology of BD.

1.4 Treatments for Bipolar Disorder

Given the many potential causes of BD outlined above, the guidance for the treatment of bipolar disorder varies greatly. For acute manic episodes, dopamine antagonists are recommended along with the discontinuation of antidepressant medications (Goodwin *et al*., 2016). For the treatment of acute depressive episodes, it is recommended that antidepressants are given along with dopamine antagonists to reduce the chance of subsequent manic episodes. Antipsychotics can also be effective in the short-term treatment of acute mania and acute depression. If the patient is already on medication, electroconvulsive therapy (the application of an electric current to the scalp of the patient to induce seizure) can be recommended, though generally not as a first-line treatment (Geddes and Miklowitz, 2013; Goodwin *et al*., 2016). For long-term treatment, lithium is recommended as the first-line pharmacotherapy, as it is effective for the treatment of mania, depression, mixed relapse, and suicide prevention. If lithium monotherapy fails, another mood stabiliser valproate is recommended. Anticonvulsants and antipsychotics can also be recommended, though the evidence of their long-term efficacy is lacking (Geddes and Miklowitz, 2013; Goodwin *et al*., 2016).

Psychotherapies can help prevent relapse and ease the prognosis of BD. The psychological treatment of bipolar depression without any pharmacological intervention is not recommended, though it can add benefits to treatment (Goodwin *et al*., 2016). Such treatments include family-focused therapy, cognitive-behavioural therapy, interpersonal and social rhythm therapy, and functional remediation (problem-solving and memory exercises). Psychoeducation can also be applied in group settings. When used in combination with pharmacotherapy, these

psychological interventions have been found to reduce relapse and improve social functioning (Geddes and Miklowitz, 2013).

Depression is responsible for much of the disease burden associated with BD, yet fewer treatments are available for bipolar depression as opposed to mania. Light therapy has therefore been employed as it has low risk of side effects and can be used in conjunction with other pharmacological treatments (Lam *et al.*, 2020). As the name suggests, light therapy involves the administration of light to a patient. This light can vary in intensity, wavelength, total exposure time, and timing of exposure (Lam *et al.*, 2020). One meta-analysis of 12 studies including 847 bipolar patients found that light intensity ≥ 5000 lux significantly improved the severity of depression. Furthermore, light therapy significantly improved depression with and without adjunctive therapy (Wang *et al.*, 2020). Another meta-analysis of seven randomized controlled trials including 259 patients found that light therapy significantly improved the Hamilton Depression Rating Scale score and clinical response in patients in the short term, but light therapy did not reduce clinical remission (Lam *et al.*, 2020). Nevertheless, the relative success of light therapy in BD further supports circadian involvement in the disorder.

1.5 The Discovery of Lithium as a Treatment for Bipolar Disorder

Among all of the treatment options previously listed, lithium remains the gold standard treatment for BD (Goodwin *et al.*, 2016). In the first half of the nineteenth century, lithium was initially known as a treatment for gout. It was in the

1870s that neurologist Silas Weir Mitchell suggested that lithium bromide could be used as an anticonvulsant and hypnotic for "general nervousness" (Shorter, 2009). One year later, William Hammond was the first to put this suggestion into practice at the Bellevue Hospital Medical College in New York, writing:

> …I have used the bromide of lithium in cases of acute mania, and have more reason to be satisfied with it than any other medicine calculated to diminish the amount of blood in the cerebral vessels, and to calm any nervous excitement that may be present (Hammond, 1871).

In the 1890s, Dutch psychiatrist Carl Lange made the connection between gout, lithium and mania. He believed that the excessive build-up of uric acid in gout had a toxic effect on the brain and was connected to periodical depression. He and his brother Frederik Lange therefore used lithium to treat gout and thus treat periodic depression. This concept, however, was eventually abandoned and lithium fell out of use (Schioldann, 2011).

The modern use of lithium as a mood stabiliser began in the 1940s with a medical officer named John Cade. Aware that lithium had been used for the treatment of gout and linked to mood alterations, Cade injected lithium carbonate into guinea pigs during an investigation into the toxicity of urine derived from manic patients. After finding that lithium carbonate produced a lethargic phenotype, Cade gave lithium citrate to 10 manic patients and found that it at least partially alleviated the symptoms of mania in every single patient without aggravating depression (Cade, 1949). His findings were subsequently replicated in follow-up studies, but it was a double-blind, placebo-controlled study led by Mogens Schou that gained traction (Shorter, 2009).

Schou designed a trial using 38 patients in typical or atypical stages of mania, divided them into a treatment group and a control placebo group and changed the groups every two weeks in a random fashion. A mixture of lithium carbonate, lithium citrate and lithium chloride were used, all with the same concentration of lithium. The researchers found that lithium had a beneficial effect on all but six patients (three experiencing typical and three experiencing atypical mania) and this positive effect disappeared with the cessation of lithium treatment. Many of the patients marked unresponsive to lithium did have some form of positive recovery with lithium treatment, but this recovery was not significant for various reasons. Schou did, however, make the important note that the dose of lithium necessary to see a clinical effect (24 to 48mEq daily) is very close to the dose at which lithium becomes toxic (Schou *et al.*, 1954). The study by Schou et al. was not fully blinded and faced some methodological criticisms (Baastrup *et al.*, 1970).

To address the methodological shortcomings, a fully double-blind study in the 1970s was conducted that had very striking results (Table 1.2). Baastrup *et al.* recruited 50 manic-depressive patients and 34 patients with recurrent depression who had already been receiving continuous lithium treatment for at least a year and assigned them to continue treatment or move to placebo in a double-blind fashion. Relapses were then measured as hospital admissions or increased therapy. The researchers saw no relapses in patients taking lithium, providing positive data for its use as a mood stabiliser. A further important finding from this study is that relapse can still occur with discontinuation of lithium even after years of treatment (Baastrup *et al.*, 1970).

—	Manic-depressive disorder		Recurrent depressive disorder		All patients	
	Placebo	Lith-ium	Placebo	Lith-ium	Placebo	Lith-ium
No. of patients completing the trial ..	22	28	17	17	39	45
No. of patients relapsing within trial period	12	0	9	0	21	0

Table 1.2 Outcome of a Completed Double-Blind Trial of Lithium Cessation. 50 Manic-depressive and 34 recurrent depressive patients were assigned to a placebo or lithium group after a year of lithium treatment. 12 manic-depressive participants receiving placebo relapsed and nine recurrent depressive patients receiving placebo relapsed during the trial. None of the patients receiving lithium relapsed. Adapted from (Baastrup et al., 1970).

With other trials showing similar positive results, lithium began to be prescribed as a mood stabiliser around the world. The United States was the last country to widely adopt it and the first to stop its widespread use, preferring valproate (an anticonvulsant used as a mood stabiliser) and antidepressants as first-line treatments (Grof and Müller-Oerlinghausen, 2009; Shorter, 2009). Much of the hesitancy surrounding lithium treatment is due to its narrow therapeutic range. The recommended serum lithium levels are 0.6-0.8 mEq/L. Concentrations of 0.8-1 mEq/L are more effective, but can be harmful if maintained long term. However, an increase of merely 50% to 1.5mEq/L is toxic; thus serum levels require close monitoring, particularly at the beginning of treatment (McKnight et al., 2012; Goodwin *et al.*, 2016). Lithium pharmacotherapy also comes with a myriad of side effects that contribute to patient noncompliance and/or discontinuation of treatment. These side effects include nephrogenic diabetes insipidus caused by reduced urinary concentrating ability, hyperparathyroidism, hypothyroidism, and

weight gain. Lithium is also widely linked to renal failure and congenital defects, though evidence for these is mixed (McKnight *et al.*, 2012). Yet the fact remains that lithium is still recommended by many as the first-line treatment in long-term therapy for BD patients, suggesting that its benefits outweigh the side effects (Goodwin *et al.*, 2016).

1.6 The Proposed Pharmacological Targets of Lithium

Though the therapeutic mechanism(s) of action for lithium remain unknown, several hypotheses have gained traction over the years. These include the inositol depletion hypothesis, the hypothesis that lithium exerts its mood stabilising effects by inhibiting glycogen synthase kinase-3 (GSK-3), the hypothesis that lithium competes with magnesium to inhibit key enzymes, and the hypothesis that lithium alters mood by altering patient circadian rhythms – all of which are discussed below.

1.6.1 The Inositol Depletion Hypothesis

Phosphoinositides are phospholipids localised in cell membranes that facilitate agonist-dependent signal transduction through the generation of second messengers involved in calcium and protein kinase C (PKC)-mediated signalling. Eukaryotic cells receive their phosphoinositide supplies though three routes: intake from the environment (i.e. diet), *de novo* synthesis, and inositol recycling (Fig 1.2) (Berridge, 1987; Berridge, Downes and Hanley, 1989).

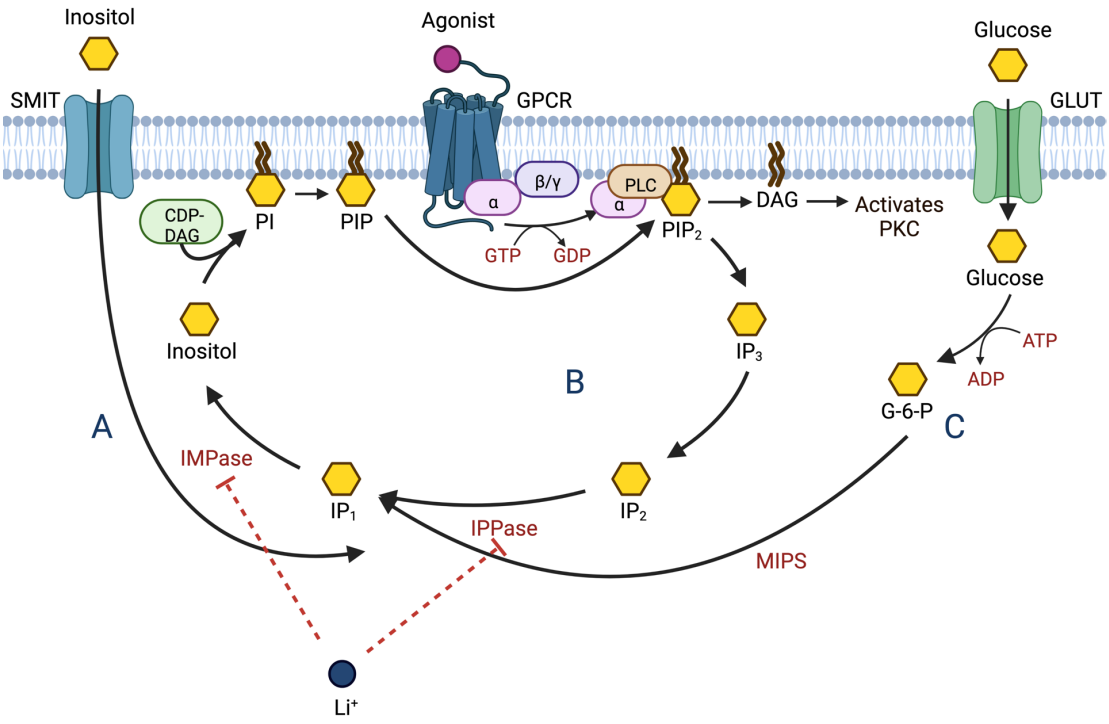

Figure 1.2 Inositol Generation. A) Inositol from the extracellular environment is imported into the cell via SMIT. B) This inositol combines with CDP-DAG to form PI, which undergoes subsequent phosphorylations into PIP_2. Upon agonist stimulation of PLC-dependent signalling, PLC hydrolyses PIP_2 into second messengers DAG and IP_3. IP_3 then undergoes successive dephosphorylations, the final of which is driven by IMPase to regenerate inositol. Lithium inhibits both IMPase and IPPase (the enzyme responsible for the dephosphorylation of IP_2), thereby reducing inositol supply. C) Inositol can be generated *de novo* from glucose. Glucose is transported into the cell and phosphorylated to G-6-P. G-6-P undergoes an isomerisation catalysed by MIPS into IP_1, which can then enter the inositol cycle to generate inositol.

Extracellular uptake of inositol is not a sufficient source for the central nervous system (CNS) as the blood-brain barrier has a low permeability to inositol and only 2% of cerebral inositol stores are acquired this way (Barkai, 1981; Berridge, Downes and Hanley, 1989). *De novo* synthesis of inositol starts with glucose. Glucose-6-phosphate (G-6-P) is converted into inositol monophosphate (IP_1) by the isomerase L-*myo*-inositol-phosphate synthase (MIPS). IP_1 is then dephosphorylated by inositol monophosphatase (IMPase) to *myo*-inositol. The majority of the brain's supply of inositol, however, comes from inositol recycling (Wong *et al.*, 1987; Berridge, Downes and Hanley, 1989).

As the name suggests, inositol recycling requires inositol as a substrate. Cytidine diphosphate diacylglycerol (CDP-DAG) combines with *myo*-inositol to form the membrane lipid phosphatidylinositol (PI). PI is then phosphorylated into seven forms, the most important of which for inositol recycling is phosphatidyl(4,5) bisphosphate (PIP_2) (Harwood, 2005; Yu and Greenberg, 2016; Saiardi and Mudge, 2018). When an agonist of inositol signalling binds its receptor, it mobilises a G-protein to bind guanosine triphosphate (GTP) and activate phosphoinositide phospholipase C (PLC). PLC then hydrolyses PIP_2 to produce the second messengers diacylglycerol (DAG) and inositol (1,4,5) trisphosphate (IP_3) that activate subsequent signalling cascades involving the release of intracellular calcium and the activation of protein kinase c (PKC). *Myo*-inositol is then regenerated through successive dephosphorylations – the second to last of which is catalysed by inositol polyphosphate phosphatase (IPPase) to produce IP_1 and the final of which is catalysed by IMPase to produce *myo*-inositol (Berridge, 1987; Harwood, 2005; Yu and Greenberg, 2016).

The inositol depletion hypothesis was suggested based on early findings that intraperitoneal injection of lithium in rats reduced levels of free *myo*-inositol in the cerebral cortex by 30% (Allison and Stewart, 1971; Berridge, Downes and Hanley, 1982). Later studies found that chronic and acute administration of lithium resulted in the accumulation of IP_1 in the cerebral cortex of rats. D- IP_1 (the form of inositol monophosphate produced by PLC-dependent signalling as opposed to the L-isomer produced in *de novo* inositol production) in particular was increased, suggesting that lithium was acting on the inositol recycling pathway (Allison *et al.*, 1976; Hallcher and Sherman, 1980; Sherman *et al.*, 1981). Further investigations into the

kinetics of lithium's interactions with IMPase revealed that lithium inhibits the enzyme in an uncompetitive fashion with a Ki of 0.8mM, thus reducing the production of *myo*-inositol at therapeutic levels (Hallcher and Sherman, 1980; Sherman *et al.*, 1981). IPPase is uncompetitively inhibited by lithium as well (Inhorn and Majerus, 1987). It was further found that lithium's inhibition of inositol recycling was signalling dependent – lithium-driven accumulation of IP_1 was much faster in insect salivary glands stimulated by serotonin and much slower in those that were not (Berridge, Downes and Hanley, 1982). Thus, lithium could impact muscarinic, histaminergic, adrenergic and vasopressin signalling by interfering with IMPase in an activity-dependent manner, explaining how it could reduce aberrant signalling in manic-depressive patients. Its alteration of signalling pathways would also be larger in the CNS as the CNS is more dependent on inositol recycling than peripheral tissues due to the blood-brain barrier (Berridge, Downes and Hanley, 1982). Interestingly, sodium valproate (another mood stabiliser prescribed in the treatment of bipolar disorder) has also been found to increase IP_1 levels and reduce *myo*-inositol in whole rat brains, suggesting a common therapeutic target (O'Donnell *et al.*, 2000).

Evidence from bipolar patients seems to support the involvement of phosphoinositide signalling in the pathogenesis of bipolar disorder. For many of these studies, platelets were deemed a suitable model because their phosphoinositide cycle is activated by analogous receptors and they are easily accessible from BD patients themselves (Brown, Mallinger and Renbaum, 1993). A small study of seven manic BD patients found elevated levels of PIP_2 compared to controls, which could increase the substrate for PLC-dependent signalling and

therefore increase signalling in monoaminergic neurons, leading to increased cholinergic activity. Cholinergic activity has been implicated in mood (Brown, Mallinger and Renbaum, 1993). A follow-up study on a single patient found increased platelet levels of PIP_2 in the manic versus euthymic stage (Soares, Dippold and Mallinger, 1997). With lithium treatment, these platelet levels of PIP_2 returned to euthymic levels; though an earlier study found that lithium did not affect PIP_2 levels in rat brains (Honchar, Ackermann and Sherman, 1989; Soares, Dippold and Mallinger, 1997). Another study found increased levels of membrane-bound PKC and increased serotonin-driven PKC activity in manic BD patient platelets. This activity was reduced in response to lithium treatment (Friedman *et al.*, 1993).

Magnetic resonance imaging has allowed real-time measurement of *myo*-inositol levels in bipolar patients' brains. It has been found that *myo*-inositol levels increased in the manic phase compared to controls, and *myo*-inositol decreased significantly more with lithium treatment in child lithium responders compared to non-responders, though results are varied across studies (Davanzo *et al.*, 2001, 2003; Yildiz *et al.*, 2001; Silverstone, McGrath and Kim, 2005).

Various studies have tried to link alterations in phosphoinositide signalling with behavioural changes yielding mixed results. Researchers using homozygous IMPase knockout mice and homozygous sodium–myo-inositol transporter 1 (SMIT1 – the transporter responsible for importing inositol into the cell) knockout mice found that these mice mimicked lithium-induced behavioural changes by decreasing immobility in the FST and increasing sensitivity to pilocarpine-induced seizures (Agam *et al.*, 2009). Another study using heterozygous SMIT1 knockout mice saw a

decrease in inositol levels (33-37% as opposed to 55-60% in the homozygous mice) but no decrease in immobility in the FST (Shaldubina *et al.*, 2006; Agam *et al.*, 2009). Hyponatraemia also reduces brain inositol without inducing lithium's behavioural effects, suggesting that the true mechanism underlying the behavioural changes are more complicated than simple inositol decrease. Therefore, one alternative that has been suggested is that there are different cellular pools of inositol which serve different functions and thus the mechanism of reduction is important (Bersudsky *et al.*, 1994).

There have been several criticisms to the inositol depletion hypothesis. One is in response to the fact that reductions in PI turnover and therefore steady-state levels are only seen after hyper-prolonged stimulation with an agonist and this stimulation is unlikely to occur naturally (Saiardi and Mudge, 2018). Others argue that though lithium reduces *myo*-inositol levels, it has no effect on PI or PIP_2 levels which are more relevant in signalling cascades. It is also unclear how inositol depletion would treat both mania and depression (Honchar, Ackermann and Sherman, 1989; Yu and Greenberg, 2016). Recently a group of researchers tried to address these discrepancies by showing that the therapeutically relevant action of lithium is not to reduce recycled inositol, but to induce the accumulation of IP_1 and reduce the rate of synthesis of PIs and PIP_2 turnover. This can be done without affecting steady-state levels of PIs if multiple pools with different rates of synthesis exist or if the rate of synthesis and hydrolysis of PIP_2 are the same (Saiardi and Mudge, 2018).

1.6.2 Glycogen Synthase Kinase Inhibition

GSK-3 is a serine/threonine kinase that has over 100 known cellular targets involved in cell cycle regulation, cell proliferation, glycogen metabolism and neuronal connectivity (Beurel, Grieco and Jope, 2015; Yu and Greenberg, 2016). There are two isoforms, α and β, that have 98% structural similarity in the kinase domain and are 51 and 47kDa, respectively (Doble and Woodgett, 2003). It is somewhat unique among the kinases in that it is constitutively active and other signalling cascades usually act to inhibit it (Beurel, Grieco and Jope, 2015). It is a negative regulator of the insulin and wingless/int-1 (Wnt) pathways. (Doble and Woodgett, 2003; Beurel, Grieco and Jope, 2015).

Wnt glycoproteins are key regulators of embryonic development, cell differentiation and cell fate. The canonical Wnt signalling pathway activates the cytosolic protein β-catenin, enabling it in turn to activate T-cell factor (TCF)/lymphoid enhancer factor (LEF)-driven expression (Fig. 1.3B) (Doble and Woodgett, 2003). The Wnt signalling cascade is activated when a Wnt ligand binds a Frizzled (Fzd) receptor and lipoprotein receptor-related protein coreceptor 5 or 6 (LRP). The scaffolding protein dishevelled (Dvl) is recruited to the complex and phosphorylates the LRP, drawing the Axin complex to the receptor. This frees β-catenin to accumulate and initiate gene transcription (MacDonald, Tamai and He, 2009).

The insulin signalling pathway is activated in response to elevated nutrients such as glucose, amino acids, and fatty acids. The hormone acts on skeletal muscle, adipose tissue, and the liver to regulate energy balance in these metabolically active

tissues. The release of insulin from β-pancreatic islet cells initiates nutrient storage pathways by binding to a receptor tyrosine kinase that autophosphorylates to recruit the binding of phosphoinositide 3-kinase (PI3K). P13K is then in the vicinity of its substrate PIP_2 (an intermediate of the inositol cycle), which it phosphorylates into phosphatidylinositol 3,4,5-trisphosphate (PIP_3). Protein kinase B (PKB, also known as AKT) binds to PIP_3, bringing it into the vicinity of kinase 3-phosphoinositide-dependent protein kinase-1 and 2 (PDK1 and 2), which phosphorylate AKT. Once active, AKT phosphorylates a number of substrates in the nucleus and cytosol involved in the storage of nutrients (Lizcano and Alessi, 2002).

Phosphorylation by AKT inactivates GSK-3, alleviating GSK-3's inhibition of glycogen synthase (GS) – the rate-limiting enzyme in glycogen synthesis (the storage form of glucose) (Lizcano and Alessi, 2002). GSK-3 also inhibits eukaryotic translation initiation factor 2B (eIF2B) by phosphorylating serine-540 (Welsh *et al.*, 1998). eIF2B is involved in the initiation of protein translation, so insulin-driven AKT phosphorylation of GSK-3 also drives the storage of amino acids in protein synthesis (Welsh *et al.*, 1998; Lizcano and Alessi, 2002). Insulin-stimulated storage of fatty acids in the form of lipids is regulated in a GSK-3-independent pathway involving peroxisome proliferator activated receptor gamma (PPARγ) and sterol regulatory element binding protein-1 (Kersten, 2001). GSK-3's inhibition of the Wnt and insulin pathways are kept separate in that the insulin signalling pathway is sensitive to the phosphorylation status of GSK-3, while Wnt signalling is not (Beurel, Grieco and Jope, 2015).

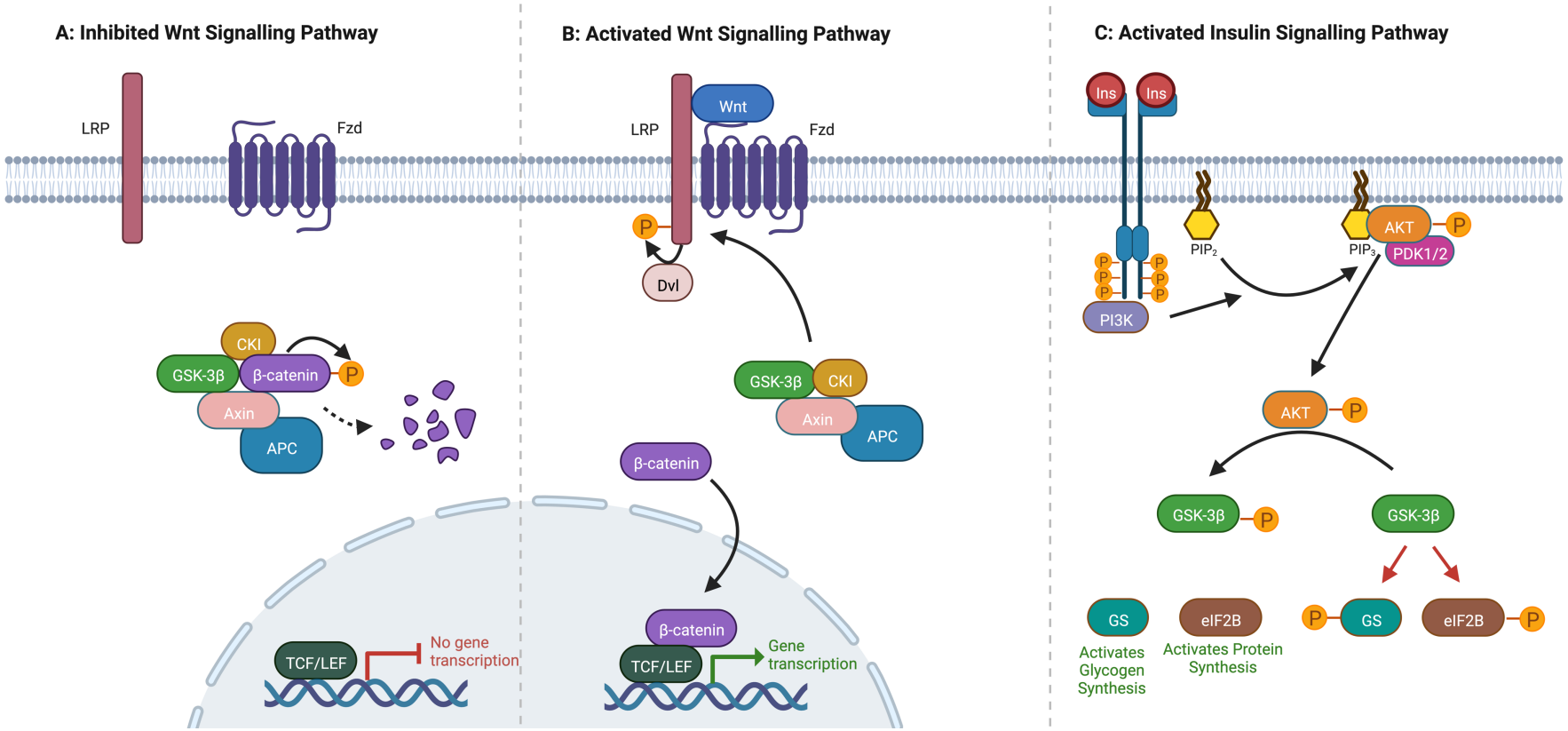

Figure 1.3 GSK-3-Mediated Signalling. A) Under basal circumstances, the Axin complex (consisting of Axin, adenomatous polyposis coli (APC), GSK-3β, and CK1) inhibits β-catenin with phosphorylation, targeting it for ubiquitin-mediated degradation. B) When a Wnt ligand binds a Fzd receptor and LRP, Dvl phosphorylates the LRP, attracting the Axin complex. β-catenin is then free to translocate to the nucleus where it activates TCF/LEF-driven transcription. C) Insulin signalling can also inhibit GSK-3β activity. Insulin binds a receptor tyrosine kinase, which autophosphorylates and binds PI3K. PI3K then phosphorylates PIP_2 into PIP_3, and AKT binds to PIP_3, leading to the phosphorylation of AKT by PDK1/2. Activated AKT then performs an inhibitory phosphorylation on GSK-3β, activating glycogen and protein synthesis. Adapted from "Wnt Signaling Pathway Activation and Inhibition", by BioRender.com (2022).

It has been shown that lithium inhibits GSK-3β *in vitro* with an IC_{50} of 2mM (Klein and Melton, 1996). It was later shown that lithium inhibits the enzyme through directly competing with magnesium (Mg^{2+}); thus the concentration of Mg^{2+} determines the potency of lithium's inhibition of GSK-3α and GSK-3β *in vivo* (Ryves and Harwood, 2001). Others have suggested that lithium activates phosphorylation of GSK-3β by activating cascades involving PKC, PI3K/AKT and protein kinase A (PKA) (Wu and Pan, 2010). Lithium increases PI3K-induced phosphorylation of AKT, thereby activating AKT and increasing GSK-3β phosphorylation, though these effects are cell type dependent (Chalecka-Franaszek and Chuang, 1999; Zhan *et al.*, 2003; Beaulieu *et al.*, 2004). It has also been suggested that lithium's activation of PI3K may increase the phosphorylation of GSK-3 through a PKC-dependent mechanism, as PKC inhibitors reduce GSK-3 phosphorylation (Kirshenboim *et al.*, 2004). PKA is another protein implicated in lithium-driven phosphorylation of GSK-3, as inhibition of the cAMP/PKA pathway blocks lithium-induced accumulation of phosphorylated GSK-3 (Liang, Wendland and Chuang, 2008).

GSK-3 has been linked to the neuroprotective effects attributed to lithium. Nerve growth factor (NGF) has been found to activate the PI3K/AKT signalling pathway, thereby inhibiting GSK-3 activity by 30-40% and increasing cell survival of rat pheochromocytoma cells (Pap and Cooper, 1998). Overexpression of GSK-3 reduces cell survival, inducing caspase-regulated apoptosis and/or apoptosis induced by heat shock and staurosporine (Bijur, Sarno and Jope, 2000). The neuroprotective effects of lithium tie into the pathophysiology of bipolar disorder, as studies of post-mortem brains of BD patients have found increased markers of

apoptosis (such as caspase 3 and 9), decreased anti-apoptotic markers (such as B-cell lymphoma-2 and BDNF), and reduced neuronal and glial density (Rajkowska, 2002; Kim, Rapoport and Rao, 2010).

Inhibition of GSK-3 has been found to have behavioural consequences. Inhibition of GSK-3 in mice with increased dopamine levels (through dopamine transporter knockout or treatment with amphetamine) reduces dopamine-driven locomotor activity (Beaulieu *et al.*, 2004). Furthermore, GSK-3β heterozygous knockout mice mimicked lithium-decreased immobility in the FST and reduced exploratory behaviour in the hole board exploratory test without impacting basal behaviour (O'Brien *et al.*, 2004). Overexpression of GSK-3β rescued lithium-induced behaviours in the heterozygous knockouts and lithium-treated controls (O'Brien *et al.*, 2011). Treatment with GSK-3 inhibitors and overexpression of β-catenin also reduced depressive and manic behaviours in mice, supporting the argument that GSK-3 plays a role in mood behaviours (Gould *et al.*, 2007; O'Brien *et al.*, 2011; Yu and Greenberg, 2016).

Due to its hypothesised role in BD and other psychiatric disorders, many GSK-3 inhibitors with different structures and mechanisms of action have been and are currently in clinical development, though few actually reach the clinical stage (Eldar-Finkelman and Martinez, 2011). These molecules have been plagued with difficulties – not the least of which has been toxicity. GSK-3 plays an essential cellular role and its widespread inhibition has many consequences, making it important to only target specific brain regions of interest (Eldar-Finkelman and Martinez, 2011; Khan *et al.*, 2017). GSK-3 inhibition could lead to continual

activation of β-catenin, increasing cancer risk. Given lithium has been used for the treatment of BD for over half a century and has not been associated with tumorigenesis, these concerns call into question whether GSK-3 is the main therapeutic target of lithium (Eldar-Finkelman and Martinez, 2011; Khan *et al.*, 2017).

It has been suggested that GSK-3 inhibition and inositol depletion are part of the same mechanism of action for lithium (Yu and Greenberg, 2016). MIPS contains a putative phosphorylation site for GSK-3, giving GSK-3 a point of regulation in inositol generation. Indeed, yeast cells lacking GSK-3 have 70% lower intracellular inositol levels than wildtypes (Azab *et al.*, 2007). This regulation appears to go both ways, as knockdown of the MIPS gene increases inhibitory phosphorylation of GSK-3 in SK-N-SH neuroblastoma cells (Ye and Greenberg, 2015). GSK-3 could also play a role in *de novo* inositol synthesis as it regulates the conversion of G-6-P to glycogen, though the directionality of this regulation is unclear (Yu and Greenberg, 2016).

1.6.3 Lithium Inhibits its Cellular Targets by Competing with Magnesium

It has been suggested that lithium's multitude of effects across diverse signalling pathways could be explained by the elegantly simple mechanism of competition with magnesium (Mg^{2+}) (Birch, 1974). Lithium (Li^{+}) and Mg^{2+} share very similar ionic radii (0.59Å and 0.57Å, respectively) when making four bonds and when making 6 bonds (0.76Å and 0.72Å, respectively) (Dudev and Lim, 2011). In fact, lithium inhibits two of its main targets of interest (GSK-3 and IMPase) along

with IPPase and adenylate cyclase by competing with Mg^{2+} (Mørk and Geisler, 1987; Leech *et al.*, 1993; Ryves and Harwood, 2001). Further studies have found that lithium also inhibits G proteins at therapeutic levels by competing with Mg^{2+} for binding sites on G proteins essential for guanine nucleotide exchange (Avissar, Murphy and Schreiber, 1991). It is specifically found to compete with the low affinity Mg^{2+} binding sites regulating Gα activation, as the high-affinity Mg^{2+} sites are highly homologous among G-proteins and lithium competition there would have disastrous effects (Srinivasan *et al.*, 2004).

The mechanism for this competition is nuanced because lithium cannot compete for vital proteins that are reliant on Mg^{2+}, otherwise lithium pharmacotherapy would be a death sentence. It has been modelled that Mg^{2+} binding sites that are lined by negative carboxylates, such as aspartic acid and glutamic acid, select for Mg^{2+} over Li^{+} because Mg^{2+} is dicationic and therefore interacts more strongly with the negative amino acids than the monocationic Li^{+}. In addition, Mg^{2+}-binding complexes with a net charge of two or less are accessible to solvent, and therefore Mg^{2+} diffusing into the solution outweighs the unfavorability of monocationic Li^{+} binding in the pocket and Li^{+} displaces Mg^{2+}. This is a calculated model, however, and the full mechanism of Li^{+}/Mg^{2+} competition has not been worked out (Dudev and Lim, 2011).

1.6.4 Lithium Alters Circadian Rhythms

Lithium has been found to normalise patient sleep and circadian rhythms and it has been hypothesised that this normalisation could underlie its mood

stabilising properties (Sanghani *et al.*, 2021). In order for lithium to exert its circadian effects, administration must be chronic rather than acute (Klemfuss, 1992). One study placed 10 students in the constant light conditions of an Artic summer and monitored their free-running circadian body temperature rhythms. Lithium carbonate induced a period lengthening which disappeared with discontinuation of treatment (Johnsson *et al.*, 1979). At the molecular level, lithium has been shown to increase period and amplitude in the SCN and peripheral clock in lung tissues and fibroblast cells (Li *et al.*, 2012). Lengthening of mouse running activity has also been observed *in vivo* (Li *et al.*, 2012). Though most studies report period lengthening with lithium treatment, some report no difference or even period shortening (Klemfuss, 1992; Li *et al.*, 2012; McCarthy *et al.*, 2019; Sanghani *et al.*, 2021).

About 40% of patients under lithium pharmacotherapy will not respond to treatment. This has prompted some to suggest that circadian biomarkers could be responsible for lithium response (Geoffroy *et al.*, 2014). Using patient fibroblasts, previous work from our lab found that though the average period of BD patients is not different from controls, BD fibroblasts have a much larger distribution of periods (Sanghani *et al.*, 2021). Those with longer periods were found to be taking more medications in addition to lithium and to have a reduced response to lithium itself. Thus the underlying circadian phenotype could predict lithium response (Sanghani *et al.*, 2021).

Studies examining the mechanisms underlying lithium's circadian effects have focused on GSK-3β with mixed results. Some researchers have proposed that

GSK-3β's action is post-translational; it stabilises REV-ERBα through phosphorylation, keeping Bmal1 repressed (Yin et al., 2006). Another study found that GSK-3β inhibition with (2′Z,3′E)-6-Bromoindirubin-3′-oxime leads to period shortening, but increases amplitude of PER2 expression in a manner similar to lithium. The researchers suggested that GSK-3β's role in amplitude enhancement was transcriptional, as they found an increase in PER2 mRNA but not PER2 protein stability. Furthermore, they found no alteration of BMAL expression (Li *et al.*, 2012). The mechanisms underlying lithium's period lengthening are still unknown, however, they are believed to separate from those underlying lithium's amplitude enhancement (Noguchi *et al.*, 2016; Sanghani *et al.*, 2021).

1.8 Aims

Lithium is the gold standard treatment of bipolar disorder, yet its mechanisms of therapeutic action and off-target effects remain largely unknown. This thesis aims to further elucidate lithium's alteration of signalling cascades to better understand its role as a mood stabiliser and the mechanisms underlying its side effects. Lithium's signalling alterations will be addressed in three ways:

1. An *in vitro* examination of lithium's kinase interactions and evaluation of the therapeutic relevance of these interactions.

One of lithium's primary targets of interest is a kinase, which it inhibits by competing with magnesium. We therefore postulated that lithium could inhibit a wider array of kinases than has previously been shown via the same mechanism. Furthermore, recent evidence suggests that kinase activity co-relates with sleep

need and sleep deprivation. Thus the first data chapter of this thesis will examine whether lithium is capable of widespread kinase inhibition and the circadian consequences of this inhibition.

2. An investigation into lithium's alteration of the circadian rhythm *in vivo* and possible sex-based differences in this chronomodulation.

Our lab has previously shown that lithium response is dependent on circadian phenotype. Using mouse models with different period lengths, the second data chapter will examine whether mice with different basal periods are behaviourally distinct and whether their basal period is associated with lithium's characterised behavioural alterations. A further investigation into sex-based differences in lithium response is included.

3. An investigation of the mechanisms underlying weight gain in certain patient populations taking lithium.

Lithium is documented to induce weight gain in certain patient populations and this weight gain is a major contributor to patient noncompliance to lithium treatment. Therefore, the final data chapter will examine the molecular mechanisms that underlie patients gain weight during lithium pharmacotherapy using *in vitro* and *in vivo* models.

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2. MATERIALS AND METHODS

2.1 General Methods

2.1.1 Cell Culture Techniques

All cell and tissue culture procedures were carried out under sterile conditions with the use of a containment level II biological safety cabinet (Gelaire). Cells were maintained in 75cm^2 or 25cm^2 cell culture flasks (Greiner) in an incubator (Panasonic) set to 37˚C and 5% CO_2 with high humidity. All experiments were performed in 96-well or 384-well clear bottom plates (Greiner) unless specified otherwise.

2.1.1.1 Cell Line Maintenance

Three immortalised cell lines were used in this thesis: human U2OS osteosarcoma cells (obtained from the ATCC), murine MMHD3 prehepatocytes (a gift from Marco Tripodi, Sapienza University of Rome, Italy), and murine 3T3-L1 preadipocytes (obtained from ZenBio). Modified MMHD3 and U2OS cells with stable expression of *Bmal*::LUC or *Per2*::LUC, respectively, were used for circadian assays. These cells were stably transfected with luciferase under the control of *Bmal1* or *Per2* promoters, respectively, enabling the detection of promotor activity through the production of photons. The *Bmal*::LUC MMHD3 cells were a gift from John Hogenesch (University of Pennsylvania, USA) and the *Per2*::LUC U2OS cells were a gift from Patrick Nolan (MRC Harwell, UK).

U2OS cells were maintained in Dulbecco's Modified Eagle's Medium with high glucose GlutaMAX™ supplement (ThermoFisher Scientific), 10% fetal bovine serum (FBS, Sigma), and 1% penicillin-streptomycin (P/S, Sigma) – hereafter

referred to as DMEM. MMHD3 cells were maintained in Roswell Park Memorial Institute media (ThermoFisher Scientific), supplemented with 10% FBS, 1% P/S, 1x GlutaMAX™ (ThermoFisher Scientific), 55ng/mL recombinant human epidermal growth factor (R&D systems), 16ng/mL insulin-like growth factor (Cambridge Bioscience), and 10µg/mL human insulin (Roche Diagnostics) – hereafter called RPMI media. MMHD3 cells were grown on flasks or plates pre-coated with rat tail collagen type 1 (Corning) diluted to 50µg/mL in 0.2M acetic acid (Sigma) for a minimum of 5h. 3T3-L1 preadipocytes were maintained in DMEM with 10% New born calf serum (NBCS, Sigma) in the place of FBS – hereafter called pre-differentiation media. For experiments involving differentiation of preadipocytes, cells were maintained on cultureware coated with rat tail collagen type 1 diluted to 50µg/mL in 30% ethanol (Sigma).

All cell lines were maintained at passage 4-30. Once 80-90% confluent, they were washed with Dulbecco's phosphate-buffered saline (PBS) without magnesium or calcium (ThermoFisher Scientific) and passaged using TrypLE™ solution (ThermoFisher Scientific). Cell counting was performed using the Countess Automated Cell Counter (Invitrogen) before cells were either passaged or used for experimentation.

2.1.2 Luminescence Detection

Recording media used for experiments measuring luminescence consisted of low glucose DMEM (D2902 Powder, Sigma), supplemented with 9.5mL/L 45% D-

glucose solution (Sigma), 10mM HEPES solution (Sigma), 3.7g/L NaHCO3 solution (Sigma), 0.5% P/S, and 1x GlutaMAX all adjusted to pH 7.3.

All assays measuring luminescence were conducted using plate readers. The plate readers used included FLUOstar Omega (BMG Labtech), Tecan Infinite M200 Pro, and Tecan Infinite F200 (Tecan). For circadian experiments photon emissions were counted from each well of sample plates every 30 minutes to one hour. For transfection experiments, photon emissions were counted every 8 minutes.

2.1.3 Lithium Dosing Regime *In Vitro, Ex Vivo,* and *In Vivo*

The recommended chronic therapeutic serum concentration of lithium in human patients generally ranges from 0.6 to 1mM (McKnight *et al.*, 2012; Goodwin *et al.*, 2016). Concentrations of 1mM lithium are enough to lengthen circadian period in the isolated suprachiasmatic nucleus (SCN) of mice bearing the Per2 luciferase circadian reporter (Noguchi *et al.*, 2016). In order to see the same period change in peripheral tissue slices, a higher concentration of 5-10mM is needed and in order to produce a similar period change in cell culture, approximately 20mM is needed (Li *et al.*, 2012; Sanghani, 2018). We speculate that this steady increase in the concentration required to produce phenotypic changes is likely due to the basal activity of the various systems (Sanghani, 2018). Indeed it has been shown that models which predict drug response based on the signalling profiles of the cells of interest are more accurate for drugs with intracellular targets (Niepel *et al.*, 2013). Thus any inhibition or activation driven by lithium would have a more immediate effect at a lower concentration in signalling systems that are more active, such as

the brain, whereas peripheral tissues would require a higher dose and cells grown in cell culture would require an even higher one. We therefore used a lower concentration of 4mM LiCl in SCN brain slices, 10mM LiCl in liver slices, and concentrations of up to 30mM LiCl in cell culture.

In vivo, all lithium administration was performed via drinking water. Given that the experimental protocols required chronic lithium administration and the half-life of lithium in mice is 3.5 hours, this method of administration avoided the chronic stress of multiple daily injections or gavages (Wood *et al.*, 1986). A half dose of lithium was given for one week to gradually build lithium levels, as is done in patients (Gould, Chen and Manji, 2004). 14mM LiCl in drinking water was chosen as the full dose for the majority of experiments based on previous research and pilot studies in our own lab which found that this concentration resulted in a serum lithium concentration of approximately 0.85mM (in the therapeutic range for patients) in C57BL/6J mice (McKnight *et al.*, 2012; Enkhuizen *et al.*, 2015; Goodwin *et al.*, 2016). This dose was reduced to 10mM LiCl in drinking water for mice in Chapter 4, matching the dose of Li *et al.* who demonstrated that 10mM LiCl lengthens circadian period of wheel-running activity (Li *et al.*, 2012).

2.1.4 Animal Maintenance

All mice were reared under controlled conditions of 20-22˚C, 40% humidity, and 12:12h light–dark (LD) cycles with standard food and water available *ad libitum*. For experiments involving administration of lithium or sodium, each was given in drinking water. For experiments involving models of obesity, a diet-induced

(DIO) model was chosen to avoid the use of genetic knockout mice. Adiposity in mice increases with fat content in the diet (not protein or carbohydrate) and maximal adiposity is achieved at 60% dietary fat (Hu *et al.*, 2018). Thus a high-fat diet (HFD – Research diets, D12492: 60% Kcal Fat) was provided *ad libitum*. All animal experiments were performed in agreement with the United Kingdom Animals (Scientific Procedures) Act of 1986.

2.1.5 General Data Analysis and Representation

Unless stated otherwise, statistical analysis was performed using Prism 9 (GraphPad). All results were represented as mean ± standard error of the mean (SEM). Calculations of the correlation between two variables were performed using the Pearson correlation coefficient. When comparing one parameter variable between two samples, an unpaired two-tailed t-test was used unless stated otherwise. When comparing one parameter across multiple samples, a one-way analysis of variance (ANOVA) with a Dunnett's post hoc test was performed. A two-way ANOVA with a Šídák's post hoc multiple comparisons test was performed when comparing two parameters across multiple samples. Significance was set at $P<0.05$. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

All figures were created using BioRender.com or Python 3.8, unless stated otherwise

2.1.6 Pathway Enrichment Analysis

Pathway enrichment analysis was performed using the online tool G:Profiler (Raudvere *et al.*, 2019). G:Profiler uses the cumulative hypergeometric test and corrects for multiple testing using the g:SCS (Set Counts and Sizes) correction method created by developers to determine KEGG pathways in which a set of given genes is significantly over-represented (Reimand *et al.*, 2007).

2.2 Methods Used in Chapter 3

2.2.1 Chemical Reagents of Interest

Compounds: All compounds were from Sigma, unless otherwise specified. 186 of the 367 kinase modulators of the PKIS library were obtained from the Structural Genomics Consortia, Oxford (Drewry, Willson and Zuercher, 2014). SMARTpool siRNA was obstained from Dharmacon. These included: non-targeting control, *Map2k1*, *Pak2*, *Map3k11*, *Tesk1*, *Src*, *Ephb3*, *Map2k2*, *Mapk7*, *Mapk12*, *Akt1*, *Stk33*, *Rps6ka5*, *Mknk2*, *Camkk2*, *Mylk*, *Phkg1*, *Gsk3b*, *Cdk9*, *Tlk1*, *Prkaa1*, *Mark2*, *Brsk1*, *Csnk1g2*, *Csnk1d*, *Ikbke*, *Tbk1*, *Pim3*, *Hipk1*, *Pak6*, *Stk3*, *Stk26*, *Ripk2*, *Oxsr1*, *Wnk1*, *Tgfbr1*, *Jak3*, *Insr*, *Hipk3*, *Plk1*, *Aurkb*, *Yes1*, *Fgfr1*, *Eef2k*, *Hipk2*, *Chek2*, *Aurkc*, *Mknk1*, *Mapk13*, *Csnk2a1*, *Lck*, *Nek6*, *Rps6ka1*, *Mapkapk2*, *Rps6ka3*, *Nek7*, *Prkcz*, *Mapk1*, *Brsk2*, *Mapk15*, *Chek1*, *Camk1*, *Prkaca*, *Ikbkb*, *Csk*, *Mapkapk3*, *Mark3*, *Epha2*, *Prkca*, *Pak7*, *Pdpk1*, *Mapkapk5*, *Pim1*, *Mapk10*, *Nek2*, *Cdk2*, *Insrr*.

2.2.2 Kinase Selectivity Profiling

The screen was conducted by the MRC Protein Phosphorylation and Ubiquitylation Unit International Centre for Kinase Profiling (Dundee, Scotland) as described previously (Hastie, McLauchlan and Cohen, 2006; Bain *et al.*, 2007). Briefly, each kinase was diluted such that its activity was linear with respect to time and enzyme concentration. Protein substrate, diluted kinase, and concentrated assay buffer (50mM Tris-HCl, 0.1mM EGTA and 10mM magnesium acetate with additional cofactors as needed for each kinase) were incubated for 5 minutes. The protein kinase reaction was initiated at room temperature by the addition of 5, 20 or 50μM [γ-^{33}P]ATP in order to be at or below the K*m* of ATP for each kinase. After the optimal incubation time, the 25.5μl reaction was stopped with 5μl of 0.5 M orthophosphoric acid and each reaction mixture was spotted onto a P81 filter plate (Whatmann) using a unifilter harvester (PerkinElmer). Assay plates were subsequently air dried. Scintillation fluid was added to each plate and radioactivity of the protein substrates, blank filter plates and ATP alone was measured with a TopCount NXT scintillation counter (PerkinElmer). Each plate was compared to quality controls assessing free ATP contamination and autophosphorylation of each kinase. The activity of undiluted kinase ($^{U}/_{ml}$) was calculated as follows: $\frac{(^{r-b}/_{sa})(d)(1.25)(200)}{10}$, where U = amount of kinase that catalyses the incorporation of 1 nmol phosphate into the protein substrate in one minute, r = counts per minute (c.p.m) of phosphate incorporation into substrate as counted by the TopCount NXT, b = c.p.m. of blank filter plates, sa = the specific radioactivity of the ATP ($^{c.p.m.}/_{nM}$), d = dilution factor of kinase, 1.25 is a correction for transfer of 80% of

reaction mixture to the plate, 200 is a correction for adding 5 µl diluted kinase to each assay and 10 is the incubation time in minutes. Each reaction was carried out in duplicate.

2.2.3 Measuring Circadian Rhythms

Bmal::LUC MMHD3 cells were chosen as a cellular model of circadian rhythms as they are a murine cell line which has been previously characterised to produce robust oscillations (Ramanathan *et al.*, 2014). In addition, previous work from our lab has found MMHD3 cells to be very responsive to chronomodulation by lithium (Sanghani, 2018). The *Per2*::LUC U2OS cell line was also chosen as it carries a different circadian reporter, is from a different species, and the impact of the knockdown of all core clock components on circadian period and amplitude has been evaluated in this model (Baggs *et al.*, 2009). Furthermore, it has been used in previous screens of small molecules and RNAi to find period and amplitude modulators, allowing cross comparison (Hirota *et al.*, 2008; Zhang *et al.*, 2009).

Cells were seeded and grown to 100% confluence in 96- or 384-well plates. Glucocorticoid receptor agonist dexamethasone was diluted to 200nM in recording media (section 2.1.2) and applied to the cells for an hour to drive circadian gene expression and synchronise the circadian clocks in individual cells. Cells were washed twice in recording media before recording media supplemented with B27 (ThermoFisher Scientific), 400µM D-luciferin potassium salt (Gold Biotechnology), and any pharmacological compound of interest was applied and bioluminescence was recorded for a minimum of 4 days in a plate reader. B27 was chosen over FBS

in these experiments to remove potential sources of variation between experimental runs, as there is great batch to batch variability in the serum (Price and Gregory, 1982; Gstraunthaler, 2003).

2.2.4 *In Vitro* siRNA Transfection

SMARTpool siRNA (Dharmacon) was obtained for genes of interest (section 2.2.1). SMARTpool siRNA were designed using the Dharmacon™ SMARTselection™ algorithm to silence specific target genes. Four siRNAs were designed per target and silencing of at least 75% of target gene expression was validated by the manufacturer using a validated positive control. siRNA were transfected into *Bmal*::LUC MMHD3 cells as follows. All plasmids were resuspended to 2µM according to the manufacturer's instructions in nuclease-free siRNA Buffer (Dharmacon). 0.4 µL Dharmafect 1 (Dharmacon) was diluted in Opti-MEM (ThermoFisher Scientific) to a final volume of 10µL per well. 2.5µL of 2µM siRNA was diluted in Opti-MEM to a final volume of 10µL per well and added to the transfection reagent. The 20µL of transfection complex was allowed to form for 20 minutes before 20,000 freshly passaged cells in P/S-free RPMI (section 2.1.1.1) were added to each well, bringing the final concentration of siRNA to 45.5nM. Cells were incubated with transfection complex for 24 hours at 37°C before the circadian assay was performed.

2.2.5 Cell Viability Assay

The PrestoBlue™ HS Cell Viability Reagent (ThermoFisher Scientific) was used to assess the viability of cells after an assay. PrestoBlue™ is a blue resazurin-based agent that is reduced to the red compound resorufin in living cells – providing a quantitative measurement of cell viability. PrestoBlue™ was diluted to 1X in media and incubated with cells of interest at 37°C for one hour. Fluorescence intensity was then measured in a plate reader with the excitation and emission filters set to 544nm and 590nm, respectively. Wells with no cells were used as negative controls (- Control) and cell viability was determined through comparison with a vehicle control.

2.2.6 Circadian Data Analysis

Circadian assays measuring luminescence were analysed using MultiCycle curve-fitting software (ActiMetrics). Data was analysed following baseline subtraction and smoothing six timepoints. The circadian parameters were calculated by fitting the data to a dampened sine wave using a Levenberg-Marquardt algorithm. For immortalised cell lines, the first 15 hours of data were excluded from analysis to account for disturbances due to cell manipulation, recovery from dexamethasone treatment, and temperature changes. The next 48 hours were then used to calculate period and amplitude. Alterations of period and amplitude were considered significant if the alteration was more than three standard deviations away from a vehicle control. This method of determining significance was chosen as

it is more stringent (accounting for 99.7% of the variability in the data) than a typical P value and thus assures that all of our hits are more likely to be true hits.

2.2.7 Assessing Protein-Protein Interactions

The STRING database was used to predict protein-protein interactions. STRING utilises previously published databases, automated text-mining of published articles and data from conserved co-expression studies, studies of gene evolution, and high-throughput lab experiments to establish primary and predicted interactions (Szklarczyk et al., 2019). The predicted interactions with a confidence level of 90% were selected.

2.3 Methods Used in Chapter 4

2.3.1 Chemical Reagents of Interest

Lithium chloride (LiCl - Sigma) and sodium chloride (NaCl - Sigma).

2.3.2 Animal locomotor activity rhythms

Cry1$^{(-/-)}$ (Cry1KO) and *Cry2*$^{(-/-)}$ (Cry2KO) mice were bred on a C57BL/6J background. Cryptochrome knockout mice were chosen as these models have relatively large changes in period in constant darkness (DD) compared to other circadian knockout mice and in addition have no significant alterations of other circadian parameters, such as amplitude of activity (Horst *et al.*, 1999; Ripperger, Jud and Albrecht, 2011). For the pilot experiment, four male and four female

C57BL/6J mice, five male and five female Cry1KO mice, four male Cry2KO mice and six female Cry2KO mice between the ages of 5-11 weeks old were individually housed with running wheels in 12:12h LD cycles for one week for entrainment purposes. After entrainment, mice were placed in constant conditions to remove zeitgebers and enable to the measurement of their endogenous circadian rhythms. Constant light conditions suppress the activity of nocturnal animals. Given that circadian parameters were being measured via wheel-running activity, DD was chosen (Carpenter and Grossberg, 1984). Animals were then placed in DD for four days, after which each animal was evaluated using a battery of behavioural tests. Following these tests, mice were left in DD for a further 10 days to enable measurement of their endogenous wheel-running circadian parameters. Their water was then replaced with 7mM LiCl and a supplementary bottle of 0.9% NaCl was also provided. After one week, the 7mM LiCl bottle was replaced with 14mM LiCl for two weeks before behavioural testing was repeated.

For the follow-up experiment, nine male and nine female C57BL/6J mice, nine male and nine female Cry1KO mice, and seven male and seven female Cry2KO mice all between the ages of 7-16 weeks old were individually housed with running wheels in 12:12h LD cycles for one week for entrainment purposes. Animals were then placed in constant DD for four days, after which each animal was behaviourally tested. After 10 further days in DD, 7mM LiCl and 0.9% NaCl were administered in drinking water for one week and then the lithium was changed to the full dose of 10mM. After two weeks of 10mM LiCl administration, behavioural testing was repeated (see Fig. 4.9 for workflow).

Wheel revolutions were collected and analysed using the ClockLab software (ActiMetrics). Baseline period of running wheel activity was calculated in the software using data from four days of running wheel activity in DD after the first round of behavioural testing. The period of wheel-running activity during lithium administration was calculated using data from four days during 10mM LiCl administration.

2.3.3 Behavioural Tests

The following tests were chosen as lithium has been validated to alter behaviour in reproducible ways in each of these tests (O'Donnell and Gould, 2007). Animals were tested once after four days of DD and once after two weeks of administration of a full dose of LiCl.

2.3.3.1 Open Field Test

The Linton AM1053 X, Y, Z IR Activity Monitor (San Diego Instruments) was used to perform the open field test (OFT). The apparatus has two parallel frames on top of each other and each frame has 8 evenly spaced infrared beams along the X axis and 16 evenly spaced infrared beams along the Y axis. Animal movement was detected by breaks in these beams and the number of beam breaks per minute was recorded for 20 minutes (Fig. 2.1A) Rearing was detected by beam breaks on the upper frame. Animals were placed in clear Perspex cages of 36 x 20 x 15cm inside the activity monitor for the duration of the test. The cages were washed with water and dried after each subject. % time spent rearing and % time spent active were

calculated using the in-built software. Emphasis was placed on % time spent rearing as this is a lithium-specific behavioural modulation which has been reproduced in previous literature (O'Donnell and Gould, 2007).

2.3.3.2 Elevated Plus Maze

Following the OFT, animals were placed in their home cages for 1-2 hours prior to commencing the elevated plus maze (EPM). The maze is elevated 47cm off the ground and consists of two opposing open arms (28 x 5 cm) and two opposing arms of the same dimensions enclosed with 30cm walls. The middle where the arms meet creates a small platform of 5 x 5 cm (Fig. 2.1B). At the start of each test the mouse was placed in the centre platform facing an open arm and its activity was video recorded for 10 minutes. The arms of the maze were cleaned with 1% ANISTEL (Tristel) and allowed to dry in between each subject. The software ANY-maze 7.10h (Stoelting Co.) was used for analysis. Analysis was automated in the software using the same parameters for each mouse to remove bias in scoring as follows. Enclosed and open regions were designated in the software and the software monitored the movement of the animals in these regions. The software was used to calculate % time spent in the open arms of the maze and the overall distance travelled.

2.3.3.3 Forced Swim Test

The forced swim test (FST) was performed one day after the OFT and the EPM. A glass cylinder 18cm in diameter and 27cm tall was filled with 3.5L water at

24 ± 1 °C, such that each animal's tail could not touch the bottom. Each mouse was placed in the water and their swimming activity was video recorded for six minutes, after which they were dried and returned to their home cage (Fig. 2.1C). The water was changed after each mouse. Video analysis was performed with ANY-maze 7.10h using the last four minutes recorded. The software was used to track movement and calculate the % time spent immobile. Analysis was automated in the software using the same parameters for each mouse to remove bias in scoring.

2.3.4 *Ex Vivo* SCN Culture

Organotypic SCN slices were harvested from homozygous male and female *mPer2*^{Luc} knockin mice (*Per2*::LUC) bred on a C57BL/6J background. Animals aged 7-9 weeks old were culled by cervical dislocation. Enucleation of the eyes was performed and the whole brain was removed from the skull. The caudal brain was glued to a stage and fixed in place with 3% low melt agar (Sigma). The stage was situated in a Compresstome VF-200 semi-automatic slicer (Precision Instruments) filled with NMDG-HEPES recovery solution (Table 2.1) and 250µm brain slices were obtained. 2-3 slices containing the SCN were collected per mouse and microdissected to isolate the bilateral SCN.

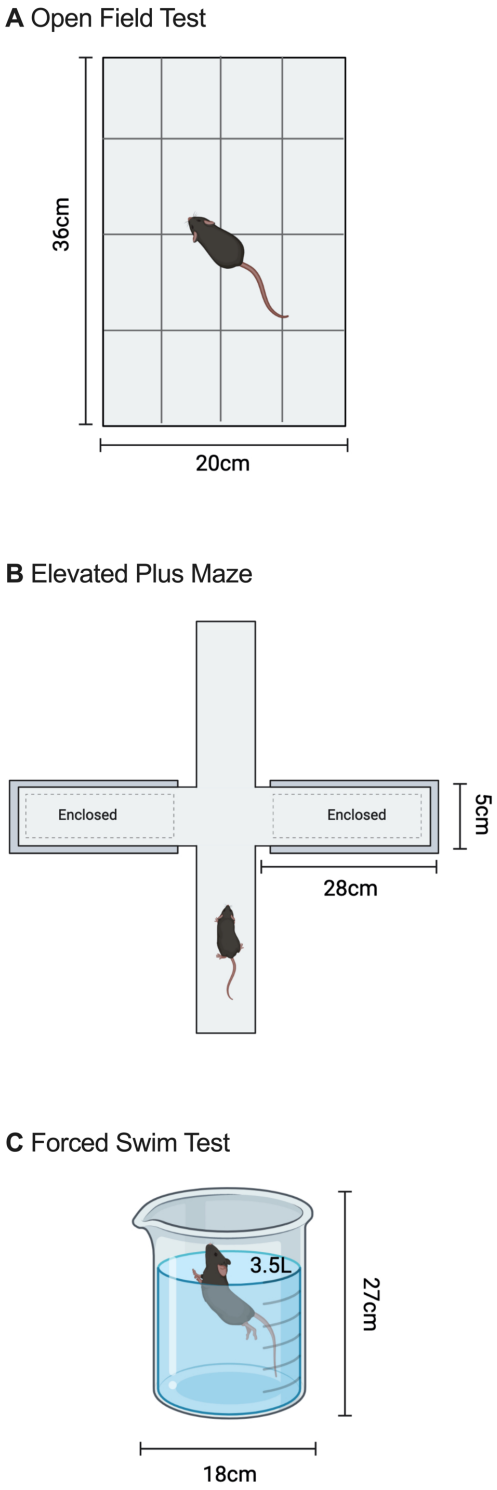

Figure 2.1 Behavioural Testing. (A) Representative diagram of the open field test setup. (B) Representative diagram of the elevated plus maze. (C) Representative diagram of the forced swim test setup.

Reagent	Supplier	Concentration (mM)
NMDG	Sigma	93
HCl	Sigma	93 (to adjust pH to 7.3)
KCl	VWR International	2.5
NaH_2PO_4	Sigma	1.2
$NaHCO_3$	Sigma	30
HEPES	VWR International	20
Glucose	Sigma	25
Sodium ascorbate	Sigma	5
Thiourea	Sigma	2
Sodium pyruvate	Sigma	3
$MgSO_4$	Sigma	10
$CaCl_2$. $2H_20$	Merk Millipore	0.5
MK-801	Abcam	0.1
GSH-EE	Sigma	3

Table 2.1 NMDG-HEPES Recovery Solution.

SCN sections were placed on 0.6cm^2 Millicell cell culture inserts (Merk Millipore) and transferred to a 24-well plate (Perkin Elmer) containing 300µL organotypic slice culture media consisting of 50% Eagle's Basal Media (Gibco) and 50% horse serum (Gibco) supplemented with 1% Glucose, 1% GlutaMAX, 1% P/S, 100nM MK-801 maleate, and 3mM glutathione reduced ethyl ester (GSH-EE). Sections were incubated at 37°C for 30 minutes before the inserts were placed in 300µL organotypic slice culture media without MK-801 and GSH-EE. Sections were incubated for one week at 37°C with 1-2 partial (1/3) media changes.

After the week incubation, the inserts were moved to 300µL recording media (section 2.1.2) supplemented with B27, 400µM luciferin, and 5% FBS and put in a

plate reader where luminescence was recorded for 2-3 days. After 2-3 days LiCl was added through a partial (1/3) media change and luminescence recording continued for 3-4 days. Period change was measured by comparing pre- and post-lithium treatment oscillations.

2.3.5 *Ex Vivo* Liver Culture

Liver sections of approximately 1000μm were taken from the same *Per2*::LUC mice whose SCN slices were analysed in section 2.3.4. These liver sections were each placed in a 0.6cm^2 Millicell cell culture insert and transferred to a 24-well plate with 300μL recording media (section 2.1.2) supplemented with B27 and 400μM luciferin. Baseline luminescence recordings were taken in a plate reader for 1-2 days before lithium was added through a 1/3 media change. Luminescence recording then resumed for a further 3-4 days. Just as for the SCN, period change was measured by comparing pre- and post-lithium treatment oscillations.

2.4 Methods Used in Chapter 5

2.4.1 Chemical Reagents of Interest

LiCl (Sigma), NaCl (Sigma), Rosiglitazone (Stratech Scientific), DMSO (Sigma), nuclease free water (Ambion), PPRE X3-TK-luc reporter plasmid (Addgene plasmid #1015 - a gift from Bruce Spiegelman (Kim *et al.*, 1998)) and SMARTpool siRNA (Dharmacon: non-targeting control and *Pparg*).

2.4.2 Transient Transfection

The U2OS cell line endogenously expresses PPARγ (the transcription factor of interest) but not C/EBPα, making it possible to examine only PPARγ-driven activity in this cell line (Sjöstedt *et al.*, 2020; The Human Protein Atlas, 2022b, 2022a). Thus they were used as the model for transfection.

2.4.2.1 Plasmid Transfection

The ViaFect transfection reagent (Promega) was used according to the manufacturer's protocol. Briefly, plasmid DNA was diluted to 1µg per 100µL of reduced serum Opti-MEM. 4µL ViaFect per 1µg of DNA was added and complex formation allowed to occur for 15 minutes. Complex containing 100ng DNA was added to each well of a 96-well plate followed by the addition of 15,000 freshly passaged U2OS cells in DMEM without P/S (section 2.1.1.1). Cells were incubated with complex at 37°C for 24 hours before the media was changed to recording media (section 2.1.2) supplemented with 400µM luciferin and the plate was placed in a plate reader. Baseline luminescence was recorded for approximately two hours before addition of lithium or vehicle. Luminescence recording continued for 32-35 hours after drug administration.

2.4.2.2 Plasmid and siRNA Co-Transfection

SMARTpool siRNA was designed as previously described (section 2.2.4). The Lipofectamine™ 3000 reagent (ThermoFisher Scientific) was used to co-transfect plasmid and siRNA into U2OS cells according to the siRNA plasmid co-transfection

protocol developed by the manufacturer for Lipofectamine™ 2000. Briefly, plasmid DNA was diluted to 0.5µg per 100µL of reduced serum Opti-MEM and siRNA was diluted to 0.1µM in Opti-MEM. Both were added in a complex with 0.4µL Lipofectamine™ 3000 per 100ng DNA. Complex formation was allowed to occur for 15 minutes. Complex containing 50ng DNA and 0.033µM siRNA was added to each well of a 96-well plate, followed by 15,000 freshly passaged U2OS cells in DMEM without P/S (section 2.1.1.1). Cells were incubated with complex for 24 hours at 37°C before their media was changed to recording media (section 2.1.2) supplemented with 400µM luciferin and baseline was recorded for approximately two hours. Drug treatment was then applied and readings taken for a further 48 hours.

2.4.3 RNA Extraction

Cellular and tissue RNA was extracted with the RNeasy plus mini kit (Qiagen) with on-column digestion of DNA using the RNase-Free DNase set (Qiagen). Briefly, cells in 96-well plates were lysed in 50µL RLT buffer (Qiagen) supplemented with 1% β-mercaptoethanol (Sigma) per well. 2.5 volumes of 100% ethanol (Sigma) were added to the lysate to precipitate total RNA. For RNA extraction from tissue, tissue samples ≤ 0.5 cm in any single dimension were lysed in 900µL QIAzol Lysis Reagent (Qiagen) using an electric homogenizer. Samples were then transferred to Phasemaker™ Tubes (ThermoFisher Scientific) and incubated for five minutes. 180µL chloroform (Sigma) was added to each sample and tubes were manually shaken vigorously for 15 seconds. The samples were then incubated with

chloroform for five minutes at room temperature, after which they were centrifuged for 15 minutes at 12,000g at 4°C. This centrifugation separated each sample into a lower red phenol-chloroform phase containing proteins, an interphase at which the Phasemaker™ gel created a barrier, and an upper aqueous phase containing RNA. The aqueous phase was collected and 2.5 volumes of 100% ethanol were added to precipitate total RNA

The RNA mixture of cell and tissue lysates were transferred to RNeasy mini elute spin columns and centrifuged for 15 seconds to allow RNA to bind the column. The columns were washed once with buffer RW1 before on-column DNA digestion was performed. One part RNase-free DNase I was added to seven parts RNase-free buffer RDD and the mix was applied to the RNeasy mini elute spin columns to digest DNA for 15 minutes at room temperature. The columns were then washed again with RW1, followed by two RPE washes with a 15 second centrifugation step after each wash. Columns were then centrifuged for two minutes to dry the columns before RNA was eluted in 30µL RNase-free-water. All centrifugation steps were conducted at 12,000g.

RNA was quantified with the Qubit® RNA BR (Broad-Range) Assay Kit (ThermoFisher Scientific). Briefly, samples and standards were prepared in Qubit® working solution and quantified in a Qubit® 2.0 Fluorometer (ThermoFisher Scientific).

2.4.4 cDNA Conversion

The qScript cDNA Synthesis kit (Quanta Biosciences) was used to convert RNA to cDNA. 300ng of RNA from each sample was mixed with 2μL 5X qScript reaction mix and 0.5μL qScript RT enzyme in a final volume of 10μL. A T100™ Thermal Cycler (Bio-Rad) was then used to implement the following polymerase chain reaction (PCR) protocol: 22°C for 5min, 42°C for 30min, 85°C for 5min, and then holding at 4°C indefinitely. This protocol was chosen as it generates one copy of cDNA per one molecule of RNA. cDNA samples were then diluted in water to 2ng/μL and stored at -80°C.

2.4.5 Real-Time PCR

Real-time PCR was performed using SYBR™ green master mix (ThermoFischer Scientific) in a 96-well PCR plate (Starlab). The SYBR™ green dye fluoresces when bound to double-stranded DNA, providing a measurement of DNA quantity. For each sample, master mix was created using 2.2μL water, 0.15μL forward primer, 0.15μL reverse primer and 7.5μL 2x SYBR green. 10ng of cDNA was then added to the master mix and the PCR plate was placed in a StepOne Real-Time PCR system (ThermoFischer Scientific). The list of primers used is provided in Table 2.2. The following PCR protocol was implemented: 95°C for 5 minutes followed by 40 cycles of 95°C for 10s, 60°C for 30s, and 72°C for 10s. After the 40 cycles, a melt curve was produced to check the specificity of the primers used.

Gene	Forward/Reverse	Sequence (5'-3')
ACTB	Forward	CATGTACGTTGCTATCCAGGC
ACTB	Reverse	CTCCTTAATGTCACGCACGAT
B2M	Forward	CCACTGAAAAAGATGAGTATGCCT
B2M	Reverse	CCAATCCAAATGCGGCATCTTCA
PPARG1	Forward	GGGATCAGCTCCGTGGATCT
PPARG1	Reverse	TGCACTTTGGTACTCTTGAAGTT
PPARG2	Forward	ACCAAAGTGCAATCAAAGTGGA
PPARG2	Reverse	ATGAGGGAGTTGGAAGGCTCT

Table 2.2 Homo Sapiens Primer Sequences Used in Chapter 5.

2.4.6 PCR Data Analysis

The Pfaffl-method was used to analyse real-time PCR data. In short, standard curves were generated and used to estimate the efficiency (E) of amplification for each primer set. E was calculated as (1+ (-slope of standards)) and is usually equal to 2. The CT values of all genes were converted to relative gene amounts by calculating E^{-CT}. Each gene of interest (GOI) was normalised to two reference housekeeping genes (REF) using the following equation: $Relative\ Gene\ Expression = \frac{(E_{GOI})^{\Delta CT\ GOI}}{GeoMean[(E_{REF})^{\Delta CT\ REF}]}$.

2.4.7 Differentiation of 3T3-L1 Preadipocytes

3T3-L1 preadipocytes are the most widely used murine cell line to study adipogenesis and have been validated to respond to a variety of metabolic agents including lipolytic compounds (Green and Kehinde, 1974; Ruiz-Ojeda *et al.*, 2016). They were differentiated according to the ZenBio protocol as follows. 3T3-L1

preadipocytes were grown to confluence in pre-differentiation media (section 2.1.1.1) in clear 24-well plates (Greiner) and further incubated for 48 hours after confluence to initiate growth arrest. Differentiation was initiated by replacing pre-differentiation media with DMEM (section 2.1.1.1) supplemented with 1µg/mL insulin (Sigma), 500µM 3-isobutyl-1-methylxanthine (Sigma), 1µM dexamethasone (Sigma), and 2µM rosiglitazone (Stratech Scientific). Three days after differentiation initiation, media was replaced with maintenance media – DMEM supplemented with 1µg/mL insulin and 1µM dexamethasone. Cells were maintained in this way with partial (1/2) media changes every 2-3 days for 7 days after differentiation initiation. On day seven after differentiation initiation, drug or vehicle treatment was added for a further 4 days.

2.4.8 Quantification of Lipid Accumulation in Mature Adipocytes

Cells were fixed in 4% paraformaldehyde (ThermoFischer Scientific) for 15 minutes and then washed with PBS. Cells were then incubated in 300nM DAPI solution (4',6-diamidino-2-phenylindole, Thermofischer Scientific) for 5-7 minutes to visualise nuclei – DAPI binds adenine-thymine rich regions within DNA. After nuclear staining, cells were washed with PBS and stained once more for 5-7 minutes in 1µg/mL of the lipid stain nile red (9-diethylamino-5H-benzo[alpha]phenoxazine-5-one, Sigma) in PBS. Three washes with PBS were performed to remove any residual dye before cells were imaged using the EVOS M7000 (ThermoFisher Scientific) at the DAPI channel (Ex 357/44, Em 447/60 nm) to visualise nuclei. They were then imaged at the RFP channel (Ex 531/40, Em 593/40 nm) to visualise nile

red staining. Fluorescence intensity was quantified using the image processing package Fiji (a distribution of ImageJ2) and normalised to nuclei count in the same software (Schindelin *et al.*, 2012).

2.4.9 Lithium Administration to DIO Mice

12 four-week-old male C57BL/6 mice were administered HFD (section 2.1.4) for 14 weeks before drinking water was replaced with 7mM LiCl and an additional supplemental bottle of 0.9% NaCl was provided for 6 mice. After one week, the 7mM LiCl was replaced with 14mM LiCl, which was provided to the mice *ad libitum* for 15 weeks. After 16 weeks of lithium administration, the mice were culled and white adipose tissue samples were collected and immediately frozen on dry ice until stored at -80°C.

2.4.10 Lithium Administration to Mice on Control Chow

12 9-week-old male C57BL/6 mice were administered standard lab chow *ad libitum*. At 16-weeks old, drinking water of 6 mice was replaced with 7mM LiCl and an additional supplemental bottle of 0.9% NaCl was provided. After one week the 7mM LiCl bottle was replaced with 14mM LiCl for 9 weeks, as it had been for the HFD mice.

2.4.11 Oral Glucose Tolerance Test and Insulin Tolerance Test

The oral glucose tolerance test (OGTT) was performed on the HFD mice after 11 weeks of lithium administration and on mice fed standard chow after 9 weeks of lithium administration. The OGTT was performed after 16 hours of fasting at zeitgeber timepoint (ZT) 2. Blood glucose levels were quantified from tail blood using the AlphaTRAK®2 blood glucose monitoring system (Zoetis). Readings were taken at baseline, 15, 30, 60, 90 and 120 minutes after an oral gavage of 1g/kg glucose (Sigma).

The insulin tolerance test (ITT) was performed within one week of the OGTT. The ITT was administered after 5 hours of fasting at ZT8. Blood glucose levels were quantified from tail blood using the AlphaTRAK®2 blood glucose monitoring system. Readings were taken at baseline, 15, 30, 60, 90 and 120 minutes after an intraperitoneal injection of 0.75U/kg insulin (Sigma).

Glucose and insulin tolerance were determined by plotting blood glucose levels over time and calculating the area under the curve in Prism 9.

2.4.12 RNA-Sequencing

RNA sample integrity was determined using a TapeStation system (Agilent Technologies) with the Sensitivity RNA ScreenTape® assay. Briefly, samples were diluted to 500 – 10,000 pg/µL in 2µL RNase-free-water and 1µL high sensitivity RNA sample buffer was added to each. Samples and a high sensitivity RNA ladder were vortexed at 2000 rpm for one minute, heated at 72°C for three minutes, placed on

ice for two minutes and loaded into the TapeStation instrument. Only samples with an RNA integrity number (RIN) ≥8 were sent for RNA sequencing.

Total RNA extracted from the white adipose tissue of DIO mice were sent to NovoGene (Beijing, China) where library construction and transcriptome sequencing were conducted for mRNA-sequencing. All samples were subjected to the company's internal quality control, which included 1% agarose gel electrophoresis, purity assessment via Nanodrop™ (ThermoFisher Scientific) and confirmation of the RIN via the Agilent 2100 (Agilent Technologies).

2.4.12.1 Non-Directional Library Construction and Sequencing

Messenger RNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation of mRNA was followed by first-strand cDNA synthesis using a random hexamer primer. Second-strand cDNA was synthesised using deoxythymidine triphosphate. End repair of the cDNA fragments was performed followed by the addition of a poly (A) tail to the 3′ ends of fragments. Illumina-compatible adaptors were ligated to the 5′ ends. cDNA fragments were amplified with PCR. Library construction was checked with a Qubit and real-time PCR for quantification and the Agilent 2100 for size distribution detection. Once quantified, libraries were pooled and sequenced on the NovaSeq 6000 platform (Illumina) according to the manufacturer's instructions and paired-end reads of 150 base pairs were generated.

2.4.12.2 Data Processing

Data from the NovaSeq 6000 platform was transformed to sequenced reads (raw data) by CASAVA base recognition. Quality control was performed on the library through NovoGene in-house perl scripts. These scripts removed reads containing adapter sequences, reads containing ploy-N, and low-quality reads.

Trim Galore! 0.6.3 was used on the clean data received from NovoGene to ensure the removal of low-quality bases, short reads, and Illumina adapters. Trim Galore! removed 0.1% of the reads. FastQC 0.11.8 was used to determine quality improvement and 99.5% of reads passed quality control. HISAT2 2.1.0 was employed to map high quality reads to the Mus musculus (mm10) reference genome and yielded an overall alignment rate of 97.12% (Kim *et al.*, 2019). featureCounts 2.0.1 was used to count the number of reads mapped to each gene (Liao, Smyth and Shi, 2014). Approximately 18 million counts were assigned per sample. FastQC, HISAT2, and featureCounts results were aggregated using MultiQC 1.9 (Ewels *et al.*, 2016).

2.4.12.3 Differential Gene Expression Analysis

Prior to performing differential gene expression analysis, genes with less than 10 counts across all samples were filtered out. The DESeq2 package was used in R 4.1.0 to detect differentially expressed genes between lithium-treated and control DIO mice (Love, Huber and Anders, 2014). An outlier that accounted for 81% of the data variance was excluded, leaving data from only two control animals (Fig.

5.10). Therefore, a non-adjusted P value of 0.05 was used as a cut-off to select up- and downregulated genes.

2.4.12.4 ChEA Analysis

Enrichr was used to identify transcription factors whose target genes were enriched among a list of genes of interest (Chen *et al.*, 2013). The ChIP-x Enrichment Analysis (ChEA) database within Enrichr contains data mined from published chromatin immunoprecipitation experiments that report the binding of specific transcription factors to DNA, listing hundreds to thousands of potential regulatory interactions (Lachmann *et al.*, 2010). Significant enrichment between this database and a gene list of interest was calculated using the Fisher exact test with the Benjamini-Hochberg correction for multiple testing. An odds ratio for the association between the enriched genes and published ChIP datasets for each transcription factor was calculated as follows. A Fisher exact test was run for random gene sets to establish a mean and standard deviation for random associations. Finally, a z score was calculated to assess the deviation of the enriched genes for a specific transcription factor from mean of the random gene set (Chen *et al.*, 2013).

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3. LITHIUM MODULATES THE ACTIVITY OF MULTIPLE KINASES

3.1 Introduction

The two targets of lithium that have historically received the most attention are a kinase and a phosphatase (glycogen synthase kinase 3 and inositol monophosphatase) – two enzymes which are both involved in the process of protein phosphorylation. It has been shown that lithium inhibits both of these targets by competing with their common cofactor magnesium (Leech *et al.*, 1993; Ryves and Harwood, 2001). Given that most kinases use magnesium as a cofactor, we theorised that lithium could induce widescale signalling alterations by changing phosphorylation patterns in widespread pathways in a manner hitherto unexplored.

3.1.1 Kinase Activity

Phosphorylation is a reversible post-translational modification of proteins that alters their activity and is responsible for changes in the majority of signal transduction pathways throughout the body. The phosphorylation of proteins is driven by protein kinases. Many kinases are themselves activated by phosphorylation in a sequence known as the activation segment, which consists of basic amino acids. The specificity of the sequence in the activation segment determines whether the kinase can autophosphorylate and activate itself in response to cellular conditions, or whether activation is performed by another kinase (Johnson, Noble and Owen, 1996). Other kinases require the binding of secondary messengers such as cyclic adenosine monophosphate (cAMP) or

Ca^{2+}/calmodulin to release the catalytic subunits and become active (Johnson, Noble and Owen, 1996).

In eukaryotes, kinases bind adenosine triphosphate (ATP) and a protein substrate to catalyse the transfer of the γ-phosphate of ATP to a serine, threonine or tyrosine of the substrate. Two magnesium (Mg^{2+}) ions stabilise ionic interactions to catalyse the phosphorylation. The first Mg^{2+} binds ATP and the second is situated in the catalytic loop of the kinase. The addition of a phosphate group in this manner can alter the activity of the substrate through allosteric conformational changes or by physically blocking access to catalytic sites in the protein (Ubersax and Ferrell, 2007; Bao, Jacobsen and Young, 2011) .

It has been estimated that roughly a third of all cellular proteins contain a phosphorylation site and each kinase has approximately 700,000 potential phosphorylation sites in a eukaryotic cell – though the actual number of phosphorylation sites is much lower and varies greatly between kinases (Ubersax and Ferrell, 2007). 518 kinases alone have been identified in the human genome. Most kinases have the same catalytic domain with a few atypical kinases breaking the pattern (Manning *et al.*, 2002).

Protein phosphatases remove phosphate groups from their substrates, providing the reversibility to phosphorylation. They do so by hydrolysing the phosphate monoesters in their substrate, thereby releasing the phosphate group. Phosphatases are more diverse than their kinase counterparts and have been grouped into three genetic families: phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs) and tyrosine phosphatases (PTPs). PPPs

contain many regulatory subunits, while PPMs are dependent on manganese (Mn^{2+}) and Mg^{2+}. Both families are largely responsible for removing phosphate groups from phosphoserine and phosphothreonine. PTPs dephosphorylate phosphotyrosine but can also remove phosphates from carbohydrates and phosphoinositides (Ardito *et al.*, 2017). Phosphatases can be activated in response to growth factors, secondary messengers, and cofactors. Their activity usually outweighs that of kinases in determining the length of a signal and they can often form inhibitory feedback loops (Nguyen *et al.*, 2013).

Lithium uncompetitively inhibits two phosphatases of interest in cellular signalling – inositol monophosphatase (IMPase) and inositol polyphosphate phosphatase (IPPase). Both are metal-dependent phosphatases that are structurally distinct except for their metal-binding catalytic sites. In fact, seven phosphatases involved in gluconeogenesis and nucleotide and inositol metabolism share this catalytic site – and all have the potential for lithium inhibition *in vivo* (Dollins *et al.*, 2021). The catalytic site coordinates Mg^{2+} and water, which then perform a nucleophilic attack on the phosphate group of the substrate. Lithium uncompetitively inhibits IMPase and IPPase by binding to the catalytic site after the substrate is bound and interfering with magnesium's coordination of the nucleophilic attack (Bone, Springer and Atack, 1992; Dollins *et al.*, 2021).

As previously mentioned, lithium also inhibits glycogen synthase kinase 3 (GSK-3) through competition with magnesium (Ryves and Harwood, 2001). Studies have shown that lithium inhibits other kinases as well, including pyruvate kinase, hexokinase, mitogen-activated protein kinase -interacting serine/threonine-protein

kinase 1 and 2, TANK-binding kinase 1, and phosphorylase kinase (Birch *et al.*, 1974; Bain *et al.*, 2007). Although lithium is known to alter the activity of select kinases and phosphatases, neither the extent of this alteration nor its ultimate effects on the general phosphoproteome and therapeutically relevant targets has been explored.

3.1.2 Sleep, Circadian Rhythms, and the Phosphoproteome

Recent advancements in the circadian field have shown that phosphorylation is a key mechanism through which sleep and the circadian rhythm are regulated. In 2018, Wang *et al.* found hyperphosphorylation in the brain proteome of sleep-deprived mice and *Sleepy* mice (a model with a gain of function mutation in salt-inducible kinase 3 (SIK3) of the AMP-activated protein kinase (AMPK) family that results in constitutively high sleep pressure). Using conditions of sleep deprivation and recovery sleep, they discovered that prolonged wakefulness drives hyperphosphorylation and sleep promotes dephosphorylation of the brain proteome. A cross comparison of the two mouse models identified a group of 80 phosphoproteins they termed the SNIPPs (synaptic sleep-need-index phosphoproteins), which are hyperphosphorylated in parallel with sleep need (Wang *et al.*, 2018).

Subsequently, it was discovered that rhythmic peaks of general synaptic phosphorylation occur in anticipation of the light changes at dawn and dusk in mice (Brüning *et al.*, 2019). Prominent among the targets undergoing daily phosphorylation changes are synaptic kinases themselves. Kinases driving excitatory activity and long-term potentiation (LTP) are activated in the wake

transition and kinases driving inhibitory activity and long-term depression (LTD) are active at the sleep transition. Sleep deprivation eliminates 98% of the rhythmic phosphorylations in the synaptic proteome, suggesting that sleep pressure plays a crucial role in these phosphorylation cycles (Brüning *et al.*, 2019). This finding provided a possible mechanism for the long-observed correlation between sleep deprivation and impaired LTP, and reinforced the idea that sleep and circadian rhythms play a crucial role in synaptic plasticity (Grønli, Soulé and Bramham, 2014).

Further evidence for the role of kinases in sleep regulation came from investigations into the kinase SIK1. Expression of SIK1 is induced in response to light input to the suprachiasmatic nucleus (SCN). In turn, SIK1 inhibits the cascade that activated it, forming a negative feedback loop which limits the rate at which mice can entrain to jetlag. Thus sleep pressure can be uncoupled from sleep induction through the inactivation of SIK1 (Jagannath *et al.*, 2013). Recent work used a SIK1 kinase-inactive transgenic mouse to show that SIK1 induces sleep in mice in the absence of sleep pressure in response to nocturnal light exposure. Specifically, it was found that light-induced changes to the phosphoproteome can causally drive sleep in mice through 17 keystone sleep phosphoproteins (KSPs). Thus, SIK1 and SIK3 appear to regulate sleep/wake timing through the phosphoproteome in response to light and sleep pressure, respectively (Taylor *et al.*, 2021).

3.1.3 Aims

Given that lithium has been shown to inhibit kinase and phosphatase activity through competition with magnesium, we theorised that it could inhibit far more kinases and phosphatases than has previously been examined. Considering the body of evidence linking kinases to circadian rhythms and sleep/wake cycles, we decided to prioritize this family in our investigations. Given that disrupted sleep/circadian rhythms are a key diagnostic marker of bipolar disorder (BD) and lithium is known to increase the length and amplitude of these rhythms, we decided to examine the circadian consequences of lithium's widespread kinase inhibition. Specifically, we set off to test the hypothesis that lithium could exert its effects across a myriad of signalling pathways via kinase inhibition.

3.2 Results

3.2.1 Lithium Alters the Activity of Genetically Diverse Kinases *In Vitro*

In order to evaluate if lithium can alter widespread signalling cascades by changing kinase activity, it was first necessary to establish that the ion is capable of altering kinase activity. An assay measuring *in vitro* kinase activity using radiolabelled [γ-^{33}P]ATP was conducted for 140 kinases in the presence of 3mM, 10mM and 20mM lithium chloride (LiCl) (Fig. 3.1). The lowest concentration of 3mM was selected because lithium inhibits GSK-3β with a Ki of approximately 2mM (Klein and Melton, 1996). Inhibition of kinase activity was considered significant if the activity of the kinase was three standard deviations away from its activity in control conditions.

The results indicated that lithium is indeed capable of altering kinase activity, as it inhibited the activity of far more kinases than has previously been reported (Fig. 3.1). At 3mM, 10mM, and 20mM, LiCl inhibited 4.29%, 23.57%, and 69.33% of the kinases tested, respectively. No kinases were inhibited at all three concentrations of lithium, but there was considerable overlap between the kinases inhibited at 10mM and 20mM LiCl (Fig. 3.2). An investigation of the genetic relatedness of the kinases inhibited by lithium revealed that lithium inhibits kinases across the phylogenetic kinase tree (Fig. 3.3). Thus lithium-driven inhibition is not specific to kinases of a specific family and is consistent with the proposed mechanism of lithium competing with magnesium.

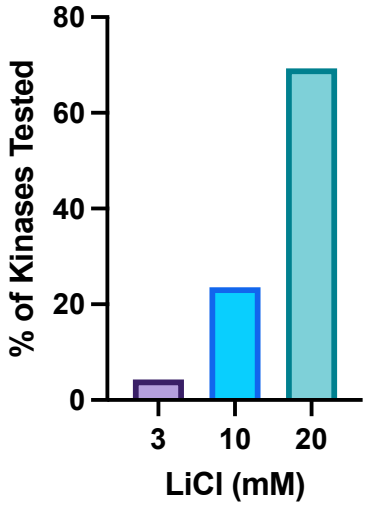

Figure 3.1 Kinases Inhibited by Lithium In Vitro. An assay of kinase activity was conducted using radiolabelled ATP for 140 kinases in the presence of 3mM and 10mM LiCl. The same assay was conducted for 75 kinases in the presence of 20mM LiCl.

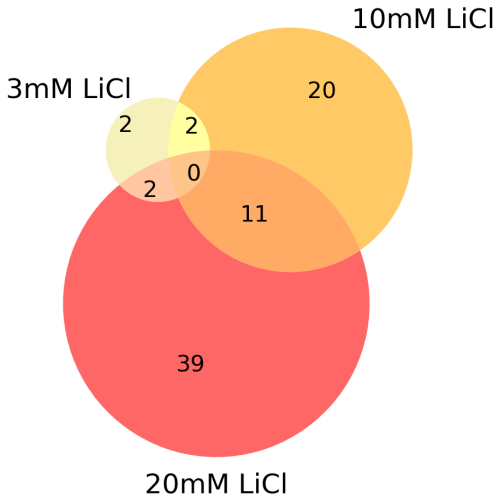

Figure 3.2 Overlap Between the Kinases Inhibited by 3mM, 10mM, and 20mM LiCl In Vitro. An assay of kinase activity was conducted using radiolabelled ATP in the presence of 3mM (yellow), 10mM (orange), and 20mM (red) LiCl. 2 kinases were inhibited by both 3mM and 10mM LiCl. 2 kinases were inhibited by both 3mM and 20mM LiCl. 11 kinases were inhibited by both 10mM LiCl and 20mM LiCl.

Of note was that lithium inhibited its well-known target GSK-3β at 10mM and 20mM by 48% and 72%, respectively. It did not have any significant effect on GSK-3β activity at 3mM – an unexpected finding given the literature-reported Ki of 2mM. Upon closer inspection, however, this Ki was determined in an assay using 4mM magnesium acetate whereas there was 10mM magnesium acetate present in our assay (Stokoe *et al.*, 1992; Klein and Melton, 1996; Ryves and Harwood, 2001; Hastie, McLauchlan and Cohen, 2006). Given that lithium competes with magnesium to inhibit GSK-3β, it is not surprising that a higher concentration of magnesium would alter the Ki and reduce the potency of lithium.

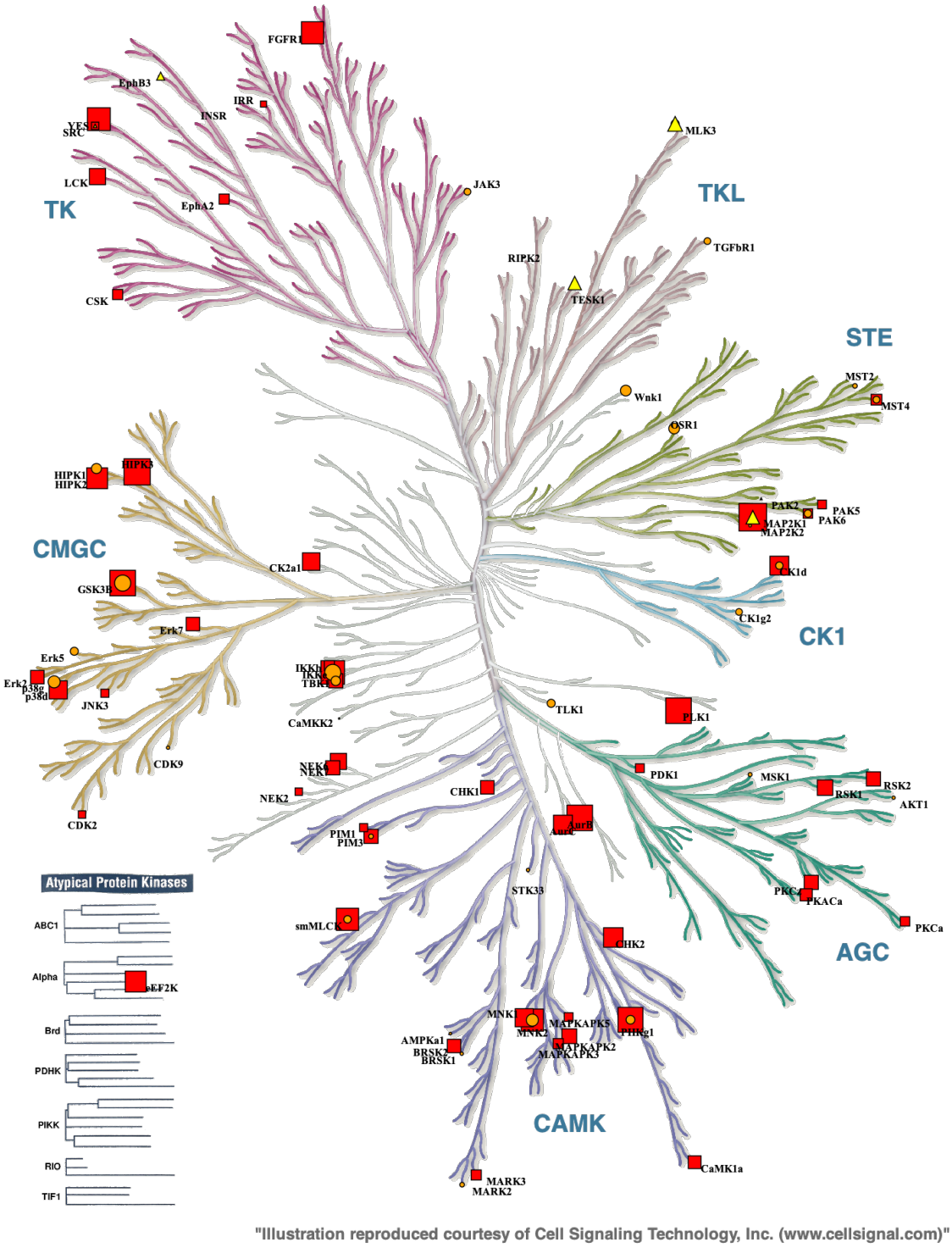

Figure 3.3 Phylogenetic Tree of Kinases Inhibited by Lithium In Vitro. An assay of kinase activity was conducted using radiolabelled ATP in the presence of 3mM (yellow triangle), 10mM (orange circle) and 20mM (red square) LiCl. LiCl significantly inhibited 6 kinases at 3mM, 33 at 10mM and 52 at 20mM. Size of markers corresponds to the percent of lithium-induced inhibition. Figure created using Kinmap (Eid et al., 2017).

Analyses of protein-protein interactions and functional enrichment of the kinases whose activity was impacted by lithium (at any concentration) were carried out to ascertain the pathways potentially inhibited by lithium (Fig. 3.4 and Table 3.1). The STRING database was used to investigate the protein-protein interactions of the lithium-impacted kinases with each other (Szklarczyk *et al.*, 2019). Functional enrichment analysis was performed on the list of kinases significantly inhibited by lithium using G:Profiler (Raudvere *et al.*, 2019). It was used to generate a list of significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, revealing which KEGG pathways lithium could alter *in vitro* through kinase inhibition.

The most enriched KEGG pathway was the mitogen-activated protein kinase (MAPK) signalling pathway with 26 kinases inhibited by LiCl (Fig. 3.5). Of particular interest is that nine kinases in this pathway are known to undergo circadian phosphorylation, providing a point of intersection between lithium's modulation of kinase activity and sleep/circadian pathways (Brüning *et al.*, 2019). Many interconnecting pathways are also inhibited by lithium *in vitro*, including the neurotrophin and the insulin signalling pathways. Thus, the results of the *in vitro* screen of lithium's impact on kinase activity revealed that lithium can inhibit a wide variety of kinases, and inhibition of these kinases could have functional effects at multiple points on signalling pathways of potential biological importance.

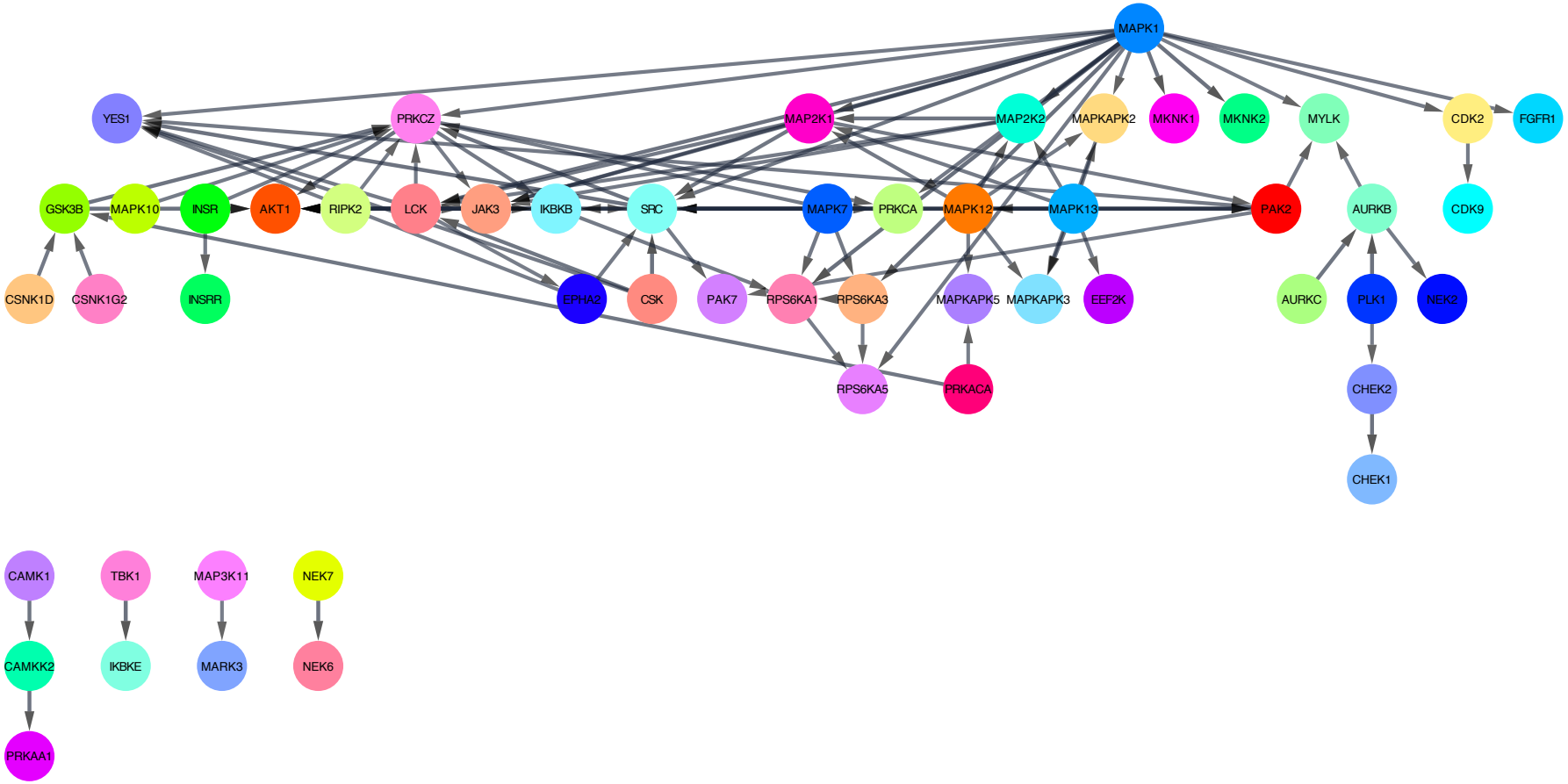

Figure 3.4 The Functional and Physical Interactions of Kinases Inhibited by Lithium In Vitro. STRING map highlighting known and predicted interactions of the kinases inhibited by lithium at 3mM, 10mM or 20mM. Arrows show the direction of interaction between kinases.

KEGG Pathway	KEGG ID	Adjusted P Value	Pathway Size	Intersection Size	Intersections (Kinases Inhibited by Lithium)
MAPK signaling pathway	KEGG:04010	4.92E-20	294	26	AKT1, EPHA2, FGFR1, IKBKB, INSR, MAP2K1, MAP2K2, MAP3K11, MAPK1, MAPK10, MAPK12, MAPK13, MAPK7, MAPKAPK2, MAPKAPK3, MAPKAPK5, MKNK1, MKNK2, PAK2, PRKACA, PRKCA, RPS6KA1, RPS6KA3, RPS6KA5, STK3, TGFBR1
Neurotrophin signalling pathway	KEGG:04722	4.91E-13	119	15	AKT1, GSK3B, IKBKB, MAP2K1, MAP2K2, MAPK1, MAPK10, MAPK12, MAPK13, MAPK7, MAPKAPK2, RIPK2, RPS6KA1, RPS6KA3, RPS6KA5
T cell receptor signalling pathway	KEGG:04660	3.83E-11	103	13	AKT1, GSK3B, IKBKB, LCK, MAP2K1, MAP2K2, MAPK1, MAPK10, MAPK12, MAPK13, PAK2, PAK6, PAK5
Hepatitis B	KEGG:05161	4.66E-11	161	15	AKT1, CDK2, IKBKB, IKBKE, JAK3, MAP2K1, MAP2K2, MAPK1, MAPK10, MAPK12, MAPK13, PRKCA, SRC, TBK1, TGFBR1
Yersinia infection	KEGG:05135	7.69E-11	136	14	AKT1, GSK3B, IKBKB, LCK, MAP2K1, MAP2K2, MAPK1, MAPK10, MAPK12, MAPK13, RPS6KA1, RPS6KA3, SRC, TBK1
Insulin signalling pathway	KEGG:04910	8.52E-11	137	14	AKT1, GSK3B, IKBKB, INSR, MAP2K1, MAP2K2, MAPK1, MAPK10, MKNK1, MKNK2, PHKG1, PRKAA1, PRKACA, PRKCZ
Cellular senescence	KEGG:04218	5.15E-10	156	14	AKT1, CDK2, CHEK1, CHEK2, HIPK1, HIPK2, HIPK3, MAP2K1, MAP2K2, MAPK1, MAPK12, MAPK13, MAPKAPK2, TGFBR1
FoxO signalling pathway	KEGG:04068	8.86E-10	131	13	AKT1, CDK2, IKBKB, INSR, MAP2K1, MAP2K2, MAPK1, MAPK10, MAPK12, MAPK13, PLK1, PRKAA1, TGFBR1
VEGF signalling pathway	KEGG:04370	1.15E-09	59	10	AKT1, MAP2K1, MAP2K2, MAPK1, MAPK12, MAPK13, MAPKAPK2, MAPKAPK3, PRKCA, SRC
ErbB signalling pathway	KEGG:04012	1.89E-09	84	11	AKT1, GSK3B, MAP2K1, MAP2K2, MAPK1, MAPK10, PAK2, PAK6, PAK5, PRKCA, SRC
Ras signalling pathway	KEGG:04014	8.77E-09	231	15	AKT1, EPHA2, FGFR1, IKBKB, INSR, MAP2K1, MAP2K2, MAPK1, MAPK10, PAK2, PAK6, PAK5, PRKACA, PRKCA, TBK1
Relaxin signalling pathway	KEGG:04926	1.3E-08	129	12	AKT1, MAP2K1, MAP2K2, MAPK1, MAPK10, MAPK12, MAPK13, PRKACA, PRKCA, PRKCZ, SRC, TGFBR1
Progesterone-mediated oocyte maturation	KEGG:04914	1.63E-08	102	11	AKT1, CDK2, MAP2K1, MAPK1, MAPK10, MAPK12, MAPK13, PLK1, PRKACA, RPS6KA1, RPS6KA3
Human immunodeficiency virus 1 infection	KEGG:05170	2.86E-08	210	14	AKT1, CHEK1, IKBKB, MAP2K1, MAP2K2, MAPK1, MAPK10, MAPK12, MAPK13, PAK2, PAK6, PAK5, PRKCA, TBK1
Oxytocin signalling pathway	KEGG:04921	1.03E-07	154	12	CAMK1, CAMKK2, EEF2K, MAP2K1, MAP2K2, MAPK1, MAPK7, MYLK, PRKAA1, PRKACA, PRKCA, SRC
IL-17 signalling pathway	KEGG:04657	1.09E-07	92	10	GSK3B, IKBKB, IKBKE, MAPK1, MAPK10, MAPK12, MAPK13, MAPK15, MAPK7, TBK1
GnRH signalling pathway	KEGG:04912	1.21E-07	93	10	MAP2K1, MAP2K2, MAPK1, MAPK10, MAPK12, MAPK13, MAPK7, PRKACA, PRKCA, SRC
Kaposi sarcoma-associated herpesvirus infection	KEGG:05167	1.25E-07	194	13	AKT1, GSK3B, IKBKB, IKBKE, MAP2K1, MAP2K2, MAPK1, MAPK10, MAPK12, MAPK13, MAPKAPK2, SRC, TBK1
Prolactin signalling pathway	KEGG:04917	1.7E-07	70	9	AKT1, GSK3B, MAP2K1, MAP2K2, MAPK1, MAPK10, MAPK12, MAPK13, SRC
Toll-like receptor signalling pathway	KEGG:04620	3.03E-07	102	10	AKT1, IKBKB, IKBKE, MAP2K1, MAP2K2, MAPK1, MAPK10, MAPK12, MAPK13, TBK1

Table 3.1 Top 20 Hits of the KEGG Enrichment Analysis of Kinases Inhibited by 3mM, 10mM or 20mM LiCl ordered by adjusted P value.

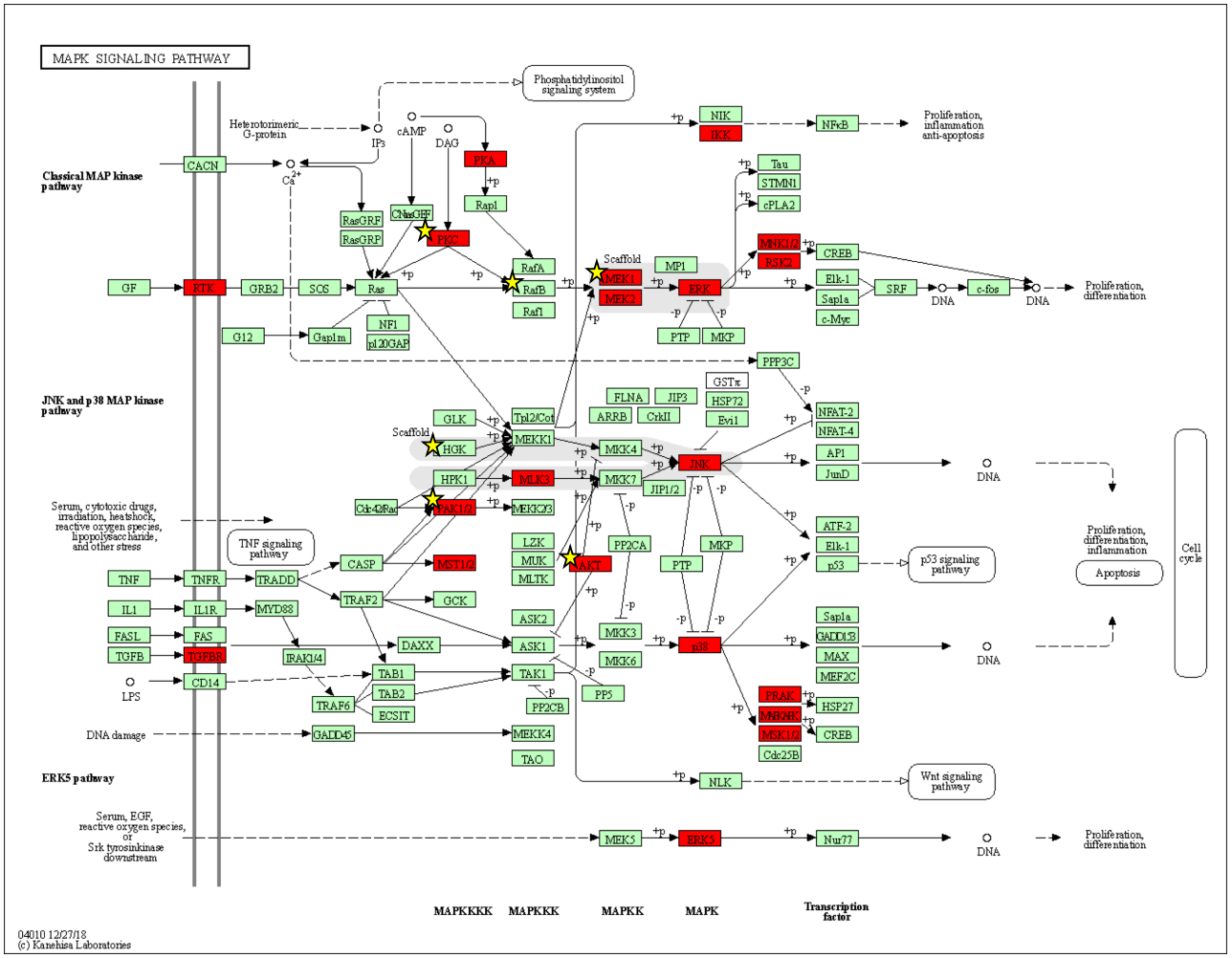

Figure 3.5 Lithium's Inhibition of the MAPK Pathway. Functional enrichment analysis of the KEGG MAPK pathway was performed with kinases inhibited by 3mM, 10mM or 20mM LiCl. Red indicates inhibited kinases and yellow stars mark synaptic kinases that are modulated by circadian phosphorylation. Figure created with GenomeNET (Kanehisa, 1997).

3.2.2 Pharmacological Inhibition of Lithium-Sensitive Kinases Alters Circadian Rhythms

Given that lithium is capable of altering the activity of many kinases at once, the functional consequence of this inhibition was examined in more detail. One of the hypothesised therapeutic targets for lithium's mood stabilising effect is the circadian rhythm (Sanghani, 2018). It was therefore determined whether any of the kinases that lithium inhibits have a circadian role in a cellular model. The Published Kinase Inhibitor Set (PKIS) of small molecule inhibitors developed by GlaxoSmithKline was chosen for this purpose. The PKIS is a set of 367 ATP-competitive kinase inhibitors that has been previously screened at 100nM and 1μM against a panel of 224 recombinant kinases (Elkins *et al.*, 2016). Molecules from the screen that inhibited our selected lithium-sensitive kinases by 50% or more at 1μM were selected and their circadian effect was determined in MMHD3 murine prehepatocytes bearing a circadian BMAL1 luciferase reporter (*Bmal*::LUC) (Fig. 3.6). Alteration of the circadian rhythm was considered significant if the inhibitor altered the period or amplitude more than three standard deviations away from the period or amplitude of the vehicle control. Cell viability was measured against a negative control without cells (- Control) and a positive vehicle control.

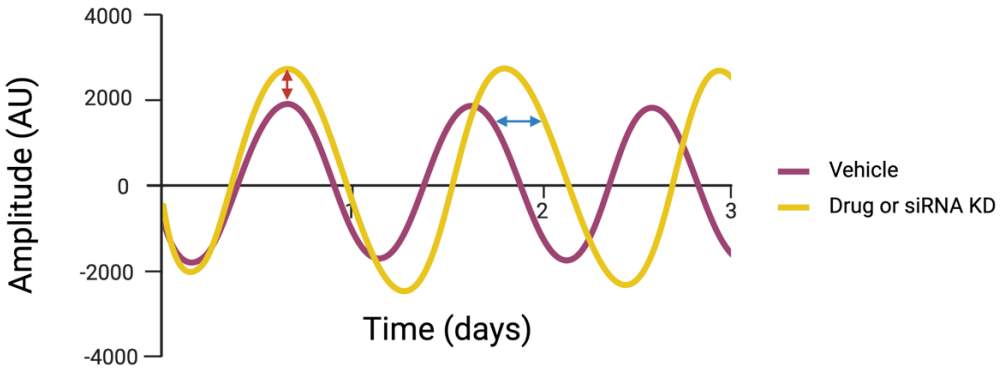

Figure 3.6 Representative Traces for Circadian Alterations. Traces demonstrating an increase in circadian amplitude in arbitrary units (AU) (red arrow) and circadian period in days (blue arrow) in response to drug or siRNA knockdown (KD).

Of the 76 kinases significantly inhibited by lithium at 3mM, 10mM or 20mM, 53 were targeted by inhibitors in the PKIS set. The kinase inhibitors of the PKIS were tested in duplicates at two concentrations (100nM and 600nM) in *Bmal*::LUC MMHD3 cells. As expected, the number of PKIS inhibitors that altered the circadian rhythm and targeted the same kinases as lithium increased with concentration – one inhibitor changed circadian period at 100nM and six inhibitors changed period or amplitude at 600nM without altering cell viability (Fig. 3.7 & Fig. 3.8). The PKIS hit found at 100nM has been shown to inhibit three lithium-sensitive kinases (aurora kinase B, aurora kinase C, and Mammalian STE20-like protein kinase 2) and the six hits at 600nM have been shown to inhibit 16 lithium-sensitive kinases (Table 3.2) (Elkins *et al.*, 2016). It should be noted that though GSK1173862A significantly altered period at 600nM, this is a by-product of the fact that the compound rendered the cells arrhythmic as is evident from the trace (Fig. 3.8C). Phylogenetic comparison of the impacted circadian kinases revealed that they reside across kinase families (Fig. 3.9). Thus, these findings supported the hypothesis that

lithium's inhibition of kinases *in vitro* could result in the alteration of circadian rhythms.

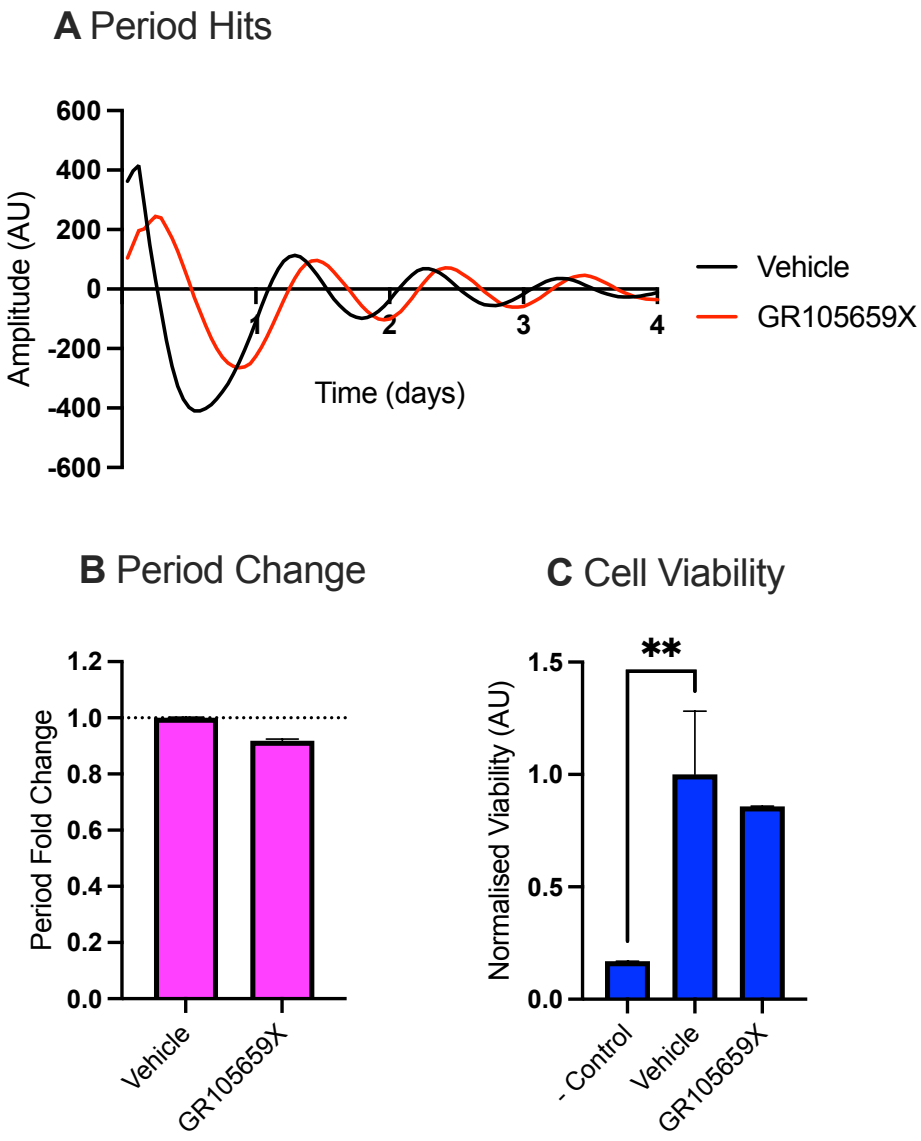

Figure 3.7 At 100nM Kinase Inhibitor GR105659X Alters Circadian Rhythms in Bmal::LUC MMHD3 Cells Through Lithium-Sensitive Kinases. The circadian phenotype of the PKIS was tested at 100nM in duplicate for each compound that targeted a lithium-inhibited kinase. Circadian period and amplitude were measured in response to each compound. Compounds that altered either parameter greater than three standard deviations away from a vehicle control were considered significant hits. Only significant hits are plotted above. (A) Normalised trace of drug treatment which significantly altered circadian period. (B) GR105659X was the only compound to significantly reduce period and (C) not alter cell viability. Significance of (C) determined using one-way ANOVA with a Dunnett's post hoc test. N=2.

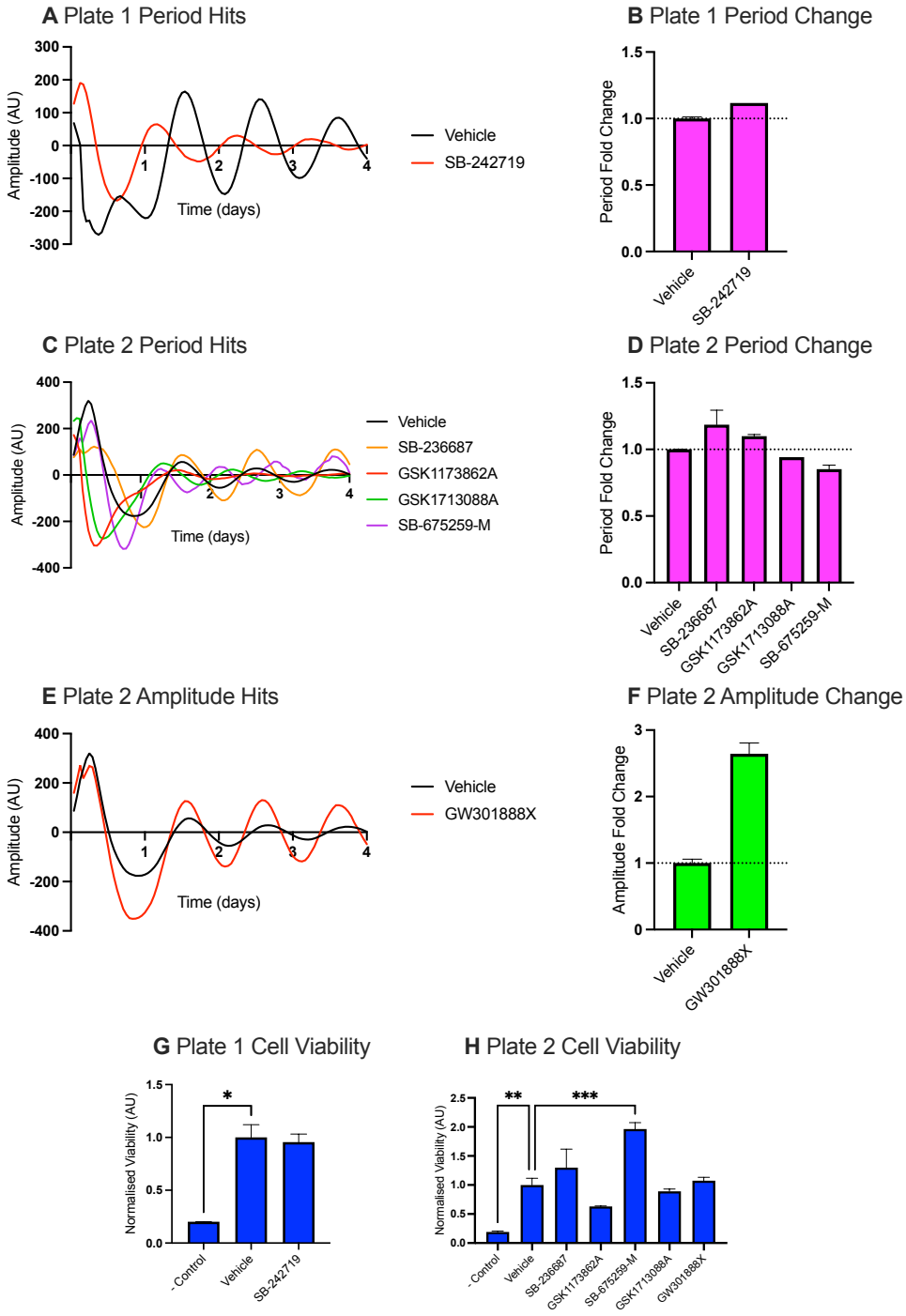

Figure 3.8 Six 600nM Kinase Inhibitors of the PKIS Alter Circadian Rhythms in Bmal::LUC MMHD3 Cells Through Lithium-Sensitive Kinases. The circadian phenotype of the PKIS was tested at 600nM in duplicate for each compound that targeted a lithium-inhibited kinase. Circadian period and amplitude were measured in response to each compound. Compounds that altered either parameter greater than three standard deviations away from a vehicle control were considered significant hits. Only significant hits are plotted above. (A) Normalised traces of inhibitors that significantly altered period in plate 1 and (B) the period fold change induced by these compounds. (C) Normalised traces of inhibitors that significantly altered period in plate 2 and (D) the period fold change induced by these compounds. (E) Normalised traces of inhibitors that significantly altered amplitude in plate 2 and (F) the amplitude fold change induced by these compounds. Cell viability after drug administration was assessed in (G) plate 1 and (H) plate 2. Significance of (G-H) determined using one-way ANOVA with a Dunnett's post hoc test. N=2.

PKIS Hit Compounds	Circadian Alteration	Total Kinase Targets	Kinase Targets
GW301888X	Amplitude	1	CSK
GSK1713088A	Period	1	INSR
GSK1173862A	Arrhythmic	10	CHK2, CK1γ2, CK2α1, HIPK1, INSR, IRR, MARK3, PHKγ1, PIM1, RSK1
SB-236687	Period	1	PKCα
SB-242719	Period	1	PKACα
SB-675259-M	Period	5	CDK2, GSK-3β, HIPK1, PIM1, PIM3

Table 3.2 Description of the PKIS Inhibitors That Significantly Alter Circadian Period or Amplitude at 600nM in Bmal::Luc MMHD3 Cells.

To confirm that the results of the PKIS screen were not specific to cell type, species or reporter gene, the screen was repeated in human U2OS cells bearing the Per2 luciferase circadian reporter (U2OS *Per2*::LUC). At 100nM, three PKIS inhibitors targeted lithium-sensitive kinases and also altered circadian period (Fig. 3.10). At 600nM this increased to 10 PKIS molecules (Fig. 3.11). Though GW805758X significantly increased period at 600nM, this is likely a by-product of the fact that the compound rendered the cells arrhythmic as is evident from the trace (Fig. 3.11A). Overall, the three 100nM PKIS inhibitors inhibited seven lithium-sensitive kinases and the 10 600nM PKIS inhibitors impacted 24 lithium-sensitive kinases (Table 3.3 & Table 3.4) across genetic kinase families (Fig. 3.12).

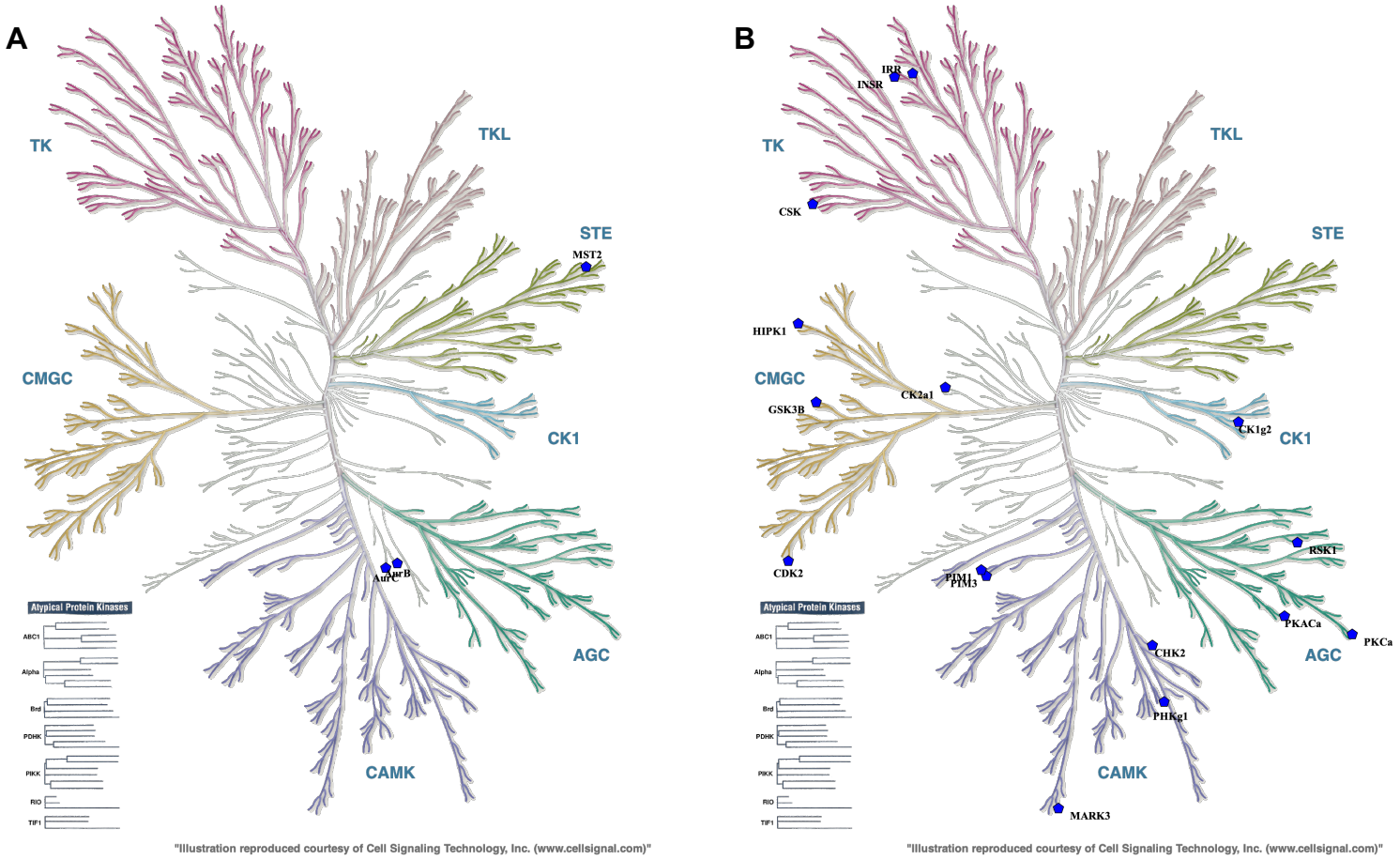

Figure 3.9 The Phylogenetic Relatedness of Kinases Targeted by PKIS inhibitors with a Circadian Phenotype in Bmal::LUC MMHD3 Cells. The circadian phenotype of the PKIS was tested at 100nM and 600nM in *Bmal::LUC* MMHD3 cells. (A) Three kinases that were targets of LiCl changed circadian period or amplitude when inhibited at 100nM by a PKIS inhibitor. (B) 16 lithium-sensitive kinases changed circadian period or amplitude when inhibited at 600nM by a PKIS inhibitor. Figure created using Kinmap (Eid et al., 2017).

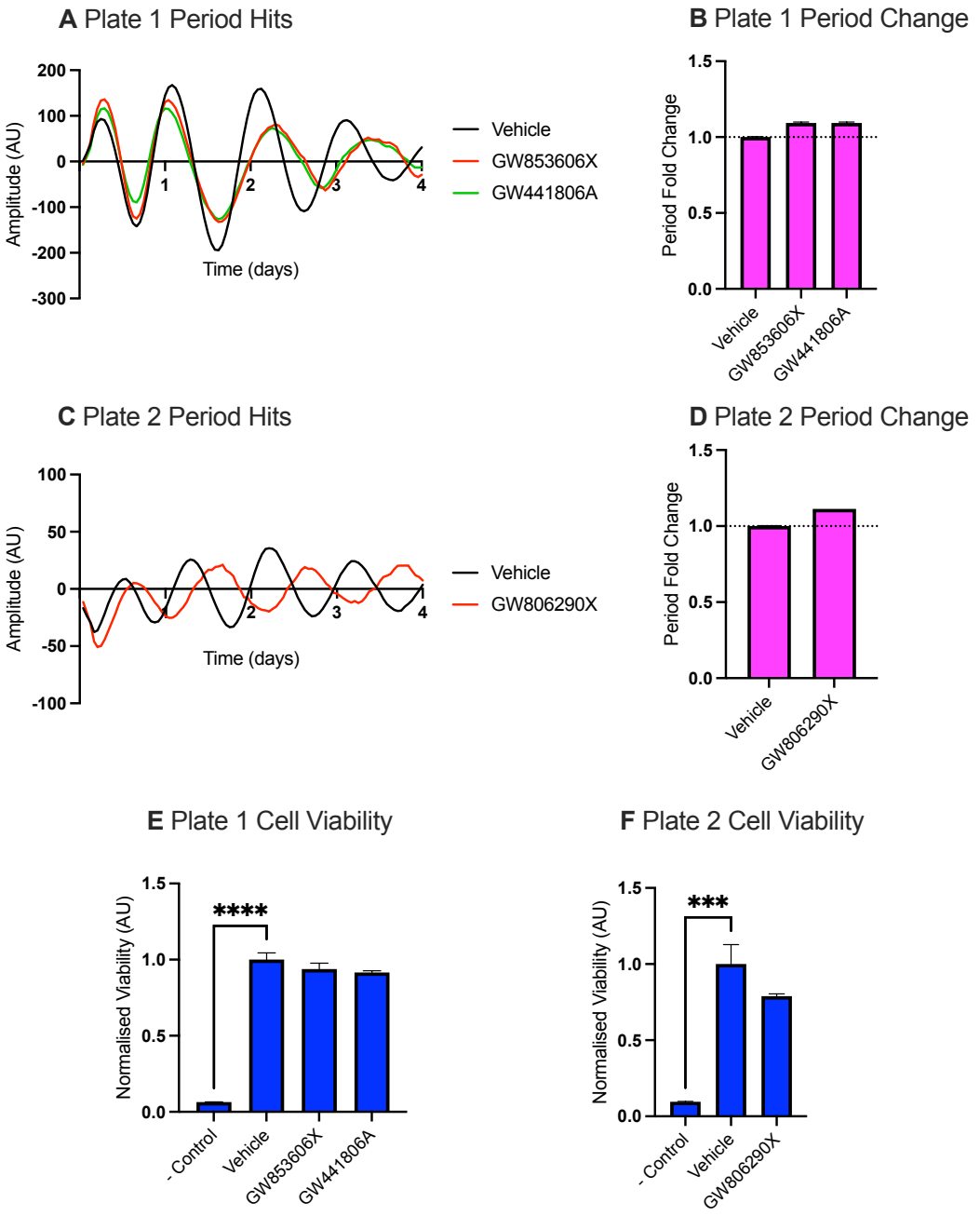

Figure 3.10 Three 100nM Kinase Inhibitors of the PKIS Alter Circadian Rhythms in Per2::LUC U2OS Cells Through Lithium-Sensitive Kinases. The circadian phenotype of the PKIS was tested at 100nM in duplicate for each compound that targeted a lithium-inhibited kinase. Circadian period and amplitude were measured in response to each compound. Compounds that altered either parameter greater than three standard deviations away from a vehicle control were considered significant hits. Only significant hits are plotted above. (A) Normalised traces of inhibitors that significantly altered period in plate 1 and (B) the period fold change induced by these compounds. (C) Normalised traces of inhibitors that significantly altered period in plate 2 and (D) the period fold change induced by these compounds. Cell viability was determined after drug administration in (E) plate 1 and (F) plate 2. Significance of (E-F) determined using one-way ANOVA with a Dunnett's post hoc test. N=2.

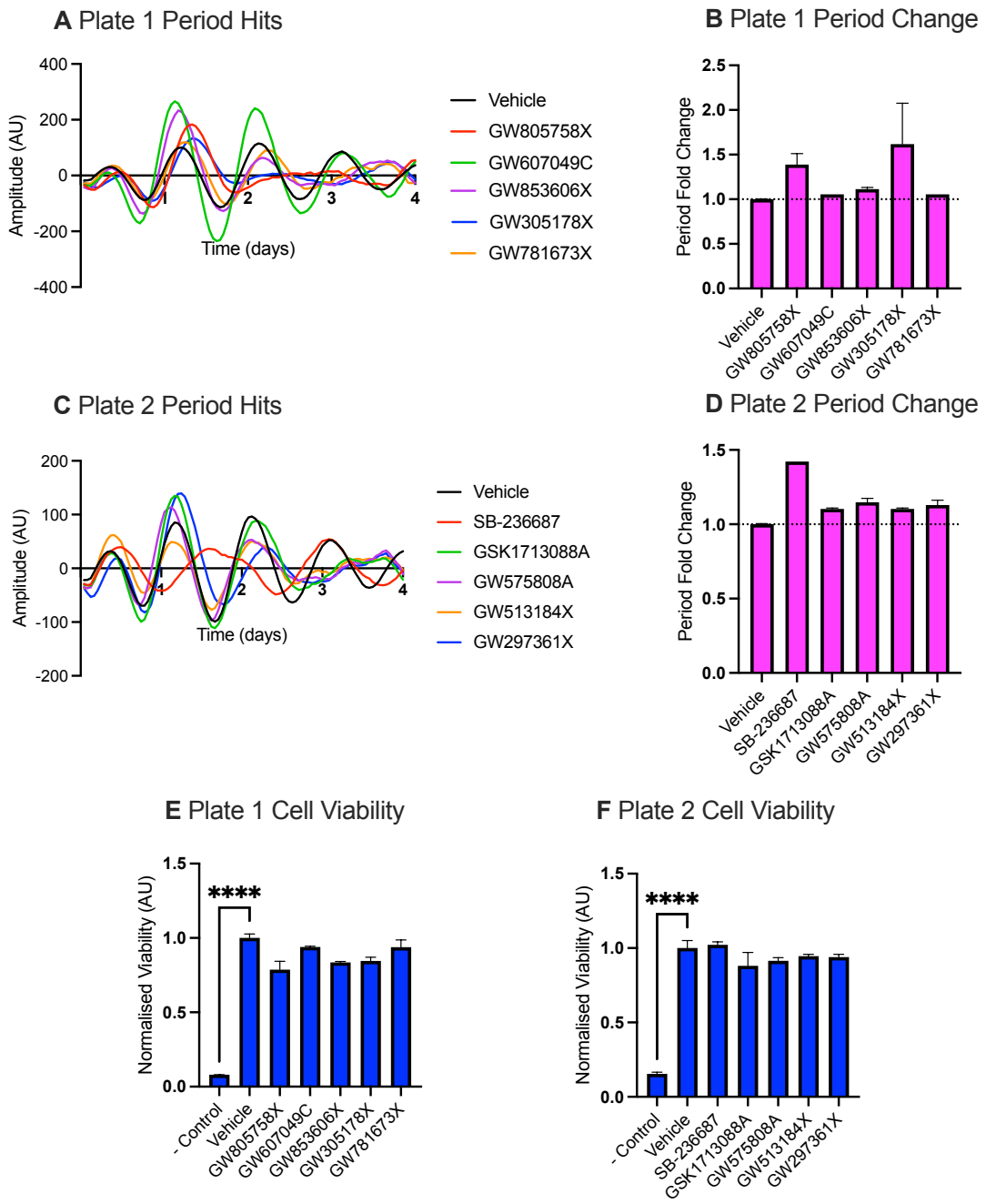

Figure 3.11 Ten 600nM Kinase Inhibitors of the PKIS Alter Circadian Rhythms in Per2::LUC U2OS Cells Through Lithium-Sensitive Kinases. The circadian phenotype of the PKIS was tested at 600nM in duplicate for each compound that targeted a lithium-inhibited kinase. Circadian period and amplitude were measured in response to each compound. Compounds that altered either parameter greater than three standard deviations away from a vehicle control were considered significant hits. Only significant hits are plotted above. (A) Normalised traces of inhibitors that significantly altered period in plate 1 and (B) the period fold change induced by these compounds. (C) Normalised traces of inhibitors that significantly altered period in plate 2 and (D) the period fold change induced by these compounds. Cell viability after drug administration was assessed in (E) plate 1 and (F) plate 2. Significance of (E-F) determined using one-way ANOVA with a Dunnett's post hoc test. N=2.

PKIS Hit Compounds	Circadian Alteration	Total Kinase Targets	Kinase Targets
GW441806A	Period	5	ABL2, EphA2, MNK1, MNK2, SRC
GW853606X	Period	1	PLK1
SB-236687	Period	1	PKCα

Table 3.3 Description of the PKIS Inhibitors That Significantly Alter Circadian Period or Amplitude at 100nM in Per2::Luc U2OS Cells.

PKIS Hit Compounds	Circadian Alteration	Total Kinase Targets	Kinase Targets
GW297361X	Period	10	AMPKα1, AurB, AurC, BRSK1, BRSK2, CDK2, GSK-3β, JAK3, MST2, MST4
GW575808A	Period	5	ABL2, AurC, LCK, SRC, YES
GW607049C	Period	2	ABL2, EphA2
GSK1713088A	Period	1	INSR
GW305178X	Period	15	AMPKα1, AurB, AurC, BRSK1, BRSK2, CDK2, GSK-3β, HIPK1, JAK3, MAP2K1, MST2, MST4, PHKγ1, SRC, TBK1
GW853606X	Period	1	PLK1
SB-236687	Period	1	PKCα
GW513184X	Period	9	ABL2, AurB, AurC, GSK-3β, LCK, MNK1, MNK2, SRC, YES
GW781673X	Period	1	HIPK1
GW805758X	Arrhythmic	4	AurC, CDK2, GSK-3β, HIPK1

Table 3.4 Description of the PKIS Inhibitors That Significantly Alter Circadian Period or Amplitude at 600nM in Per2::Luc U2OS Cells.

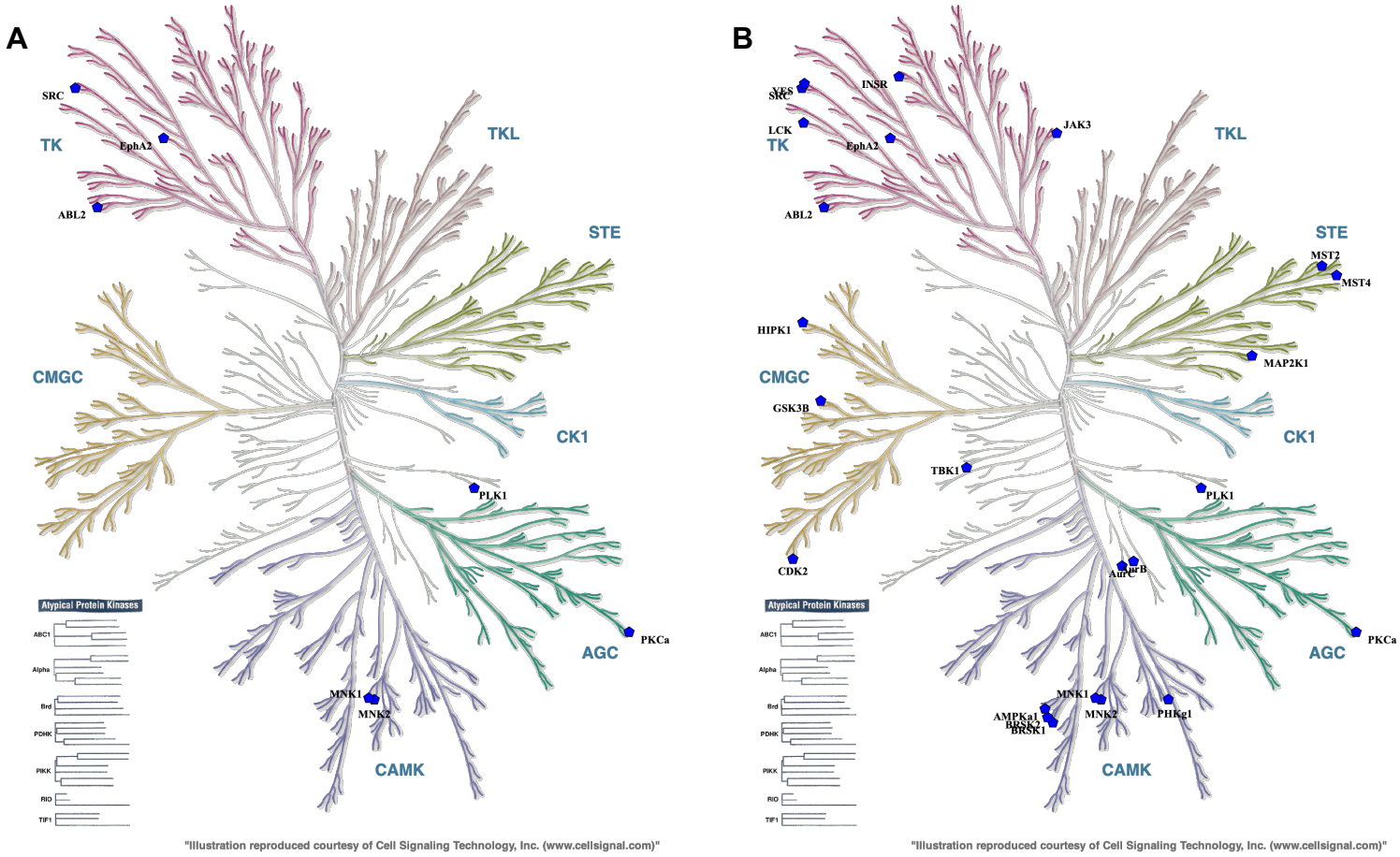

Figure 3.12 The Phylogenetic Relatedness of Kinases Targeted by PKIS inhibitors with a Circadian Phenotype in Per2::LUC U2OS cells. The circadian phenotype of the PKIS was tested at 100nM and 600nM in *Per2::LUC* U2OS cells. (A) Seven kinases changed circadian period when inhibited at 100nM by a PKIS inhibitor and were also significantly inhibited by LiCl. (B) 24 kinases changed circadian period when inhibited at 600nM by a PKIS inhibitor and were also significantly inhibited by LiCl. Figure created using Kinmap (Eid et al., 2017).

None of the circadian hit kinases detected with 100nM PKIS molecules in U2OS cells were also significant in the 100nM PKIS screen in MMHD3 cells. At 600nM, six of the 16 kinases found to be circadian hits in MMHD3 cells were also hits in U2OS cells. Altogether, the PKIS screens conducted demonstrate that many of the kinases that lithium inhibits *in vitro* can alter the circadian rhythm when pharmacologically inhibited with a small molecule. In addition, many kinases that lithium is capable of inhibiting have a circadian role across species and cell type. Thus, lithium could exert its circadian phenotype by inhibiting kinase activity.

3.2.3 siRNA Knockdown Validated the Role of Lithium-Sensitive Kinases in Modulating Circadian rhythms

The PKIS screen provided encouraging support of the hypothesis that lithium can exert changes to therapeutically relevant pathways by altering widespread kinase activity. Therefore to confirm the findings from the PKIS screen and to build evidence that inhibition of lithium-sensitive kinases leads to alterations in circadian rhythms, a circadian reporter assay was performed using *Bmal*::LUC MMHD3 cells in the presence of siRNAs to ablate lithium-sensitive kinases.

MMHD3 cells were treated with siRNA targeting a kinase of interest for 24 hours before they were synchronised and their circadian luminescence was measured. Casein kinase I δ (*Csnk1δ* or *Csk1δ)* was used as a positive control for period lengthening (though incidentally it was also significantly inhibited by lithium in the original cell-free *in vitro* screen of kinase activity) and a non-targeting siRNA was used as a negative control (NTC) (Etchegaray *et al.*, 2009, 2010). Alteration of

the circadian rhythm was considered significant if knockdown altered the period or amplitude more than three standard deviations away from the NTC.

17 of the 76 lithium-sensitive kinases altered circadian period when knocked down and nine altered circadian amplitude. Of these, ephrin type-A receptor 4 (EphA4), extracellular signal-regulated kinase 7 (ERK7), C-terminal Src kinase (CSK), Ribosomal S6 kinase 2 (RSK2), and insulin receptor (INSR) impacted both period and amplitude (Fig. 3.13). Examination of the shared genetics of the hit kinases revealed that the circadian kinases fall across the kinase phylogenetic tree, much like the hit kinases from the PKIS screen and the cell-free *in vitro* examination of kinase activity (Fig. 3.14).

A cross comparison of the kinases that altered period or amplitude when pharmacologically inhibited at 600nM in MMHD3 cells with those that altered period or amplitude when genetically knocked down with siRNA in the same cell line revealed four kinases that had an effect both conditions: CSK, INSR, Protein Kinase C α (PKCα), and Microtubule Affinity Regulating Kinase 3 (MARK3) (Table 3.5). GW301888X targeted CSK and increased amplitude by 264.5±16.25% (Fig. 3.8F). siRNA-mediated knockdown of *Csk* reduced amplitude by 26.84±0.12% and reduced period by 5.43±0.67% (Fig. 3.13D,F). Though these two methods of kinase inhibition had opposing effects, it is worth noting that GW301888X inhibited other non-lithium sensitive kinases by 50% or more, including 100% inhibition of Receptor tyrosine-protein kinase erbB-4. INSR was targeted by two molecules in the PKIS set: GSK1713088A and GSK1173862A. GSK1713088A shortened period by 5.83±0% while GSK1173862A rendered cells arrhythmic (Fig. 3.8C&D).

GSK1173862A inhibited 10 lithium-sensitive kinases by 50% or more, giving a possible explanation for the arrhythmic response (Table 3.2). siRNA knockdown mimicked the phenotype driven by GSK1713088A, as it reduced period by 6.76±0.67% (Fig. 3.13D). Knockdown of *Insr* also reduced amplitude by 39.08±0.82% without reducing cell viability (Fig. 3.13F,H). In MMHD3 cells, PKCα was targeted by 600nM SB-236687 in the PKIS, which increased period by 18.5±10.95% (Fig. 3.8D). siRNA knockdown of *Prkca* (the gene encoding PKCα) also increased period by 7.6±0.63% (Fig. 3.13B). Finally, MARK3 was targeted by GSK1173862A in MMHD3 cells, though GSK1173862A rendered the cells arrhythmic (Fig. 3.8C). *Mark3* siRNA knockdown increased period by 7.0±2.53% (Fig. 3.13B).

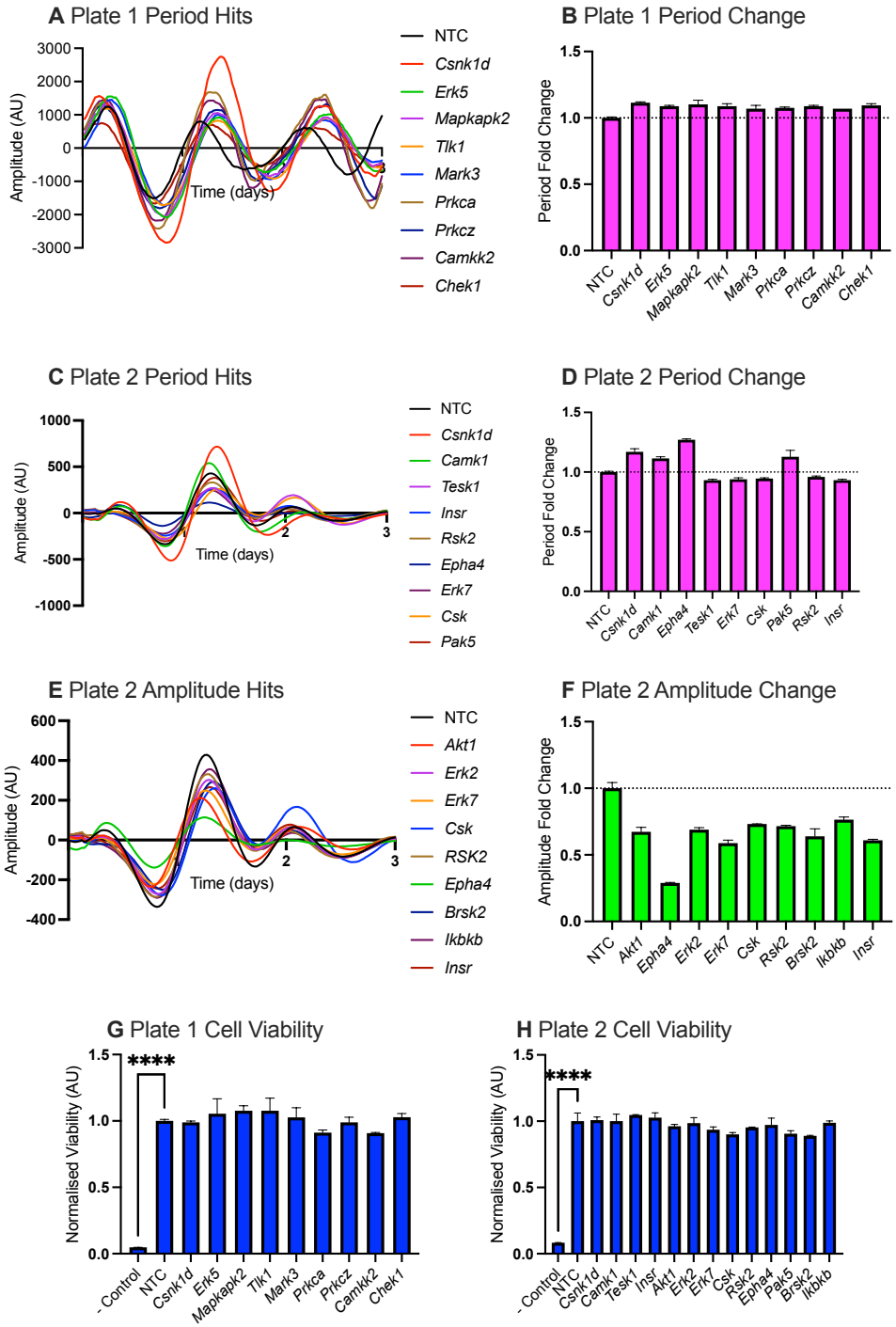

Figure 3.13 The Circadian Impact of siRNA-Mediated Knockdown of Lithium-Sensitive Kinases in Bmal::LUC MMHD3 Cells. 76 lithium-sensitive kinases were independently knocked down in *Bmal*::LUC MMHD3 cells and luminescence was measured to assess their role in circadian period and amplitude. Compounds that altered period or amplitude greater than three standard deviations away from a vehicle control were considered significant hits. Only significant hits are plotted above. (A) Normalised traces of kinase targets that significantly altered period in plate 1 and (B) the period fold change induced by knockdown of these kinases. (C) Normalised traces of kinase targets that significantly altered period in plate 2 and (D) the period fold change induced by knockdown of these kinases. (E) Normalised traces of kinase targets that altered amplitude in plate 2 and (F) the amplitude fold change induced by knockdown of these kinases. Cell viability after drug administration in (G) plate 1 and (H) plate 2. Significance of (G-H) determined using one-way ANOVA with a Dunnett's post hoc test. N=2.

siRNA Hits	Circadian Alteration	Total PKIS Molecules Targeting Kinase	PKIS Assays in Which PKIS Molecules Were Significant	PKIS Molecules That Targeted Silenced Kinase
AKT1	Amplitude	0	N/A	N/A
BRSK2	Amplitude	2	600nM U2OS	GW297361X, GW305178X
CaMK1α	Period	0	N/A	N/A
CaMKK2	Period	0	N/A	N/A
CHK1	Period	0	N/A	N/A
CK1δ	Period	0	N/A	N/A
CSK	Amplitude, Period	1	600nM MMHD3	GW301888X
EphA4	Amplitude, Period	0	N/A	N/A
Erk2	Amplitude	0	N/A	N/A
Erk5	Period	0	N/A	N/A
Erk7	Amplitude, Period	0	N/A	N/A
IKKβ	Amplitude	0	N/A	N/A
INSR	Amplitude, Period	2	600nM MMHD3, 600nM U2OS	GSK1713088A, GSK1173862A
MAPKAPK2	Period	0	N/A	N/A
MARK3	Period	1	600nM MMHD3	GSK1173862A
PAK5	Period	0	N/A	N/A
PKCα	Period	1	600nM MMHD3, 100nM U2OS, 600nM U2OS	SB-236687
PKCζ	Period	0	N/A	N/A
RSK2	Amplitude, Period	0	N/A	N/A
TESK1	Period	0	N/A	N/A
TLK1	Period	0	N/A	N/A

Table 3.5 Description of the kinases which altered circadian period or amplitude when knocked down with siRNA in Bmal::LUC MMHD3 cells.

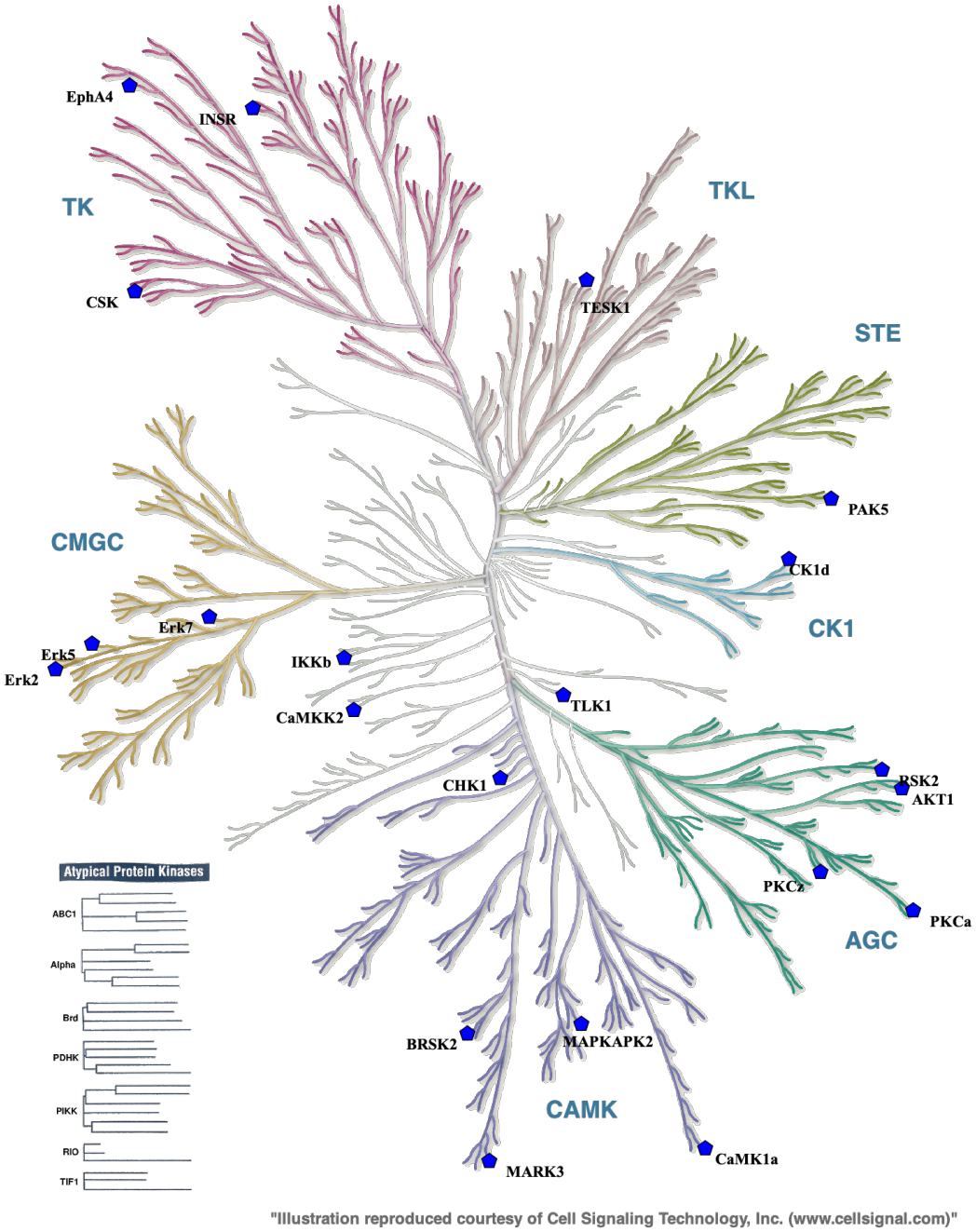

Figure 3.14 The Phylogenetic Relationship Between Lithium-Sensitive Kinases that Alter Circadian Phenotype When Genetically Knocked Down. The siRNA-mediated knockdown of 21 kinases that lithium inhibits *in vitro* altered the circadian period or amplitude of *Bmal*::LUC MMHD3 cells. These kinases are genetically diverse, falling across the phylogenetic tree. Figure created using Kinmap (Eid et al., 2017).

3.2.4 Pathway Enrichment Analysis of Lithium-Sensitive Kinases

Pathway enrichment analysis using G:Profiler was performed on the kinase hits from our 600nM PKIS MMHD3 screen, our 600nM PKIS U2OS screen and our siRNA MMHD3 circadian screen. KEGG pathway enrichment analysis revealed the significant overrepresentation of the insulin signalling pathway, the MAPK signalling pathway, the mammalian/mechanistic target of rapamycin (mTOR) signalling pathway, and the thyroid hormone signalling pathway in all three screens.

3.3 Discussion

Our investigation aimed to test the hypothesis that lithium induces widespread signalling alterations by inhibiting a wider range of kinases than previously envisaged. Here we show that lithium can indeed alter the *in vitro* activity of as many as 76 kinases. Furthermore, we demonstrate that inhibition of many of these kinases has functional consequences as exemplified by alterations of circadian period and amplitude. Given that the majority of the small molecules in the PKIS library inhibited more than one kinase, we further refined our targets using an *in vitro* siRNA screen. This dual approach of small molecule and siRNA, in addition to giving us chemical starting points to investigate lithium's molecular targets, has also identified a range of kinases' roles in circadian rhythms. It is probable that lithium is capable of inhibiting kinases beyond the hits that we identified as our initial *in vitro* cell-free assay used 10mM magnesium acetate, which would reduce lithium's potency in the competition for magnesium binding sites.

3.3.1 Lithium's *In Vitro* Kinase Inhibition Could Alter Circadian Phenotype

Comparing our list of lithium-sensitive kinases which have a circadian role to known circadian kinases revealed that seven of our 50 lithium-sensitive kinases are known to have circadian rhythms of activity which are ablated by sleep deprivation (Brüning *et al.*, 2019). These seven kinases included ABL Proto-Oncogene 2 Non-Receptor Tyrosine Kinase (ABL2), protein kinase B (AKT – an activator of the insulin pathway), brain-selective kinase 1 (BRSK1), BRSK2, GSK-3β, dual specificity mitogen-activated protein kinase kinase 1 (MAP2K1), and PKCα. In fact, two of these (ABL2 and BRSK1) are known to be phosphorylated with increased sleep need (Wang *et al.*, 2018). Given that lithium lengthens circadian period and amplitude and also increases slow wave sleep (a sleep marker of increased sleep need/pressure), these kinases provide a mechanism through which lithium could alter both circadian rhythms and sleep architecture (Borbély *et al.*, 1981; Friston *et al.*, 1989; Li *et al.*, 2012). An examination of the KEGG pathways enriched in the set of circadian kinases inhibited by lithium revealed that these kinases participate in similar pathways in human and mouse tissue, in disparate cell types and under different methods of kinase inhibition. Four KEGG pathways were significantly enriched among the kinase hits from the 600nM PKIS MMHD3 screen, the 600nM PKIS U2OS screen, and the siRNA-mediated knockdown screen in MMHD3 cells. These pathways were the insulin signalling pathway, the MAPK signalling pathway, the mTOR signalling pathway, and the thyroid hormone signalling pathway.

3.3.1.1 The Insulin Signalling Pathway

The insulin pathway has a well-documented impact on the circadian rhythm. In fact, a genome-wide study of the knockdown of 22,468 genes in U2OS cells found that the insulin signalling pathway was overrepresented among the genes that altered circadian period or amplitude (Zhang *et al.*, 2009). The circadian rhythm drives and responds to feeding patterns, and insulin largely mediates this interaction. Insulin is secreted in response to food intake and it increases translation of *Per2* transcripts through activation of the PI3K pathway and mTOR (Crosby *et al.*, 2019). *Per2*$^{(-/-)}$ mice have been shown to have shorter periods than WT in DD and constant light (LL), possibly explaining how our siRNA knockdown of *Insr* and inhibition with 600nM GSK1713088A led to period shortening in MMHD3 cells (Tamiya *et al.*, 2016). Inhibition of INSR with 600nM GSK1173862A led to arrhythmicity in MMHD3 cells, though GSK1173862A has nine other lithium-sensitive kinase targets which are likely responsible for this phenotype. Inhibition of INSR by GSK1713088A led to period lengthening in U2OS cells at 600nM, which is contrary to the literature mentioned above. However, it is important to note that lithium is ineffective in altering the circadian rhythms of U2OS cells at all viable concentrations (Sanghani, 2018). Thus cell type differences may contribute to the different actions of GSK1713088A on period in MMHD3 and U2OS cells at 600nM. It is likely that lithium's modulation of circadian rhythms through INSR would mimic the short period phenotype seen in MMHD3 cells.

Insulin and AKT have been shown to phosphorylate CLOCK at Ser^{845}, negatively regulating its nuclear translocation. Insulin-stimulated AKT activation also leads to the nuclear exclusion of BMAL1 and *Akt*$^{(-/-)}$ knockout mice have

dampened amplitude of wheel-running activity. Correspondingly, mice with a mutation in Ser^{845} also exhibit reduced amplitude of hepatic expression of *Per2*, *Rev-erbα*, and *Dbp* (Lizcano and Alessi, 2002; Dang *et al.*, 2016; Luciano *et al.*, 2018). Our siRNA screen supports these results, showing a reduction in hepatocyte circadian amplitude upon *Akt* and *Insr* knockdown. Amplitude dampening was also observed on knockdown of inhibitor of nuclear factor kappa-B (NF-κB) kinase β (IKKβ). siRNA-mediated knockdown of IKKβ does not alter amplitude in U2OS cells and evidence for the impact of the pharmacological inhibition of IKKβ on amplitude is mixed, as some inhibitors increase and others decrease amplitude at various doses in U2OS cells (Shen *et al.*, 2021). Given that IKKβ inhibits insulin signalling, it is likely that IKKβ influences amplitude through the mTOR pathway discussed below, the ablation of which reduces amplitude (Gao *et al.*, 2002; Ramanathan *et al.*, 2018). IKKβ was not targeted by the PKIS. In a related role, BRSK2 helps activate glucose-stimulated insulin secretion (Nie *et al.*, 2012). Though it has not been reported to have a circadian role, it is plausible that knockdown of the kinase would reduce amplitude through reducing insulin signalling – as was shown in our siRNA screen.

An important output of insulin signalling is activation of the PI3K/AKT pathway, which results in inhibitory phosphorylation of GSK-3β and activation of glycogen synthesis (Lizcano and Alessi, 2002). As previously discussed in Chapter 1, GSK-3β alters the activity of key circadian proteins such as REV-ERBα and is even among the kinases found to undergo circadian phosphorylation (Yin *et al.*, 2006; Brüning *et al.*, 2019). siRNA knockdown and pharmacological inhibition of GSK-3β is known to reduce period, and indeed pharmacological inhibition of the kinase via 600nM SB-675259-M resulted in a 14.95±3.04% reduction in period in MMHD3

cells (Hirota *et al.*, 2008; Li *et al.*, 2012). At 600nM, four PKIS hits (GW513184X, GW297361X, GW305178X, and GW805758X) that targeted GSK-3β lengthened circadian period in U2OS cells. However, each of the inhibitors had other targets and GW805758X induced arrhythmicity overall, demonstrating that the overall pattern of kinase activity is more important in ultimately determining circadian phenotype than the activity at a single target.

We identified the insulin signalling pathway as a newly discovered target for lithium's circadian modulations. Importantly, it feeds into and influences the MAPK pathway and mTOR signalling pathway, which were also pathways significantly altered by lithium (Boucher, Kleinridders and Kahn, 2014). Furthermore, it has been suggested that insulin and noncanonical thyroid hormone receptor signalling converge on the PI3K/AKT pathway, which was also significantly enriched in the hits from each 600nM PKIS assay and the hits from siRNA-mediated knockdown in MMHD3 (Hönes *et al.*, 2017).

3.3.1.2 The MAPK Signalling Pathway

Interrogating LiCl against 140 kinases *in vitro* revealed that lithium is capable of modulating 26 kinases of the MAPK signalling pathway. The MAPK pathway is activated by mitogens that elicit intracellular responses which regulate cell differentiation, cell proliferation, inflammatory response and apoptosis (to name a few) (Wei and Liu, 2002). The MAPK signalling cascade involves the sequential phosphorylation and activation of kinases. Initially the MAPK kinases (MAPKKs) are phosphorylated and activated in response to an external stimulus.

These in turn activate MAPKs through phosphorylation, which then modulate the expression of 200-500 genes. Four major MAPK pathways have emerged – the extracellular-signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), p38, and ERK5 pathways – and the first three have been tied to the circadian clock (Plotnikov *et al.*, 2011; Goldsmith and Bell-Pedersen, 2013).

The ERK pathway has been shown to mediate entrainment of the SCN to light stimuli. Photic stimulation during the dark phase leads to the accumulation of activated phosphorylated ERK1/2 in the SCN and circadian phase shift in mice on running wheels. Application of a MAPK pathway inhibitor reduces this light-induced phase shift by over 90%. Calcium/calmodulin kinase (CAMK) inhibitors reduce light-induced activation of ERK, indicating that CAMK kinases work upstream of the MAPK pathway (Butcher *et al.*, 2002). Calcium/calmodulin-dependent (CAM) protein kinase kinase 2 (CAMKK2) is a CAM kinase that activates CAMK1 by phosphorylating it on its activation loop (Corcoran and Means, 2001; Racioppi and Means, 2012). In the fungus *Neurospora*, CAMK1 knockout has been linked to large phase shifts and modest period lengthening (Yang *et al.*, 2001). These results are contended as another group found the CAMK1 knockout *Neurospora* strain to be arrhythmic (Wang *et al.*, 2021). We reproduced the former finding in mammalian cells, showing that knockdown of both CAMKK2 and its target CAMK1 produce period lengthening effects. (Um *et al.*, 2011; Racioppi and Means, 2012).

Evidence also suggests that the protein kinase A (PKA) signalling cascade converges with MAPK to activate cyclic adenosine monophosphate response element binding protein (CREB) in cultured SCN neurons. Light exposure drives

rapid phosphorylation of CREB at Ser133, and this is a MAPK and PKA-dependent process (Dziema *et al.*, 2003; Wheaton *et al.*, 2018). Mice with mutations at Ser133 display longer periods and reduced activity amplitude of endogenous wheel running activity in DD (Wheaton *et al.*, 2018). Knockdown of PKAα did not significantly alter circadian period in our hepatocytes while inhibition with 600nM SB-242719 in MMHD3 lengthened period. This is likely through a MAPK pathway-dependent mechanism, as SB-242719 inhibited P38α and P38β with higher potency than PKA.

Consistent with the role of MAPK in CREB activation, it has been shown in the SCN of mice bearing a *Per2::*LUC reporter that inhibition of ERK1 and 2 reduces amplitude (Hamnett *et al.*, 2019). We replicated this finding as siRNA-mediated knockdown of *Erk2* reduced amplitude by 31.11±2.17% in MMHD3 cells. This result illustrates that lithium may modulate some pathways with more efficacy than others. As lithium is a well-known amplitude enhancer and ERK2 inhibition on its own leads to amplitude reduction, this observation reinforces our view that summated activity at the level of the kinome dictates the ultimate circadian phenotype. However, these results also indicate that lithium could alter light shifting responses. This alteration of light response could have consequences for mood as well as circadian rhythms because light therapy is prescribed to alleviate depression (Oldham and Ciraulo, 2014).

Ribosomal S6 kinase 2 (RSK2) is a kinase that is activated by ERK1/2. In turn, RSK2 inhibits ERK1/2 signalling, creating a negative feedback loop (Chan *et al.*, 2021). In Drosophila melanogaster, RSK2 has been found to inhibit Shaggy (an orthologue of GSK-3β). GSK-3β inhibition results in amplitude increase, so it is

possible that RSK2 knockout would have the opposite effect of amplitude dampening. Indeed, RSK2 knockout in the X chromosome has been linked to increased PER accumulation in Drosophila (Beck *et al.*, 2018). Though inhibition of RSK2 by molecules in the PKIS did not alter circadian phenotype, we did see amplitude dampening of 28.47±0.90% in our siRNA screen. It is likely that *Rsk2* knockdown decreases amplitude through a pathway that involves GSK-3β.

A role for the p38 MAPK pathway was also implicated in lithium response, as knockdown of the downstream MAPK-activated protein kinase 2 (MAPKAPK2) lengthened period. Accordingly, lengthening has been reported in mouse fibroblasts upon administration of specific p38 inhibitor VX-745 (Goldsmith *et al.*, 2018).

Importantly, our knockdown assay revealed new MAPK participants in circadian regulation. It has been noted that ERK5 is downregulated after four days of jetlag in human peripheral blood mononuclear cells; however, a role for ERK5 in modulating the molecular clock is yet to be established (Kervezee *et al.*, 2018). Here we show that the fourth MAPK pathway is also tied to the circadian clock and knockdown of *Erk5* results in period elongation. In addition, we show that ERK7, a poorly understood autoactivating MAPK, also plays a role in the circadian system (Abe *et al.*, 1999).

3.3.1.3 The mTOR Signalling Pathway

mTOR acts as an intracellular sensor of nutrient status and translates these signals into cellular metabolism and growth. As an indicator of nutritional status, insulin is one of the upstream activators of mTOR signalling. Accordingly, the

overlap of lithium-sensitive kinases with a circadian role in the insulin signalling pathway and circadian lithium-sensitive kinases in the mTOR-signalling pathway was substantial. Indeed, almost every kinase that was enriched in the insulin signalling pathway after siRNA knockdown was also enriched in the mTOR signalling pathway. The only hit not in the insulin signalling pathway was *Rsk2*. RSK2 illustrates crosstalk between MAPK signalling and mTOR signalling. This crosstalk is seen in the circadian action of mTOR, as mTOR has also been shown to alter entrainment of the SCN to light stimuli. Light increases the accumulation of activated phosphorylated mTOR in the SCN and this accumulation is blocked by mTOR inhibitor rapamycin. Rapamycin also attenuates the entrainment of mice to light pulses (Cao *et al.*, 2010).

Knockdown of mTOR directly has been shown to decrease amplitude and increase period (Ramanathan *et al.*, 2018). We saw decreased amplitude, but also decreased period upon knockdown of mTOR pathway components *Rsk2* and *Insr*. We did, however, see period lengthening on pharmacological inhibition of PKCα with 600nM SB-236687 in U2OS and MMHD3 and upon siRNA-mediated knockdown of *Prkca*. PKCs have been shown to activate mTOR signalling (Liu *et al.*, 2017). Thus mTOR knockdown likely lengthens period through a PKC-mediated pathway and knockdown of *Rsk2* must not impact period through mTOR, but likely by disrupting PER2 rhythms as hypothesised for INSR.

3.3.1.4 The Thyroid Hormone Signalling Pathway

Thyroid hormones regulate a variety of biological processes from metabolism to cell differentiation. Circadian rhythms have a substantial influence on thyroid hormone signalling, as the SCN projects directly to hypothalamic thyrotropin-releasing hormone (TRH) neurons, which regulate thyroid hormone production by producing thyroid stimulating hormone (TSH). There is therefore a substantial amount of research on how circadian rhythms influence thyroid hormone actions (Ikegami *et al.*, 2019).

The majority of studies on how thyroid function in turn affects circadian rhythms have been performed in rat. These have shown shortened behavioural wheel-running period and ablated circadian oscillation of *Per2*, *Nr1d1*, and *Rorα* expression in heart ventricles after thyroidectomy (Schull *et al.*, 1988; Peliciari-Garcia *et al.*, 2018). All of the thyroid hormone signalling pathway hits in our PKIS and siRNA screens (PKA, PKCα, GSK-3β, MAP2K1, sarcoma kinase (SRC), ERK2, and AKT1) were also hits in our other hit KEGG pathways (notably the insulin signalling pathway), except SRC. SRC was targeted in U2OS cells by 600nm GW575808A, GW513184X, and GW305178X, which all lengthened circadian period. Given that siRNA-mediated knockdown of *SRC* has been shown to have no effect on period in *Bmal*::LUC U2OS cells, it is likely that the PKIS inhibitors exerted their circadian phenotype through a combination of the other kinases that they also targeted (Tamai *et al.*, 2018). This, in combination with the fact that lithium still lengthens the wheel-running rhythms of thyroidectomised mice, illustrates that lithium likely does not exert its circadian effects solely through the thyroid signalling pathway and

highlights the importance of lithium's effect across multiple interconnected signalling pathways (Schull *et al.*, 1988).

3.3.1.5 Other Circadian Lithium-Sensitive Kinases

The discovery of these lithium-sensitive pathways and circadian kinases highlights the myriad of processes that control and in turn are controlled by circadian signalling pathways. In addition to the four KEGG pathways which were significantly inhibited by lithium in all three of our circadian screens, we identified further kinases which, to the best of our knowledge, have not yet been reported to alter circadian rhythms. These kinases include CSK, testis-specific protein kinase 1 (TESK1), Tousled-like kinase 1 (TLK1), MARK3, and p21-activated kinase 5 (PAK5).

Previous work from our lab has demonstrated that U2OS cells do not respond to lithium's characteristic chronomodulation of increasing period and amplitude (Sanghani, 2018). Though the circadian rhythm of our U2OS cells were altered by four of the same pathways as those enriched for MMHD3 cells, the specific kinases that were altered in each pathway differed. Indeed, 6 of the 16 kinases inhibited by 600nM PKIS inhibitors in MMHD3 cells were also inhibited in U2OS with a circadian consequence. The overlap between kinase hits in our siRNA knockdown screen and U2OS cells showed three common kinases (PKCα, INSR, and BRSK2). The differences in the kinases impacted in these pathways between MMHD3 and U2OS cells could underlie lithium's inability to alter circadian rhythms in its characteristic fashion in U2OS cells and thus warrant further investigation.

3.3.2 Lithium-Sensitive Kinases Are Implicated in the Pathophysiology of BD

Lithium is capable of inhibiting 76 kinases *in vitro* and we have successfully shown that pharmacological inhibition and/or siRNA-mediated knockdown of 50 of these kinases can alter the circadian rhythm of peripheral clocks. To evaluate if any of the pathways that lithium-inhibited kinases are involved in regulate mood, we investigated the mood-stabilising roles for the three most significantly enriched KEGG pathways for all the 76 kinases that lithium inhibits: the MAPK, the neurotrophin, and the T-cell receptor signalling pathways.

3.3.2.1 The MAPK Signalling Pathway

There is a wealth of evidence tying the MAPK signalling pathway to mood stabilisation and lithium. Lithium and valproate (two structurally dissimilar mood stabilisers) have been linked to the activation of the MAPK pathway and the accumulation of phospho-ERK in the brain. It has been proposed that valproate activates ERK through the phosphorylation of PKA and lithium activates ERK through inhibition of GSK3. Accordingly, inhibition of the ERK pathway has been associated with antidepressant and manic behaviours (Einat, Yuan, Todd D. Gould, *et al.*, 2003; Ludtmann, Boeckeler and Williams, 2011). Stimulation of the MAPK pathway has been linked to increased neurite growth and cell survival via activation of the AP-1 transcription factor, linking the MAPK pathway to lithium's neuroprotective effects (Yuan *et al.*, 2001). We have shown additional levels at which lithium can activate the MAPK pathway by inhibiting PKA, AKT and CSK which all activate ERK signalling (Zimmermann and Moelling, 1999; John M Schmitt and

Stork, 2002). The intracellular signalling environment, however, is very complex. When stimulated by hormones, PKA is activated. PKA can either activate ERK through the GTPase Ras or inhibit ERK through the GTPase Rap1, depending on which GTPase is more prominently expressed in the cell type (Stork and Schmitt, 2002). It therefore seems that the effect of lithium's inhibition of MAPK pathway activity would be highly cell type dependent. This, in fact, makes sense given that the MAPK pathway is essential for basic cell functions including cell proliferation and division, and thus activation of its activity at the whole organism level would have catastrophic effects.

3.3.2.2 The Neurotrophin Signalling Pathway

The neurotrophin signalling pathway is highly interconnected with MAPK signalling and has also been implicated in the pathology of bipolar disorder. Brain-derived neurotrophic factor (BDNF) in particular plays an important neuroprotective role in the survival of neurons and is found at lower levels in BD patients (Kim, Rapoport and Rao, 2010). Congruently, reductions in brain volume and cell atrophy in certain brain regions have been reported in BD (Selvaraj *et al.*, 2012; Wang *et al.*, 2019). BDNF has been shown to activate three major pathways relevant to brain function: the ERK pathway, the PI3K/AKT pathway, and the phospholipase C (PLC) pathway. The activation of ERK signalling promotes cell growth, as discussed previously. PLC-dependent signalling activates the release of intracellular calcium. This activates the CAMK signalling cascade which activates CREB transcription. CAMK signalling is inhibited by PKA-driven phosphorylation of

CAMKK2 (Shaltiel, Chen and Manji, 2007; Numakawa *et al.*, 2010). We demonstrated that lithium can inhibit CAMK1 and CAMKK2 in addition to the ERK pathway and thus lithium's impact on neurotrophin signalling is likely also cell type dependent.

3.3.2.3 The T-Cell Receptor Signalling Pathway

The third most significantly enriched KEGG pathway that lithium inhibits the kinase activity of is the T-cell receptor signalling pathway. In addition to reduced expression of neuroprotective factors like BDNF, BD patients also have increased markers of inflammation, including higher serum levels of tumour necrosis factor (TNF)-α and interleukins, and abnormal T-cell activation (Panaccione, Spalletta and Sani, 2015). Many of the same pathways involved in neurotrophic signalling are involved in T-cell receptor activation, including the ERK pathway, the PI3K/AKT pathway, and the PLC pathway. In addition, we show that lithium inhibits IKKβ, the overactivation of which is tied to carcinomas (Hu *et al.*, 2004). PAK5 is activated downstream of T-cell receptor activation and lithium also inhibits this kinase (Kanehisa, 1997). Therefore, once again there are multiple points for lithium to interfere with T-cell receptor signalling through kinase activity.

3.3.3 Conclusion

In this chapter we explored the hypothesis that lithium inhibits a myriad of kinases. We supported the feasibility of this hypothesis with an *in vitro* cell-free kinase screen demonstrating that lithium significantly inhibits at least 76 kinases. Through the use of a cell-based circadian reporter assay we established that as many

as 50 of the kinases that lithium inhibits play a role in regulating the molecular circadian clock. Several of these kinases have already been shown to play a role in the circadian rhythm, validating our approach. In addition to this we reveal novel circadian roles for kinases hitherto unreported. These findings could be relevant to lithium's therapeutic action, as lithium is a potent chronomodulator and it has been hypothesised that this chronomodulation is necessary for its mood stabilising properties. Thus we present evidence that lithium can induce widespread signalling alterations through general inhibition of kinase activity, uncovering a new mechanism of action for the mood stabiliser.

The implications of lithium's alteration of the phosphoproteome extend far beyond circadian period and amplitude. Kinases are promiscuous proteins and often have multiple targets, participating in a myriad of pathways. Accordingly, the kinases that lithium inhibits have widespread roles, which helps explain how lithium simultaneously alters a variety of biological processes. These range from cell differentiation and inflammatory responses to insulin signalling and calcium signalling. Many of these pathways, including inflammation and MAPK signalling, have been tied to the pathophysiology of BD (Yuan et al., 2001; Einat, Yuan, Todd D Gould, et al., 2003; Panaccione, Spalletta and Sani, 2015). It is tempting, therefore, to speculate this could be a new framework for mood modulation through inhibition of kinase activity. Future studies should examine the action of other bipolar treatments on the phosphoproteome and then narrow the affected kinases down to a few therapeutic targets.

3.4 References

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4. AN INVESTIGATION OF THE CIRCADIAN AND BEHAVIOURAL EFFECTS OF LITHIUM IN MICE

4.1 Introduction

Lithium is a well-described chronomodulator and it has been hypothesised that this modulation underlies lithium's mood stabilisation (Roybal *et al.,* 2007; Kirshenbaum *et al.,* 2011; Timothy *et al*., 2018; Sanghani *et al*., 2021). It has also been suggested that lithium response in patients is determined by the underlying molecular circadian phenotype of the patient. In addition, structurally distinct chronomodulators which mimic lithium's alteration of the molecular clock also alter the behaviour of mice in a similar manner to lithium (Sanghani *et al.,* 2021). Nevertheless, it has not yet been shown whether lithium's circadian modulation is a pre-requisite for its behavioural alterations (McCarthy *et al*., 2019; Sanghani *et al*., 2021).

4.1.1. The Connection Between Circadian and Behavioural Modulation

Disruptions in circadian rhythms of activity, hormone secretion and monoamines have been linked to mood disorders since the 1950s. It is now widely accepted that circadian rhythms and mood are interconnected, as sleep disruptions are a diagnostic criterion for virtually all mood disorders and circadian disruptions are well documented in patients with mood disorders such as bipolar disorder (BD) (Murray and Harvey, 2010; McClung, 2013). Many mood stabilising treatments including total sleep deprivation, bright light therapy, agomelatine and lithium itself impact the circadian system in some manner (Wirz-Justice, 2006; McClung, 2007). Nevertheless, the mechanism and directionality of the relationship between circadian disruptions, sleep, and mood disorders has yet to be established.

Recent work has indicated that individual molecular circadian phenotypes may predict responsiveness to the mood stabilising properties of lithium in patients. Two studies using bipolar patient skin fibroblasts showed that lithium responders are more likely to have shorter basal circadian periods of the core transcription/translation feedback loop (TTFL) (McCarthy *et al.,* 2019; Sanghani *et al.,* 2021). Furthermore, fibroblasts from patients with longer basal periods (25+ hours) show a muted response to lithium-induced period lengthening. These results suggest that lithium's alteration of circadian period may be linked to the overall success of lithium pharmacotherapy, as patients with longer periods were administered a wider range of drugs (Sanghani *et al.*, 2021).

Ex vivo work has also examined whether lithium can alter the circadian properties of the suprachiasmatic nucleus (SCN) and peripheral cells derived from circadian mutant mice (Noguchi *et al.*, 2016). Common mouse models used to generate mice with different period lengths are mice with homozygous deletions of cryptochrome 1 and/or 2 (*Cry1* and/or *Cry2*), which form the negative arm of the primary circadian TTFL (Fig. 1.1). *Cry1* homozygous knockout mice (*Cry1*$^{(-/-)}$ or Cry1KO) have a short basal circadian period of wheel-running activity that is approximately 22.5 hours in constant darkness. *Cry2* homozygous knockout mice (*Cry2*$^{(-/-)}$ or Cry2KO) have longer basal circadian rhythms of approximately 24.6 hours and *Cry1*/*Cry2* double knockout mice (*Cry1*$^{(-/-)}$/*Cry2*$^{(-/-)}$) are behaviourally arrhythmic (Horst *et al.*, 1999). Noguchi *et al.* used wildtype (WT), Cry1KO, and Cry2KO mice all bearing a PER2 luciferase reporter (*Per2*::LUC) to determine whether lithium lengthens period regardless of basal period length. The researchers found that 1mM lithium increased period in the SCN of all three genotypes by about

an hour, but did not increase amplitude (Noguchi *et al.*, 2016). Lithium's impact on amplitude may be dose dependent, as earlier work demonstrated a period and amplitude increase in the SCN of *Per2*::LUC WT mice with 2mM lithium (Li *et al.*, 2012). In contrast to this effect on the SCN, Noguchi *et al.* found that 10mM lithium had no effect on period length in the peripheral clocks of mouse tail fibroblasts, though it did increase the amplitude in all three genotypes (Noguchi *et al.*, 2016). These results confirmed that *Cry* genotype is not involved in lithium-driven period lengthening or amplitude increase, while also demonstrating that choice of peripheral tissue is paramount, as lithium has been demonstrated to lengthen period in other peripheral tissues in WT mice (Li *et al.*, 2012). Thus, while lithium's chronomodulation in human peripheral clocks is determined by periodicity of the circadian clock, the master clock of mice seems to lengthen irrespective of basal period as a result of *Cry* disruption (Li *et al*., 2012; Noguchi *et al*., 2016; Sanghani *et al*., 2021).

As discussed in Chapter 1, disruption of the molecular clock leads to altered behaviour that mimics mania in mice. ClockΔ19 mice show manic behaviours, such as hyperactivity, decreased sleep, and decreased time spent immobile in the forced swim test (FST), and lithium rescues many of these back to WT levels. It has not been shown, however, if lithium also normalises the circadian rhythm in these mice and therefore it remains unclear if lithium's chronomodulation underlies its behavioural alterations (Roybal *et al*., 2007; Kristensen, Nierenberg and Ostergaard, 2018).

Recent evidence supports the use of chronomodulation to alter mood. From a nonpharmacological standpoint, manipulation of the circadian cycle via sleep deprivation, phase advancement, and light therapy are all known therapeutic treatments for improving mood (Bunney and Bunney, 2000). Previous work from our lab demonstrated that nobiletin and CGS-15943 (two chronomodulators with different molecular targets) phenocopy lithium's behavioural modulation in mice (Sanghani *et al.*, 2021). Nobiletin is a natural polymethoxylated flavone that activates retinoid acid receptor-related orphan receptors and preferentially increased amplitude in BD fibroblasts with short/medium circadian periods (He *et al.*, 2016; Sanghani *et al.*, 2021). CGS-15943, a nonspecific adenosine antagonist, preferentially increased period in the same (Ongini *et al.*, 1999; Sanghani *et al.*, 2021). When administered via intraperitoneal injection for 11 days, both compounds increased entries into open areas during the elevated plus maze (EPM) and decreased rearing during the open field test (OFT) in WT mice in a lithium-like fashion (Sanghani *et al.*, 2021). Chronomodulation, therefore, seems a promising route for pharmacological mood stabilisation.

4.1.2 Sex-based Differences in Circadian Regulation

As research progresses it is becoming more apparent that there are differences in circadian regulation between biological males and females. Given the connection between circadian regulation and mood, these differences need to be considered when examining mood stabilisers that function as chronomodulators. Hormonal rhythms are known to be circadian and it has been shown that human

SCN neurons of the master clock express estrogen receptors α and β and progesterone receptors. Thus sex hormones can have a direct input to the master clock (Kruijver and Swaab, 2002). Indeed, the expression of c-fos (a marker of neuronal activity) in the SCN is oestrogen-dependent in females (Peterfi *et al.*, 2004). Furthermore, there is a clear sex-based difference in susceptibility to sleep disorders. Biological females are as much as 41% more likely to experience insomnia than their male counterparts – a percentage that increases at puberty, suggesting hormonal regulation of sleep (Paul, Turek and Kryger, 2008).

Gonadal steroids have been demonstrated to alter the amplitude, period and phase of the circadian rhythm in rodents (Fitzgerald and Zucker, 1976; Morin, Fitzgerald and Zucker, 1977; Davis, Darrow and Menaker, 1983). At the behavioural level of wheel-running activity, female hamsters have an earlier circadian onset of activity while males can entrain to longer days. These differences are removed only following long-term castration, suggesting a role for sex hormones in light-induced phase shifting and entrainment (Davis, Darrow and Menaker, 1983). In humans, dysregulation in circadian body temperature, sleep, and mood occur during menopause in females (Kruijver and Swaab, 2002). Female mice also exhibit larger phase shifts than males (47 minutes) in wheel-running activity after a pulse of light in the subjective evening – a behaviour that is dependent on oestrogen responsiveness via oestrogen receptor subtype 1 in females but not males (Blattner and Mahoney, 2013). A study of intrinsic circadian period in humans also found a sex-based difference. Woman were found to have a shorter circadian period than men and a greater proportion of women had periods shorter than 24 hours (34% vs 14% in men) (Duffy *et al.*, 2011).

In terms of sleep, woman have been found to experience greater amounts of slow wave sleep (SWS) and slow wave activity (SWA) – all indicative of higher sleep pressure in females. Females also accumulate sleep pressure faster than males after sleep deprivation, as indicated with higher SWA (Paul, Turek and Kryger, 2008). Work in mice has demonstrated that female mice spend approximately 1.5 more hours awake per day than males and generally have a more consolidated sleep-wake cycle with fewer arousals and less sleep fragmentation. Removal of the gonads reduces and/or eliminates these sex-based differences in murine sleep (Paul *et al.*, 2006).

It has been proposed that gonadal hormones influence sleep/wake rhythms through the hypothalamic-pituitary-adrenal (HPA) axis. Androgens inhibit and oestrogen excites HPA function. As a result, females have higher levels of corticosterone release – a marker of stress and HPA activity that is circadian in nature (McCormick *et al.*, 2002). HPA activity has been shown to modulate sleep, as corticotropin-releasing hormone (a regulator of the HPA) receptor antagonists increase SWS and reduce waking during the active period of rats (Chang and Opp, 1998). In agreement with these findings, it has been shown that corticotropin releasing factor and adrenocorticotropic hormone (HPA intermediates) increase wakefulness (Chastrette, Cespuglio and Jouvet, 1990). Thus, sex hormones have been shown to influence sleep and circadian rhythms independently and given the cross-talk between the two processes, it is possible that sex hormones could influence one through the other (Borbély, 1982).

4.1.3 Sex-based Differences in Mood Regulation and Lithium Response

Biological sex appears to play a crucial role in mood disorders as well. In a nationally representative face-to-face household survey of 9,282 Americans, researchers found that women were significantly more at risk for anxiety and mood disorders while men had significantly worse impulse-control and substance abuse problems (Kessler *et al.*, 2005). The finding that women across cultures are more prone to depression has been criticised as merely reflecting the fact that women seek medical attention for more minor illnesses while men do not and are therefore more likely to die from suicide. Community surveys seem to indicate that there is a biological component, however, as the majority of the participants have not sought medical attention and women are still more likely to be depressed in these surveys (Weissman and Klerman, 1977).

Women's higher risk of mood disorders could hold true for BD. Though no difference between male and female circadian rhythms was found in BD fibroblasts, a recent meta-analysis including studies from the last decade with more than 1000 subjects reported a larger proportion of women in all BD samples (Dell'Osso, Cafaro and Ketter, 2021; Sanghani *et al*., 2021). This matches previous findings that bipolar women have higher rates of cycling, depressive episodes and suicide (Diflorio and Jones, 2010). The majority of studies included in this meta-analysis collected their subjects in BD speciality clinics, however, introducing bias from only including those receiving treatment (Dell'Osso, Cafaro and Ketter, 2021). In addition, the findings do not match earlier studies which found no sex difference in BD diagnosis. Thus, the sex-based prevalence of BD is still contended (Grant *et al.*, 2005; Merikangas *et al.*, 2007).

No consistent associations between biological sex and lithium response have been reported. A recent within-individual study of Swedish national registries in which patients were used as their own controls found no difference between male and female responses to lithium; both males and females experienced reduced rehospitalisations upon lithium maintenance treatment (Ragazan, Eberhard and Berge, 2020). A metanalysis of 16 papers found that 8 reported a better lithium response in females and 8 reported a better response in males, while the metanalysis' authors own analysis yielded no difference (Viguera, Tondo and Baldessarini, 2000). A study using black swiss mice found that males and females have similar behavioural responses to lithium in a battery of behavioural tests including the FST, EPM, and OFT, yielding them both appropriate models for the study of BD and lithium (Ene, Kara and Einat, 2015). Thus, the evidence seems to suggest there is no sex-based difference in lithium's mood stabilisation.

Though both sexes appear to respond to lithium treatment, sex-based differences in lithium metabolism have been reported. A study in Wistar/ST rats found that at 60 μg/min lithium infused into the femoral artery with a catheter for two hours, female rats had a higher plasma concentration of lithium. In addition, the fractional tubular reabsorption of lithium in the kidney was higher in females at high clinical lithium plasma concentrations (1.3mM to 1.6mM). This reabsorption was found to be largely dependent on sodium-dependent phosphate transporters (Uwai, Yamaguchi and Nabekura, 2021). Further, a study using vizcachas (nocturnal rodents in Argentina) caught from the wild found that daily injections of 1mM lithium per kilogram of bodyweight resulted in a significantly higher accumulation of lithium in the tissues of male vizcachas as compared to females. Unlike the more

recent study of Uwai, Yamaguchi and Nabekura (2021) using rats, these researchers found that male serum levels of lithium were higher than females (García *et al.*, 1999). Thus, species could be an important factor determining sex-based differences in serum lithium levels, along with method of lithium administration and animal procurement (as the vizcachas were simply caught in the wild and thus could have a whole host of confounding factors). Given all of the above, it is clear that further research is needed into the mechanisms underlying possible sex-based differences in lithium pharmacokinetics and response.

4.1.4. Aims

Previous work using BD fibroblasts has demonstrated the importance of underlying circadian phenotype in determining patient response to lithium prophylaxis. To determine if these results were purely due to basal rhythm length, we wanted to see if we could reproduce these results *in vivo* using mutant mice with different basal periods. It has also been hypothesised that lithium's mood stabilising abilities are tied to its circadian alterations. We therefore also wanted to examine whether circadian modulation is necessary for behavioural modulation *in vivo*. Finally, based on the evidence that sex-differences may be evident in sleep and circadian rhythms, BD symptomology, and lithium pharmacokinetics, we included male and female mice in our studies to determine if there are sex-based differences in lithium response in mice.

4.2 Results

4.2.1 Lithium Differentially Alters the Wheel-Running Behaviours of Male and Female Mice

Lithium is a potent mood stabiliser in BD and circadian mutant mice exhibit behaviours that resemble bipolar mania. Lithium returns these manic behaviours, including decreased time spent immobile in the FST and hyperactivity, back to WT levels, but whether lithium corrects the circadian phenotype along with mood is unknown (Roybal *et al.*, 2007). It has further been found that basal circadian period length can predict lithium response in BD patient fibroblasts (McCarthy *et al.*, 2019; Sanghani *et al.*, 2021). We wanted to evaluate this finding in a circadian model using mice with different basal period lengths. We therefore chose WT, Cry1KO, and Cry2KO mouse models, as Cry1KO mice have shorter rhythms than WT and Cry2KO mice have longer rhythms than WT (Horst *et al.*, 1999). Male and female mice were included to determine if sex could play a role in lithium's chronomodulation and behavioural alterations.

WT, Cry1KO, and Cry2KO animals were housed in a 12:12 light–dark (LD) cycle on running wheels for one week to acclimatise to their environment. They were then released into constant darkness (DD) for two weeks and their wheel running activity was monitored to determine basal period length. A half-dose of 7mM lithium chloride (LiCl) was administered in the drinking water for one week to gradually build the dose, as is done in human patients (Gould, Chen and Manji, 2004). A full dose of 14mM LiCl in drinking water was then administered for two weeks – a dose which results in low therapeutic serum concentrations of 0.46 ± 0.14 mM LiCl in C57BL/6J mice (van Enkhuizen *et al.*, 2015). Lithium treatment often

results in diabetes insipidus – a side effect we observed during our pilot experiments. This necessitated a change in bedding every day and the provision of a supplementary bottle of 0.9% sodium chloride (NaCl) to all lithium-treated animals to mitigate the loss of sodium and toxic side effects (Timmer and Sands, 1999; Gould, Chen and Manji, 2004). The 14mM dose of lithium in water, however, proved to be intolerable for our Cry1KO male mice, with some consuming up to 90mL of 14mM LiCl a day. This resulted in 80% of the Cry1KO males reaching the ethical endpoint (losing more than 10% body weight) (Table 4.1). Indeed, similar observations of lithium intolerance have been reported in Cry1KO mice. Schnell *et al.* reported poor tolerance for lithium in Cry1KO mice (presumably male but not specified) (Schnell *et al.*, 2015). However, to our knowledge lithium intolerance being a sex-dependant phenomenon has not been reported previously. In light of this finding, we reduced our full dose of lithium to match that of Li *et al.* (10mM in drinking water) – a dose that induces period lengthening in C57BL/6 mice (Li *et al.*, 2012).

	WT Female	WT Male	Cry1KO Female	Cry1KO Male	Cry2KO Female	Cry2KO Male
Animals That Reached Ethical Endpoint	0	0	1	4	0	0
Total Animals	4	4	5	5	4	6

Table 4.1 Animals That Reached Ethical Endpoints During Administration of 14mM LiCl in Drinking Water.

Baseline recordings of our second cohort revealed that our WT, Cry1KO, and Cry2KO mice indeed had significantly different basal periods. WT females had an endogenous period of 23.58±0.08 hours, Cry1KO females had an endogenous period of 21.28±0.06 hours, and Cry2KO females had an endogenous period of 24.12±0.11 hours (Fig. 4.1). Males showed similar period differences. Male WT mice had an average basal period of 23.63±0.08, Cry1KO male mice had an endogenous period of 21.09±0.06, and Cry2KO males had a basal period of 23.98±0.14 (Fig. 4.2). The basal period of Cry2KO males seemed to be slightly shorter than their female counterparts. Cry2KO males were only 21 minutes longer than WT while female Cry2KO mice were 32.4 minutes longer than their WT counterparts.

After two weeks of DD, the mice were given access to 7mM LiCl in their drinking water for a week and then the full dose of 10mM LiCl in drinking water for two weeks. To prevent Cry1KO animals from drinking too much lithium, they were limited to 30mL 10mM LiCl a day if their previous day's consumption exceeded this volume. The cap of 30mL was chosen based on observations from the pilot that animals which drank more than 30mL a day started showing signs of lithium toxicity. Based on our lab's previous work with BD fibroblasts, we predicted that male and female mice with short and medium periods would exhibit period lengthening on lithium while Cry2KO mice would display a muted response to lithium-induced period lengthening. Contrary to our hypothesis, however, all three female genotypes displayed lithium-induced period lengthening, while the males completely failed to show any response whatsoever. We saw increased lengthening of 30.75±2.27 minutes in our WT females (Fig. 4.3D). The period of female Cry1KO

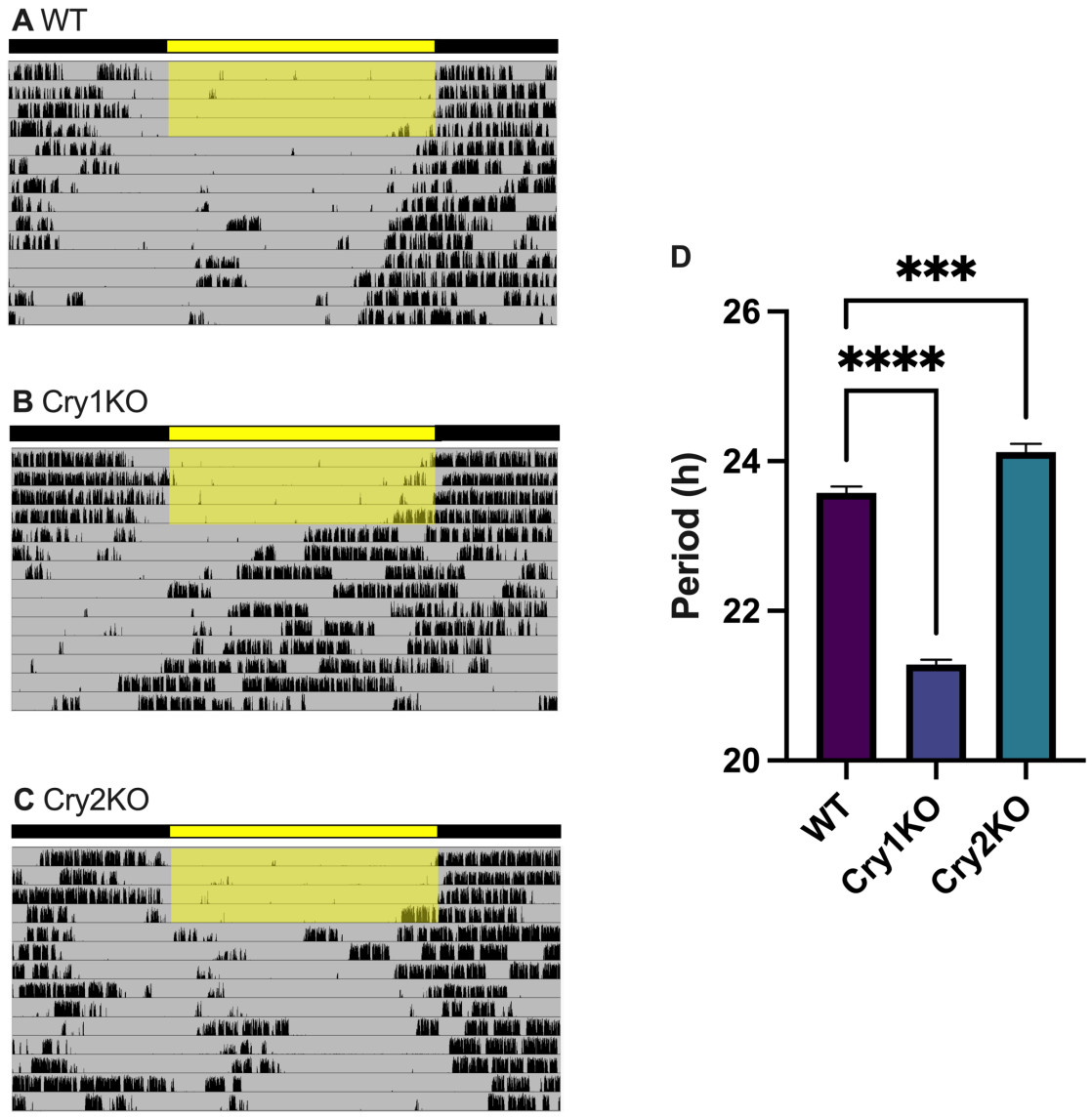

Figure 4.1 The Endogenous Circadian Period of Wheel-Running Activity in Female WT, Cry1KO, and Cry2KO Mice. Representative double-plotted actograms of wheel-running activity from: A) WT, (B) Cry1KO, and (C) Cry2KO female mice plotted over 24 hours during 12:12 LD entrainment and DD. Yellow denotes light applied during entrainment. (D) Endogenous period measured over four days in in DD. Significance determined using one-way ANOVA with a Dunnett's post hoc test. N=6-9.

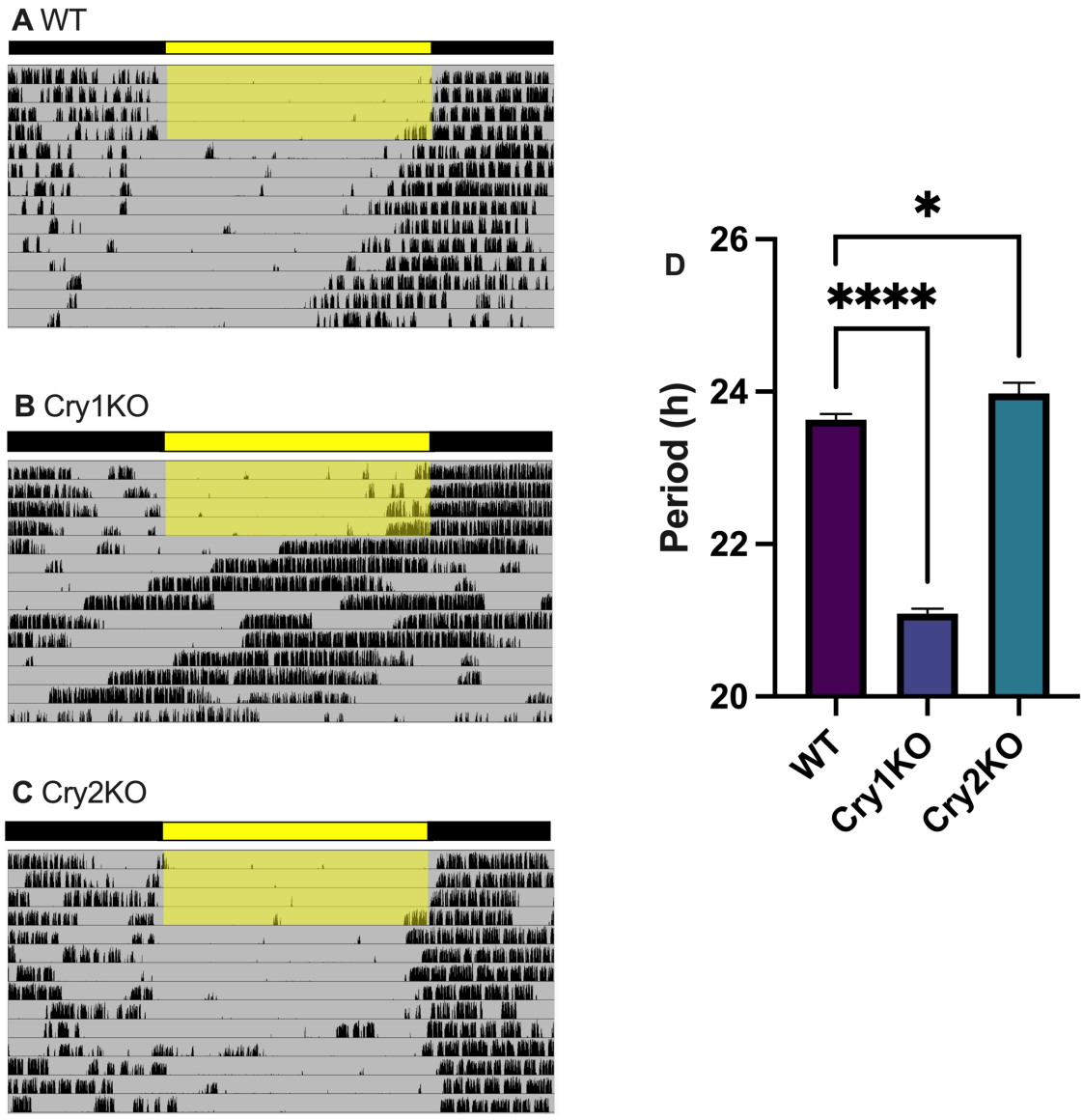

Figure 4.2 The Endogenous Circadian Period of Wheel-Running Activity in Male WT, Cry1KO, and Cry2KO Mice. Representative double-plotted actograms of wheel-running activity from: (A) WT, (B) Cry1KO, and (C) Cry2KO male mice plotted over 24 hours during 12:12 LD entrainment and DD. Yellow denotes light applied during entrainment. (D) Endogenous period measured over four days in in DD. Significance determined using one-way ANOVA with a Dunnett's post hoc test. N=5-7.

mice trended toward significant lengthening of 14.73±5.28 minutes. Female Cry2KO mice also showed a period-lengthening response to lithium of 36.20±11.58 minutes, though this was accompanied with a larger standard error of the mean, as the amount of lengthening ranged from three minutes to 63 minutes Our data

therefore suggests that at the behavioural level of wheel-running activity, the underlying circadian period driven by CRY1 or 2 deletion does not determine lithium response.

The most surprising result of this study was that all three male genotypes failed to demonstrate period-lengthening in their wheel-running activity following lithium treatment (Fig. 4.4). Under the new treatment regime, four Cry1KO males were removed from the study due to lithium-induced weight loss. The baseline periods of male versus female mice were not significantly different for all genotypes. The data of male and female mice were combined to see if combining sexes could mask the sex-specific of lithium (Fig. 4.5). WT animals experienced significant lithium-induced lengthening of 20.44$\pm$4.69 minutes, Cry1KO animals lengthened by 9.43$\pm$4.03 minutes and Cry2KO animals significantly lengthened by 17.17$\pm$7.40 minutes, indicating that studies combining sexes could miss the sex-specific lengthening in females.

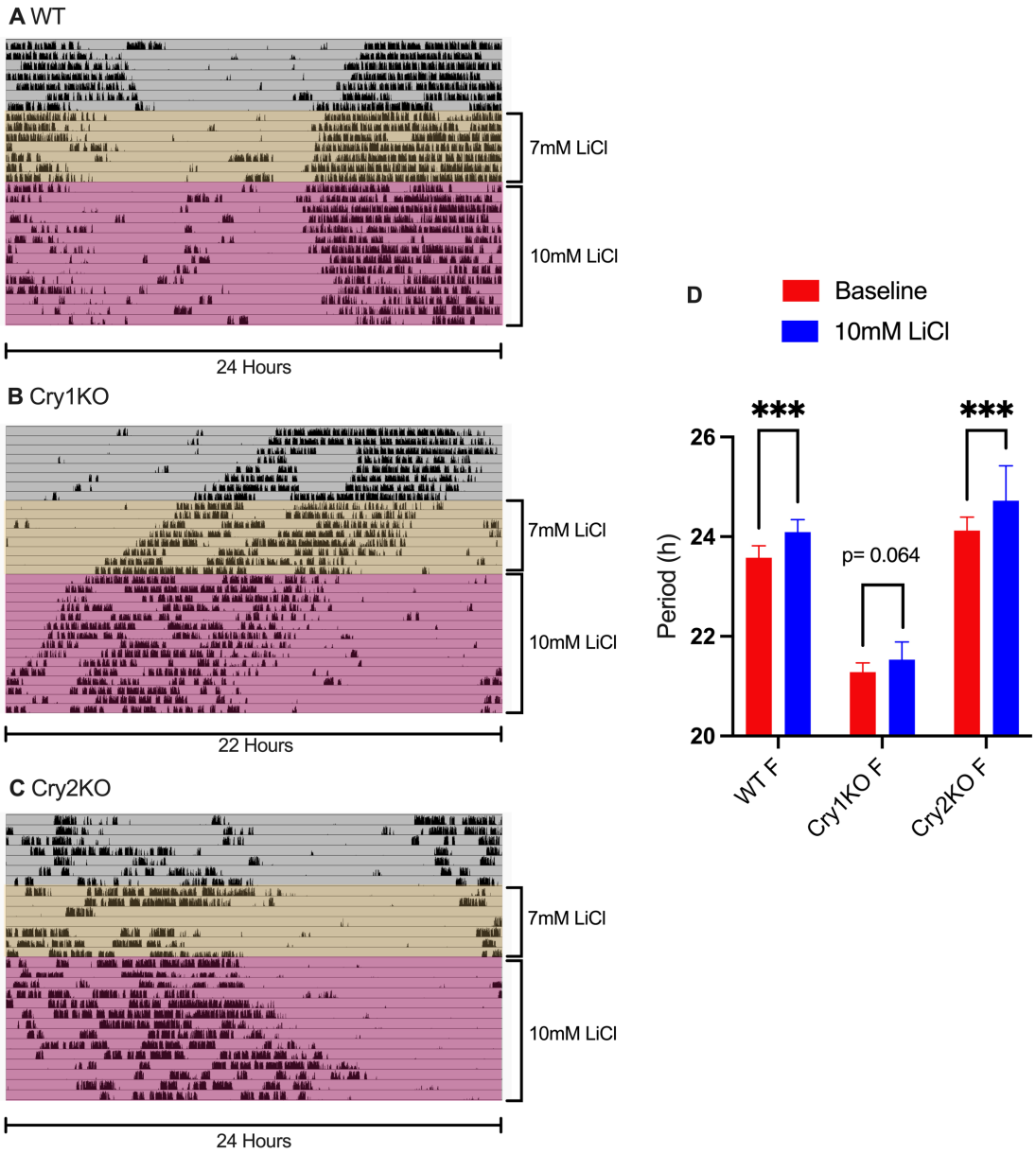

Figure 4.3 Lithium Lengthens Period of Behavioural Rhythms in Female Mice with Short, Medium, and Long Basal Rhythms. Representative double-plotted actograms of wheel-running activity from: (A) WT, (B) Cry1KO and (C) Cry2KO female mice in DD. One week of 7mM lithium administration in drinking water (tan colouring) was followed by 2 subsequent weeks of 10mM lithium administration in drinking water (pink colouring) in DD. (D) Lithium lengthened the period of all three genotypes. Significance determined using a two-way repeated measures ANOVA with a Šídák's post hoc test. N=6-9.

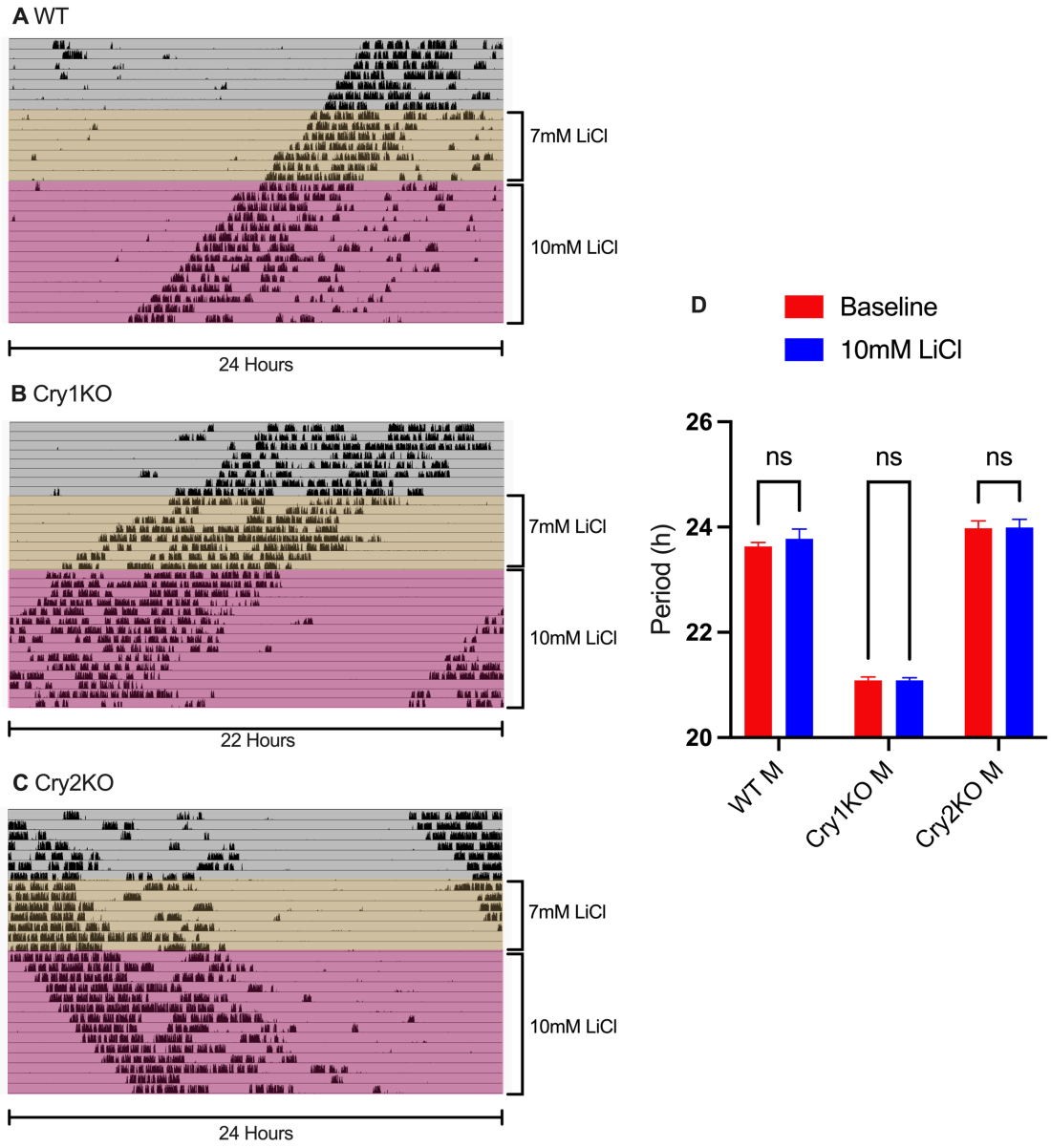

Figure 4.4 Lithium Does Not Lengthen Period of Behavioural Rhythms in Male Mice with Short, Medium or Long Basal Rhythms. Representative double-plotted actograms of wheel-running activity from: (A) WT, (B) Cry1KO and (C) Cry2KO male mice in DD. One week of 7mM lithium administration in drinking water (tan colouring) was followed by 2 subsequent weeks of 10mM lithium administration in drinking water (pink colouring). (D) Lithium did not lengthen the period of all three genotypes. Significance determined using a two-way repeated measures ANOVA with a Šídák's post hoc test. N=5-7.

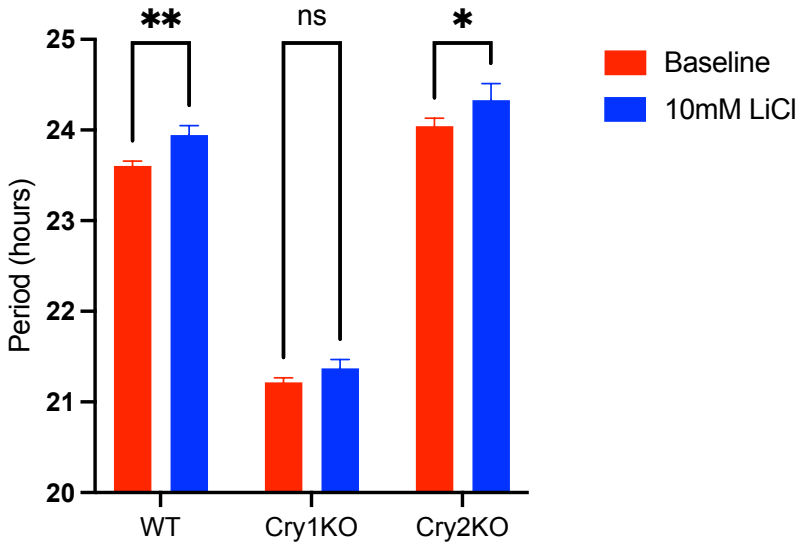

Figure 4.5 Lithium-Induced Period Lengthening Is Significant When Male and Female Mice are Combined. Wheel-running activity was monitored in male and female WT, Cry1KO, and Cry2KO mice. Baseline period was measured during DD and period under lithium treatment was measured during two weeks of 10mM LiCl administration in drinking water. Significance determined using a two-way repeated measures ANOVA with a Šídák's post hoc test. N=13-15.

As the discrepancy in period lengthening could be explained by different drinking behaviours between males and females, drinking behaviour was monitored throughout the two-week administration of 10mM LiCl in drinking water (Fig. 4.6). A two-way ANOVA with Šídák's post hoc test comparing the area under the curve of lithium consumed revealed that only WT females drank significantly more than their male counterparts (P=0.050). There was no significant difference in sodium consumption between sexes of the same genotype. It is important to note that when two WT male mice did consume as much lithium as their female counterparts (up to 20-30mL a day), this led to an acute and immediate weight loss, reaching the ethical endpoint. These results, therefore, indicate that male WT mice cannot tolerate lithium as well as their female counterparts and as a result consumed less lithium. Females across genotypes on average drank more lithium

than males, though this was only significant for WT mice. It is possible, therefore, that the lack of behavioural period lengthening in male mice is due to worsened lithium tolerance and thus lower doses of lithium.

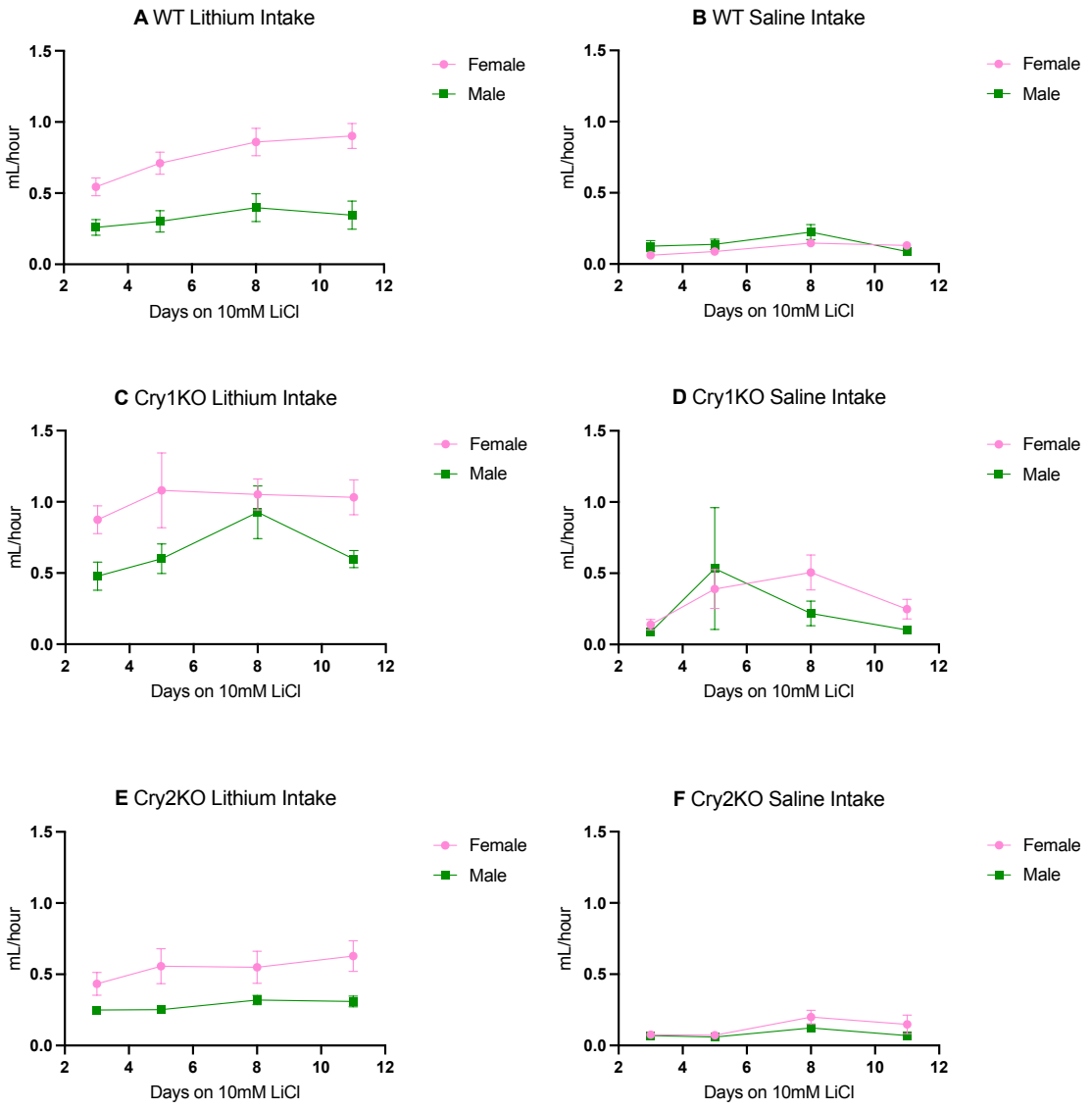

Figure 4.6 WT Female Mice Drink More LiCl Than WT Male Mice. Lithium and saline consumption were measured every 2-3 days during 10mM LiCl administration in drinking water and the amount consumed per hour was calculated. Lithium consumption was monitored for (A) WT, (C) Cry1KO, and (E) Cry2KO animals. Sodium consumption was monitored for (B) WT, (D) Cry1KO, and (F) Cry2KO animals. N=5-9.

Given the sex-based difference in behavioural period change with lithium treatment, we wanted to evaluate if this difference was also apparent *ex vivo* at the level of the master clock (SCN) and peripheral clocks. First, we examined sex-based differences in lithium-induced period lengthening in the SCN. For this investigation, isolated organotypic SCN sections from *Per2*::LUC mice were subjected to 4mM lithium treatment – a concentration selected based on previous work from our lab (Fig. 4.7) (Sanghani, 2018). The period of female SCN slices lengthened by 1.04 ± 0.15 hours and the period of male SCN slices lengthened by 1.28 ± 0.41 hours in response to 4mM LiCl. An unpaired t-test found no statistically significant difference between period-lengthening in female and male SCN (P=0.63). Thus, the sex-based difference in behavioural period-lengthening upon lithium treatment does not occur at the level of the master clock.

To further investigate if there was a sex-based difference in lithium-induced period lengthening in peripheral clocks, we applied lithium to liver slices of the same *Per2*:LUC animals used for SCN work (Fig. 4.8). Period change upon 10mM LiCl treatment was virtually identical in female and male liver slices. Female liver slices lengthened by 1.72 ± 0.27 hours and male liver slices lengthened by 1.72 ± 0.20 hours (Fig. 4.8C). Thus, we found no evidence for a sex-based difference in the response of murine peripheral clocks to lithium treatment.

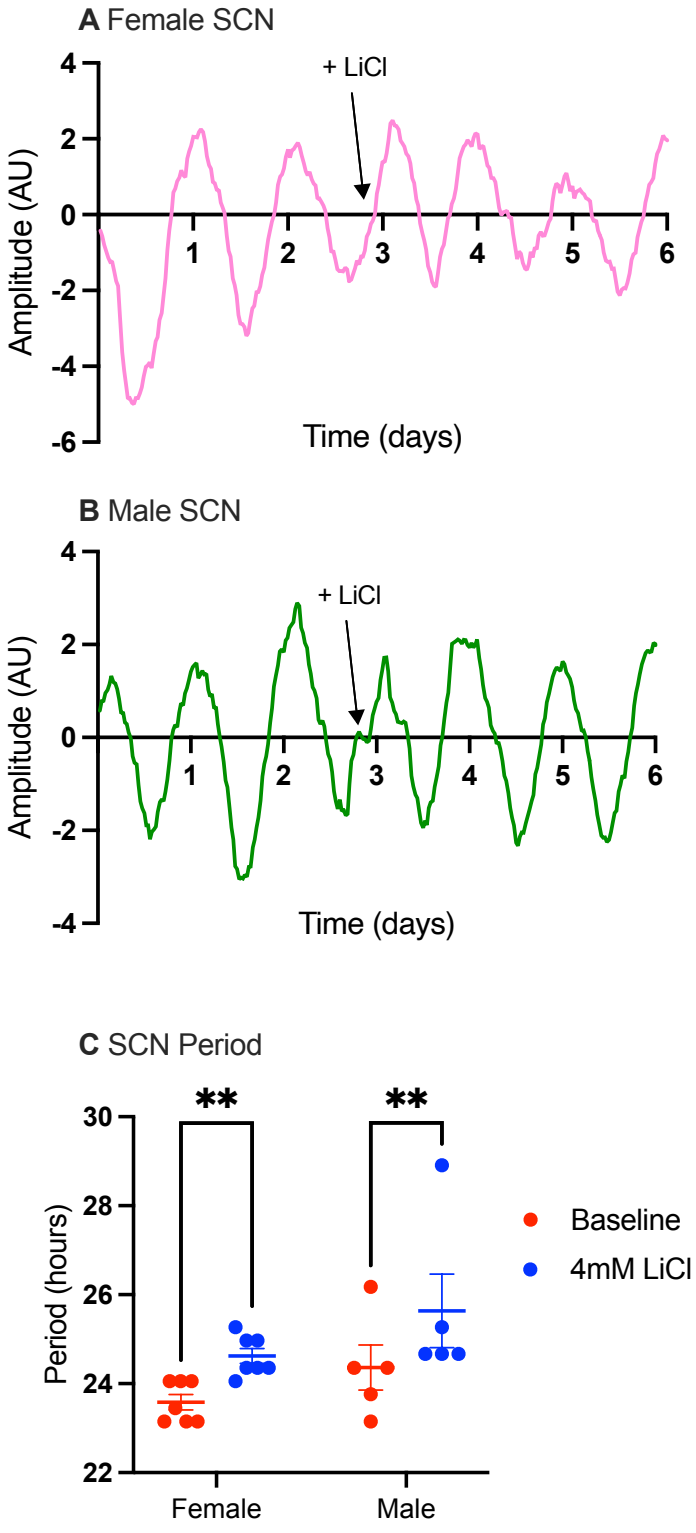

Figure 4.7 4mM Lithium Induces Period Lengthening in Male and Female Organotypic SCN Sections. The circadian luminescence of *Per2*::LUC knockin female and male organotypic SCN sections was measured for 1-3 days to establish a baseline period and then 4mM LiCl was applied. (A) Representative trace from a female SCN section. (B) Representative trace from male SCN section. (C) Baseline period and period after 4mM LiCl treatment in female and male SCN. Significance in (C) determined using a two-way repeated measures ANOVA with a Šídák's post hoc test. N=5-7.

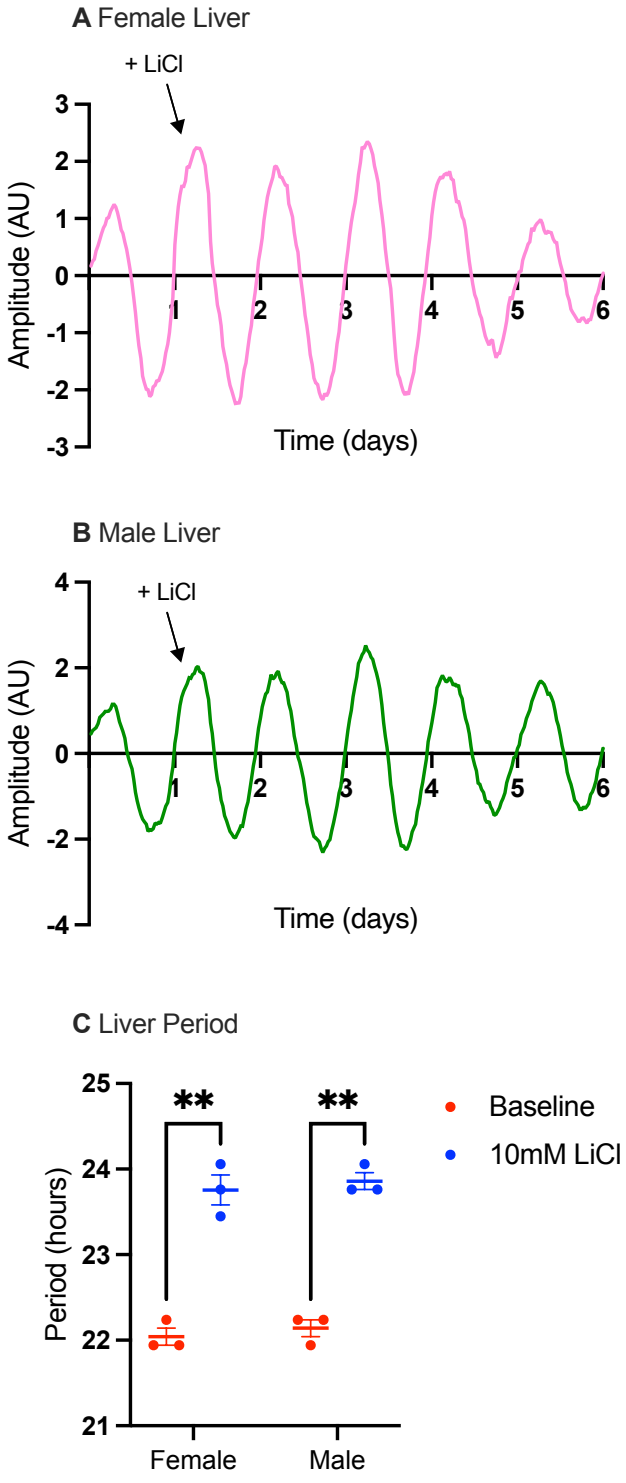

Figure 4.8 10mM Lithium Induces Period Lengthening in Male and Female Liver Sections. The circadian luminescence of *Per2*::LUC knockin female and male liver slices was measured for 1-3 days to establish a baseline period and then 10mM LiCl was applied. (A) Representative trace from female liver section. (B) Representative trace from male liver section. (C) Baseline period and period after 10mM LiCl treatment in female and male liver. Significance in (C) determined using a two-way repeated measures ANOVA with a Šídák's post hoc test. N=3.

4.2.2. Lithium's Modulation of Behaviour in *Cry* Circadian Phenotypes

Given the sex-based difference in wheel-running behaviour after lithium treatment, we wanted to see how these differences corresponded to behavioural outcomes. We also wanted to see if basal period length mediated lithium-associated behavioural changes. We therefore performed behavioural assays on the same Cry mice from our circadian investigations. The FST, EPM, and OFT were chosen as mice under lithium administration display well-validated lithium-specific behavioural changes during these tests (O'Donnell and Gould, 2007). We examined lithium's alteration of immobility in the FST, exploration of open arms in the EPM, and rearing behaviour in the OFT in WT, Cry1KO, and Cry2KO male and female mice.

The same CryKO and WT mice that had been used for the assessment of lithium-induced behavioural period lengthening were used for the FST, EPM, and OFT. To establish a baseline of behaviour for each strain, the OFT and EPM behavioural tests were administered 4 days into DD and the FST was performed 5 days into DD – prior to the initiation of lithium treatment. The tests were then repeated after two weeks of 10mM lithium administration in drinking water (Fig. 4.9).

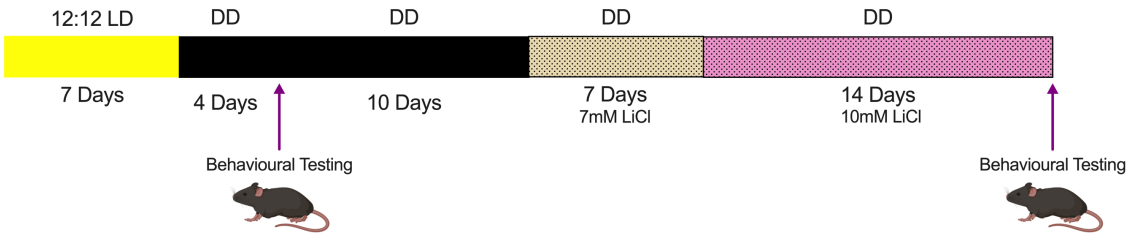

Figure 4.9 Timeline for Behavioural Testing of WT, Cry1KO, and Cry2KO Mice.

4.2.2.1 Lithium Decreases Helpless Behaviour According to Underlying Circadian Phenotype

The FST has historically been used to measure the effects of antidepressants. Improvement in depression-like or helpless behaviour is interpreted as less time spent immobile during the test (Kara, Stukalin and Einat, 2018). The results of the FST indicated that underlying circadian phenotype does indeed affect the antidepressant action of lithium (Fig. 4.10). Lithium improved helplessness to the greatest extent in short period Cry1KO female mice – significantly reducing the time spent immobile by 28.03±8.02%. This was followed by WT female mice whose immobility was significantly reduced by 17.07±5.28%. Meanwhile the long period Cry2KO female mice did not show a statistically significant reduction in immobility. In males, only short period Cry1KO mice showed a significant reduction in time spent immobile (29.41±11.76%). Thus, the results of the FST supports the hypothesis that lithium's modulation of helpless behaviour mirrors its modulation of molecular circadian clocks in BD fibroblasts and has a greater impact in shorter period mice *in vivo*.

4.2.2.2 Lithium's Modulation of Exploratory Behaviours

Previous work from our lab demonstrated that lithium and lithium-like chronomodulators increase the exploration of open anxiety-provoking spaces and also decrease rearing behaviour in WT mice (Sanghani *et al.*, 2021). We therefore hypothesised, as for the FST, that underlying circadian period would mediate lithium's alteration of exploratory behaviour in the EPM and rearing behaviour in the OFT, and these alterations would be greater in females than males.

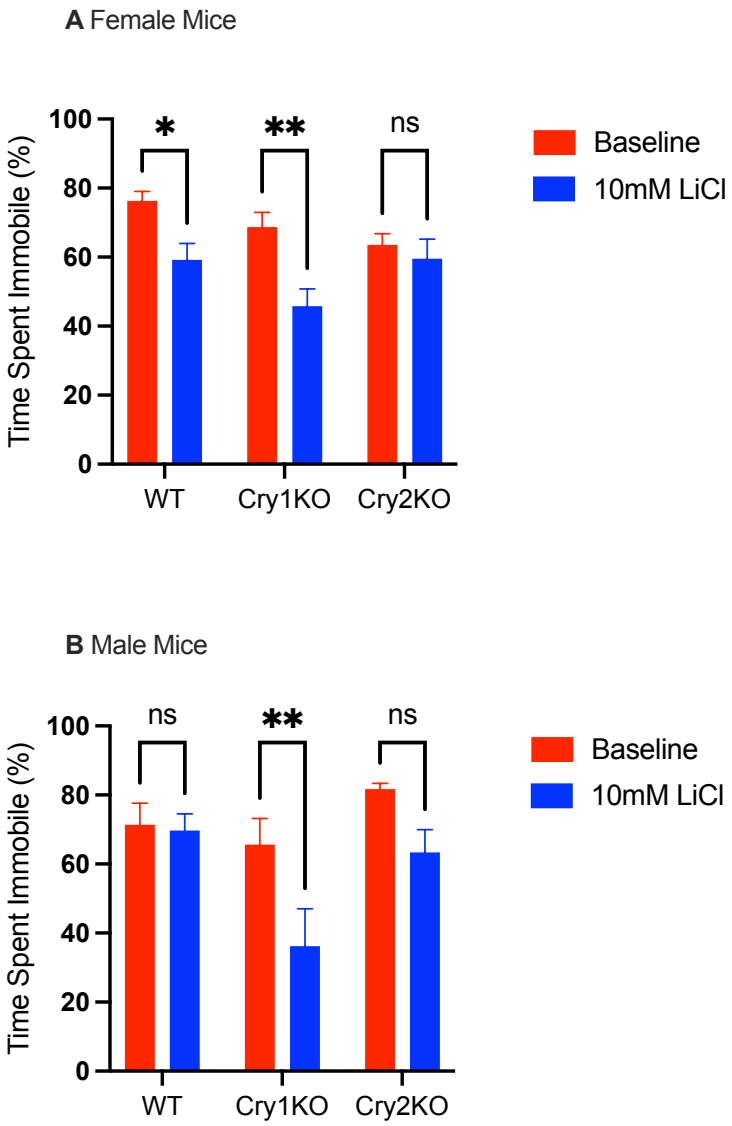

Figure 4.10 Lithium's Antidepressant Action is Dependent on Underlying Cry Circadian Phenotype. The FST was performed on (A) female and (B) male WT, Cry1KO, and Cry2KO mice before and after 10mM LiCl administration in drinking water. The percent of time spent immobile was scored using ANYmaze software. Significance calculated using two-way repeated measures ANOVA with a Šídák's post hoc test. N=5-9.

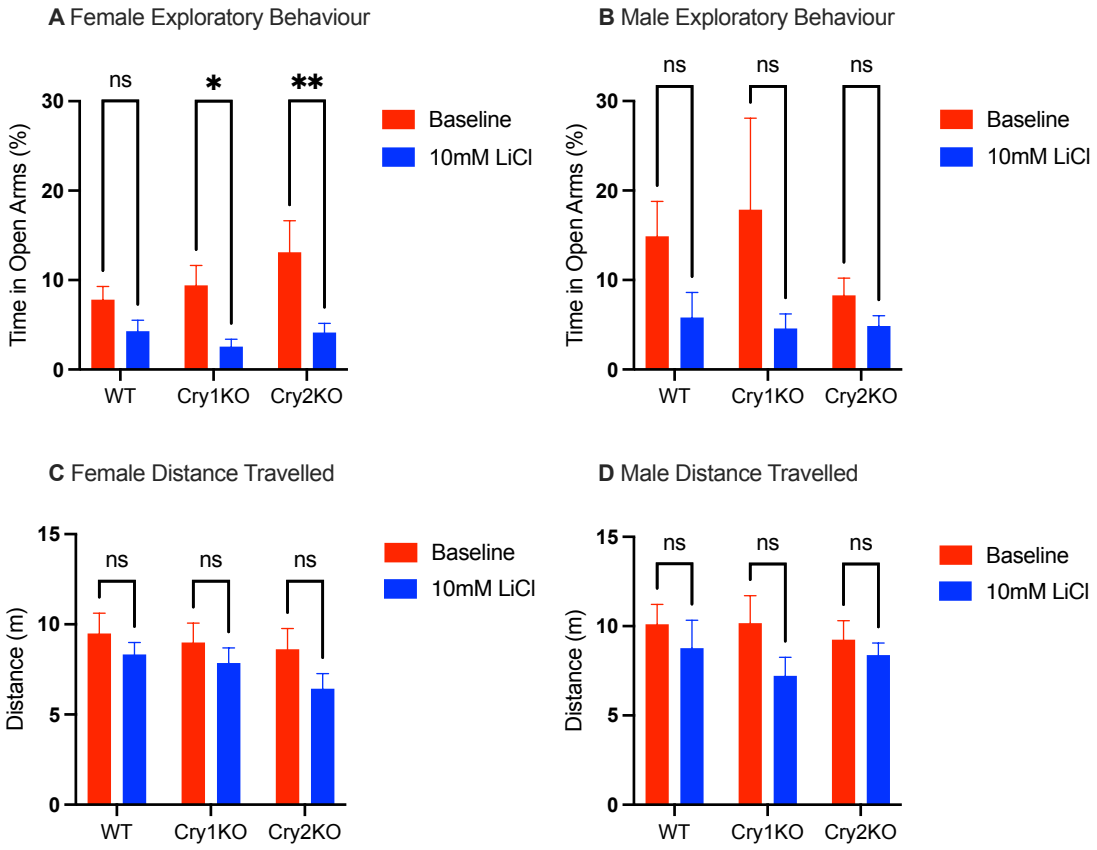

Figure 4.11 Lithium's Anxiolytic-Like Effects. The EPM was performed on (A) female and (B) male WT, Cry1KO, and Cry2KO mice before and after 10mM LiCl administration in drinking water. Distance travelled for (C) female and (D) male mice was measured as a proxy for activity. The percent of time spent in the open arms of the EPM and distance travelled were scored using ANYmaze software. Significance calculated using two-way repeated measures ANOVA with a Šídák's post hoc test. N=4-8.

The EPM is often used to measure anxiolytic properties of compounds of interest (Hogg, 1996). Previous work from our lab showed that lithium increases exploratory behaviour by increasing time spent in the less sheltered areas of the EPM (Sanghani *et al.*, 2021). Our results, however, showed a decrease of open arm exploration behaviour upon lithium administration. Cry1KO and Cry2KO female mice significantly reduced time spent in the open arms of the EPM by 6.84±2.46% and 8.98±2.63%, respectively (Fig. 4.11A), without any significant reduction in

overall activity or distance travelled (Fig. 4.11C,D). This corresponds to a reduction of 72.74±26.13% and 68.42±20.03%, respectively, relative to baseline conditions.

Lithium has been demonstrated to reduce rearing in the OFT (O'Donnell and Gould, 2007; Sanghani *et al.*, 2021). We therefore hypothesised that circadian period would correlate to reduction in rearing and females would rear less than males in response to lithium. However, none of the mice experienced a significant alteration of rearing behaviour, except for WT male mice, who actually increased their time spent rearing after lithium administration (Fig. 4.12B). This, however, could be explained by the fact that their general level of activity increased with lithium administration (Fig. 4.12D).

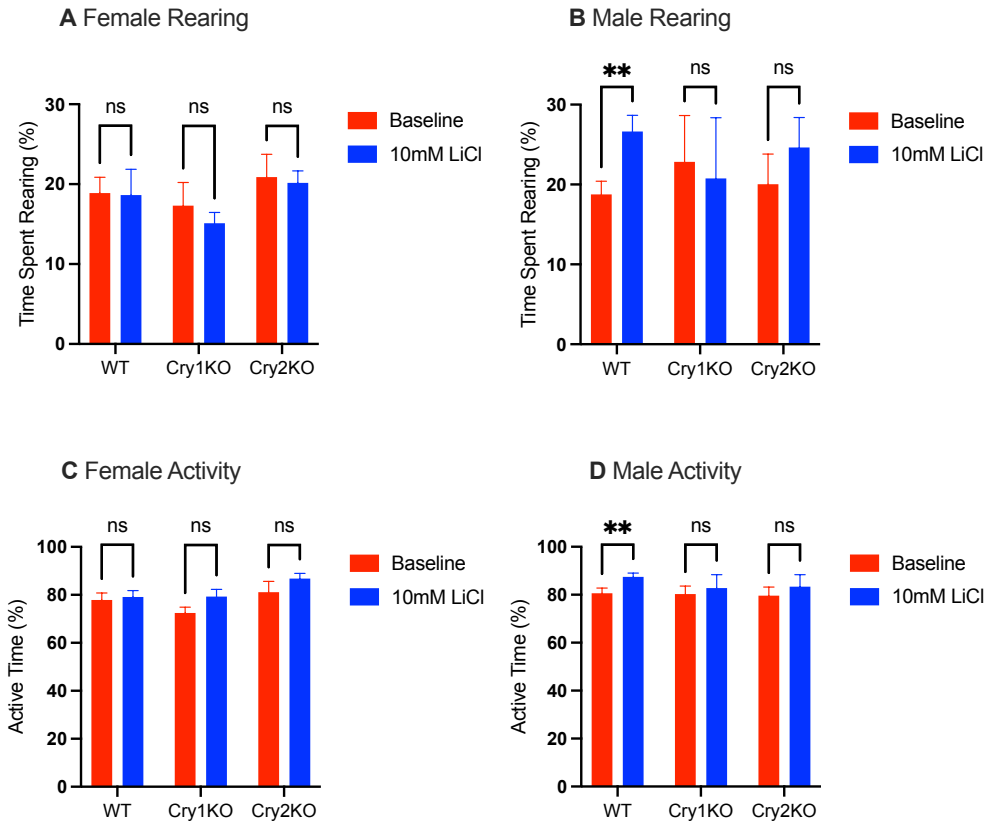

Figure 4.12 Lithium's Impact on Rearing Behaviour. The OFT was performed on (A) female and (B) male WT, Cry1KO, and Cry2KO mice before and after 10mM LiCl administration in drinking water. The percent of time spent active was tracked for (C) female and (D) male mice. Time spent rearing and active was scored using ANYmaze software. Significance calculated using two-way repeated measures ANOVA with a Šídák's post hoc test. N=4-7.

4.3 Discussion

4.3.1 *Cry* Genotype Does Not Determine Lithium-Induced Behavioural Period Lengthening, But Sex Does

Lithium is the gold standard treatment for BD. It is also a potent chronomodulator and it has been suggested that this manipulation of the circadian clock could underlie its mood stabilising effects (McClung, 2013). Previous work has shown that lithium lengthens the circadian clock of BD patient fibroblasts of short and medium length more effectively than fibroblasts with long circadian periods (McCarthy *et al.*, 2019; Sanghani *et al.*, 2021). In addition, it was found that

chronomodulators which phenocopy lithium's lengthening of the circadian clock produce similar behavioural alterations in the EPM and OFT (Sanghani *et al*., 2021). We therefore investigated whether lithium was also more effective at lengthening the behavioural period of mice with short and WT rhythms than the behavioural period of mice with long rhythms *in vivo*. Furthermore, the literature suggests that there are differences in sleep and circadian rhythms between males and females so we wanted to see if these differences were apparent in the circadian response to lithium in male and female mice (McCormick *et al.*, 2002; Paul, Turek and Kryger, 2008; Duffy *et al.*, 2011).

Previous work has demonstrated that it takes approximately three weeks for the basal period of WT female mice to fully lengthen upon lithium administration and we found the same in our WT female mice (Fig. 4.3) (Li *et al.*, 2012). The authors reported an average period lengthening of 20.4 minutes in WT females while our female WT cohort lengthened to a total of 30.75±2.27 minutes. Our results revealed that lithium lengthens the period of wheel-running behaviour in female mice with short, medium, and long Cry-related rhythms. It is worth noting that the "long" rhythms of the fibroblasts in previous studies were much longer than the Cry2KO mice in our study (>25.8 hours in McCarthy *et al.*, and >25 hours in Sanghani *et al.*, versus 23.98 and 24.12 hours in our Cry2KO males and females, respectively) (McCarthy *et al*., 2019; Sanghani *et al.,* 2021). This is to be expected, as the period of peripheral fibroblasts, though correlated to *in vivo* behavioural rhythms in mice and humans, are longer than the actual behavioural rhythms (Brown *et al.*, 2005; Pagani *et al.*, 2010). Thus, it is possible that if we had used a different genotype with a longer rhythm, lithium would not have lengthened the behavioural wheel-running period.

Our findings match those of Noguchi *et al.*, who showed that lithium lengthens the SCN period of WT, Cry1KO mice, and Cry2KO mice, and therefore *Cry* genotype does not play a role in lithium's period lengthening (Noguchi *et al.*, 2016).

The somewhat surprising result of our circadian investigations, however, was that lithium only lengthened the rhythms of female mice. Our data suggests that the sex-based difference in lithium response occurred only at the level of the behavioural clock, as male and female master and peripheral clocks exhibited similar period lengthening upon 4mM and 10mM LiCl administration, respectively (Fig. 4.7 and Fig. 4.8). To our knowledge, all previous mouse studies of lithium's impact on wheel-running activity have used female mice – a telling choice as generally studies default to male mice (Possidente and Exner, 1986; Li *et al.*, 2012).

In our study, male mice seemed to have a lower tolerance for lithium, particularly male Cry1KO mice. On average males drank less lithium than their female counterparts, though this was only statistically significant in WT. Currently, the dose of lithium is prescribed for patients on an individual basis with constant monitoring of serum levels until the therapeutic index is reached. A perusal of the literature revealed no different recommendations for lithium dose based on sex, except with regards to pregnancy (Goodwin *et al.*, 2016; Tondo *et al.*, 2019).

Lithium is widely used in conditioned taste aversion tests. When lithium is given after consumption of another substance, such as sucrose, animals learn to avoid the first substance because it is associated with lithium (Loy and Hall, 2002). No sex-based difference has been found in conditioned taste aversions induced by intraperitoneal injection of the same dose of lithium in C57BL/6J mice (Ingram and

Corfman, 1981). It has been found, however, that the minimal effective dose of lithium to induce an aversion is lower in male rats than female rats in a paradigm in which lithium was intraperitoneally injected (Dacanay *et al.*, 1984). Thus it is possible that the male mice in our study were more sensitive to the taste of lithium at 10mM in drinking water and therefore avoided drinking it. Cry1KO mice showed a particular intolerance to lithium – an observation that has previously been reported (Schnell *et al.*, 2015). This intolerance was likely not driven by taste aversion as their consumption of lithium had to be limited to avoid toxicity. These results therefore suggest that CRY1 plays a protective role against lithium toxicity, particularly in male mice, which warrants further exploration.

4.3.2 *Cry* Genotype Does Not Predict Behavioural Alterations Induced by Lithium

Our second focus in this chapter was to establish 1) the relationship between circadian phenotype induced by *Cry1/2* knockout and the mood alterations associated with lithium and 2) whether circadian alterations induced by lithium are necessary for its mood alterations. The FST is a behavioural assay to assess depressive behaviour, and lithium is well documented to reduce time spent immobile in this test – a behavioural modulation that is seen as antidepressant (O'Donnell and Gould, 2007; Kara, Stukalin and Einat, 2018). Lithium has also been documented to impact exploratory behaviour, which has been measured using the EPM (Pellow *et al.*, 1985; Youngs *et al.*, 2006; Sanghani *et al.*, 2021). Lastly, lithium has been well characterised to reduce rearing in the OFT (O'Donnell and Gould, 2007). Previous results from our lab demonstrated that two structurally distinct

chronomodulatory compounds (Nobilitin and CGS-15943) preferentially lengthen the molecular clocks of short and medium length BD fibroblasts. These compounds were also found to mimic lithium's modulation of exploratory and rearing behaviour in WT C57BL/6J mice with a modest correlation between amount of period-lengthening induced by LiCl, nobiletin, and CGS-15943 and the extent of behavioural modulation (Sanghani, 2018; Sanghani *et al*., 2021). We therefore hypothesised that lithium-induced behavioural modulation would be stronger in Cry1KO and WT mice.

Lithium reduces time spent immobile in the FST. Importantly lithium dose is not correlated to effect size so variations in drinking behaviour should have no effect (Can *et al.*, 2011; Kara, Stukalin and Einat, 2018). Our prediction, therefore was that Cry1KO mice would spend the least time immobile, followed by WT mice, and Cry2KO mice. Given that lithium had successfully lengthened period in female mice alone, we expected these results to be more pronounced in the female mice. The FST matched our prediction such that short and medium period female mice responded to lithium-driven improvement of helplessness while long period female mice did not. In male mice this response seemed to be shifted such that only short period male mice responded – again an indication that males are less sensitive to lithium. Thus, lithium's modulation of the behavioural wheel-running clock did not predict its alteration of helpless behaviour, as Cry1KO male mice did reduce their time spent immobile despite having the same period as baseline conditions before lithium treatment. Furthermore, Cry2KO females did respond to lithium's behavioural period lengthening, but did not alter their time spent immobile in the FST during lithium treatment.

Schnell *et al.* previously conducted the FST on Cry1KO and Cry2KO mice and demonstrated that both genotypes respond to lithium's reduction of helplessness at ZT18, but Cry1KO do not respond at ZT6. The authors conducted the FST on animals in a 12:12 LD cycle every day for three successive days during baseline treatment and then every day for three successive days after 10-14 days of lithium treatment (Schnell *et al.*, 2015). The difference in lighting conditions between our study, where mice were in DD, and theirs, where mice were in a 12:12 LD cycle, is likely crucial for explaining why we did not see a response in our Cry2KO female mice. In rats, DD has been shown to induce morphological alterations in the brain characteristic of depression and specifically to increase immobility in the FST (Gonzalez and Aston-Jones, 2008). Thus, it is possible that lithium's reduction of helpless behaviour is weaker in Cry2KO female mice and therefore not strong enough to overcome the depression-inducing environment of DD in our study.

In our test of lithium's impact on exploratory behaviour in CryKO mice, the behaviour of WT mice did not significantly change. This is a rather odd result given that previous work from our lab using the exact same setup showed an increase of open arm entries after 10-12 days of repeated intraperitoneal injections of lithium (Sanghani *et al*., 2021). Other previous research has shown either a reduction or an increase in exploratory behaviour – but a change nonetheless (Youngs *et al.,* 2006; Chen *et al*., 2013). It is clear that lithium was at an effective concentration in our female CryKO mice at the very least because their period elongated in DD. Furthermore, the altered behaviour could not have been due to lithium toxicity because the amplitude of the wheel-running activity of all of the mice remained more or less constant during lithium treatment.

In the case of the EPM, it has been found that the behaviour of animals exposed to the maze twice could change with repeated exposure. However, the nature of the alteration of behaviour is contended in the literature. Rats exposed to two trials of the EPM have been shown to avoid the open arms of the maze in a second trial – an effect seen in the absence of any drug administration (Bertoglio and Carobrez, 2000). Mice also avoid the open arms upon a second exposure – even under the influence of anxiolytics like diazepam (Rodgers and Shepherd, 1993). However, another study which performed up to 16 repeated trials of the EPM in rats found that repeated exposures led to a trend of increased open arm entries. Furthermore, the same study demonstrated that light has a pronounced impact on behaviour in repeated trials with bright light increasing open arm avoidance (Schrader *et al.*, 2018). Light levels therefore could have affected the results of our tests as well, given that the second test was conducted in light after 5 weeks of DD and the first was conducted in light only 4 days into DD. We could not perform our behavioural tests in the dark, however, as the behaviour of the mice needed to be video recorded. In addition, we were limited by mouse numbers and had to perform our behavioural tests on the same mice used to measure lithium's period elongation; therefore the mice had to be kept in constant darkness.

Much like the results from the EPM, the results of the OFT were not significant except in WT males, and this was likely due to increased overall activity (Fig. 4.12). Interestingly, male C57BL/6 trend towards increased activity when kept in constant darkness for four weeks, so it is possible this would become significant after 5 weeks as we observed (Zhou *et al.*, 2018). However, rearing is consistently reduced by lithium even without an increase in locomotion (O'Donnell and Gould,

2007). Previous work from our lab using the same apparatus has replicated this finding in mice (Sanghani *et al*., 2021). We, however, did not. In rats, increased rearing activity has been linked to higher levels of anxiety and less time spent in the open arms of the EPM (Borta and Schwarting, 2005). Constant darkness increases freezing behaviour of mice in the OFT and increases in light intensity during the OFT reduces their exploratory behaviour (Martin-Arenas and Pintado, 2014; Namgyal *et al.*, 2021). Therefore, it is likely that by keeping the mice in constant darkness and then performing the OFT and EPM in light, the change in light had a greater effect on the behaviour of the mice than lithium administration. Our investigations of lithium's alteration of kinase activity in Chapter 3 revealed that it inhibits a number of kinases involved in circadian entrainment to light pulses. Thus it is alternatively possible that lithium did significantly alter the behavioural response to the light pulses during testing, but we did not see it because all mice were under lithium treatment. Consequently, existing literature of lithium's mood modulation with these behavioural tests likely does not provide an appropriate comparison for our results, as to our knowledge none of these tests have previously been performed in DD.

4.3.3 Conclusion

The investigations of this chapter had two aims: 1) to examine lithium's chronomodulation of mice with different basal circadian periods *in vivo* and 2) to evaluate if chronomodulation is a prerequisite for lithium induced alterations in behaviour. Towards the first aim, we demonstrated that CRY1/2 circadian

phenotype does not play a role in lithium's period elongation, as lithium lengthens the behavioural wheel-running rhythms of short, medium, and long period female mice of different *Cry* genotypes. We also demonstrated, for the first time, a conclusive sex-based difference in response to chronomodulation by lithium. This sex-based difference was not apparent *ex vivo* at the level of the master clock (SCN) or peripheral clocks and warrants further exploration. Our investigations of the second aim revealed that lithium's modulation of helpless behaviour is dependent on *Cry* genotype and males are less sensitive to lithium's reduction of helpless behaviour. Additionally, lithium-induced period elongation was not necessary for the *Cry* genotype-dependent modulation of helpless behaviour. Our examination of lithium's modulation of exploratory and rearing behaviours was potentially impacted by factors including light cycles and repeated exposure to behavioural tests.

4.4 References

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5. LITHIUM ALTERS METABOLISM THROUGH A PPARγ -DEPENDENT MECHANISM

5.1 Introduction

While lithium is the gold standard treatment for bipolar disorder (BD), its use is associated with many side effects that lead to patient noncompliance to treatment and can eventually force cessation of treatment. Prominent among these side effects is weight gain (McKnight *et al.*, 2012). Under lithium pharmacotherapy, the odds ratio for clinically significant weight gain (>7%) has been calculated as 1.89 (Volkmann, Bschor and Köhler, 2020). Intriguingly, it has been observed that weight gain is prominent among patients with pre-existing obesity before lithium treatment. They gain an average of 6.1 kg over a year as opposed to nonobese patients on lithium who only gain 1.1 kg (Bowden *et al.*, 2006). No mechanism has been proposed to explain this selective weight gain. If the mechanism(s) were understood, they could be addressed to reduce patient nonadherence to lithium pharmacotherapy.

5.1.1 Mechanisms of Weight Gain

Weight gain, the excessive accumulation of fat, is becoming very common in the modern world with mass consumption of energy-dense food. Fat storage is increased when energy intake is greater than energy expenditure. Humans are considered overweight when the body mass index (BMI – an index of height and weight) is greater than or equal to 25 (World Health Organization, 2021). Since 1975, worldwide obesity (BMI $\geq$ 30) has almost tripled and as of 2016, 39% of adults were overweight and 13% were obese. High BMI affects more than aesthetics,

as it increases the risk of cardiovascular disease (the leading cause of death in 2012), diabetes, and some cancers (World Health Organization, 2021).

Lipid accumulation occurs when the average size of adipocytes (fat cells) increases (a process known as hypertrophy) and/or the number of adipocytes increases (a process known as hyperplasia). A study of 687 adults found that the number of adipocytes in adipose tissue stabilizes after adolescence and remains constant even after weight loss during adulthood. Furthermore, the relationship between adipocyte size and number was nonlinear, suggesting that both hypertrophy and hyperplasia are responsible for weight gain (Spalding *et al.*, 2008). When excess energy is absorbed through the diet, dietary lipids are taken up by adipocytes, which esterify them into triglycerides. When energy is required by the body or under conditions of cellular inflammation (as occurs in obesity), these energy stores are mobilised in a process called lipolysis in which the triglycerides are hydrolysed and secreted from adipocytes as free fatty acids (FFAs) which can be used for adenosine triphosphate (ATP) production. When excess FFAs are circulating in the body, they begin to accumulate ectopically on peripheral tissues causing insulin resistance in these tissues (Nielsen *et al.*, 2014; Sears and Perry, 2015).

Approximately 10% of adipocytes are turned over in humans each year (Spalding *et al.*, 2008). The new adipocytes are generated through a process called adipogenesis. Pluripotent mesenchymal stem cells have the capacity to differentiate into adipocytes, osteoblasts, chondrocytes, and myocytes, and cellular signalling determines which fate they commit to. Mesenchymal stem cells commit to the

adipocyte lineage through bone morphogenetic protein signalling, after which they are called preadipocytes. These preadipocytes then arrest their growth and initiate a cascade of transcription factors that results in the formation of mature adipocytes in a process called terminal differentiation (Fig. 5.1) (Lowe, O'Rahilly and Rochford, 2011; Ghaben and Scherer, 2019). Wnt signalling is crucial at this step, as activation of the Wnt pathway inhibits preadipocytes from terminally differentiating into mature adipocytes (Ross *et al.*, 2000).

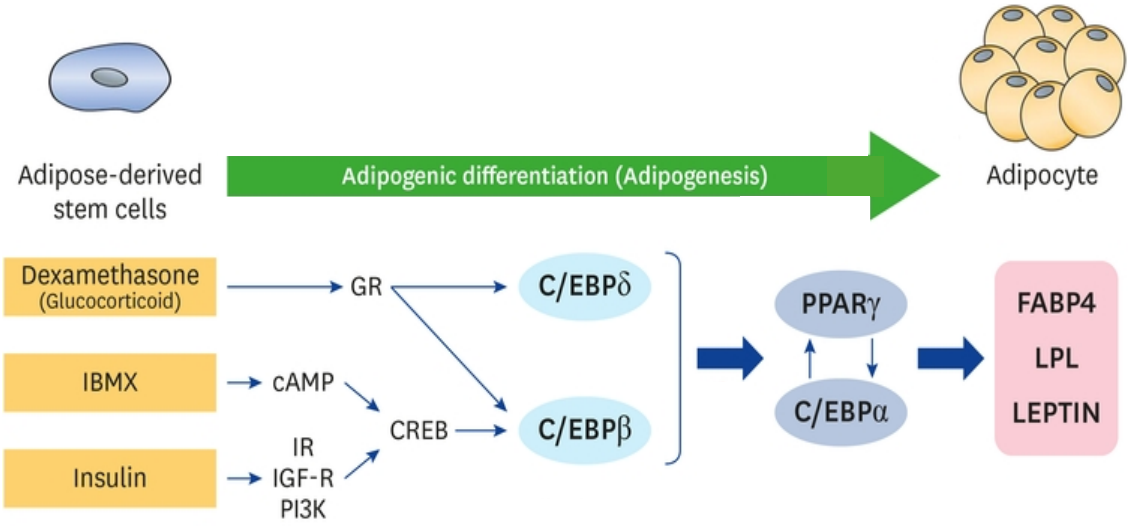

Figure 5.1 The Mechanism of Adipogenesis. Adipose-derived stem cells are induced to differentiate into mature adipocytes by glucocorticoid, 3-isobutyl-1-methylxanthine (IBMX), and insulin signalling, which activate C/EBPβ and C/EBPδ. C/EBPβ and C/EBPδ in turn activate PPARγ and C/EBPα, which form a positive feedback loop and drive the expression of genes characteristic of mature adipocytes. Adapted from (Kim et al., 2021).

Several CCAAT-enhancer-binding protein (C/EBP) transcription factors participate to promote terminal differentiation. C/EBPβ and C/EBPδ expression is induced after growth arrest and these in turn drive the expression of C/EBPα and the nuclear hormone receptor peroxisome proliferator activated receptor γ

(PPARγ). C/EBPα forms a positive feedback loop with PPARγ and together they drive the expression of genes characteristic of mature adipocytes, such as adipocyte protein 2 (AP2, also known as FABP4) (Rosen and MacDougald, 2006). It has been shown that over 60% of the genes upregulated during adipogenesis by PPARγ have a C/EBPα binding site in close proximity and only 3% are bound by PPARγ alone (Lefterova *et al.*, 2008). Ectopic expression of C/EBPα or PPARγ has been shown to promote differentiation of in a variety of fibroblast cells (Freytag, Paielli and Gilbert, 1994; Tontonoz, Hu and Spiegelman, 1994). An examination of protein expression in 3T3-F442A adipocytes during the differentiation process revealed that PPARγ accumulates much sooner (day one after growth arrest) than C/EBPα (day three after growth arrest) (Tontonoz, Hu and Spiegelman, 1994). It has also been shown that ectopic PPARγ can promote differentiation in C/EBPα-deficient cells, but ectopic C/EBPα cannot promote differentiation in PPARγ-deficient cells. Thus, PPARγ is considered the master regulator of adipogenesis (Rosen *et al.*, 2002).

5.1.2 PPARγ-Stimulated Weight Gain

Peroxisome proliferators are chemicals that are known to lower plasma triglycerides and modulate gene expression. The receptors at which these chemicals act (PPARs) were first cloned in 1990 and bind to DNA response elements termed peroxisome proliferator responsive elements (PPREs) that consist of two repeats of the sequence AGG(A/T)CA separated by one nucleotide (also called a direct repeat spaced by one nucleotide – DR1). The PPARs form a heterodimer with retinoid X receptor α (RXRα) when binding to PRREs, and together the nuclear receptors drive

the expression of genes involved in lipid storage and catabolism (Issemann and Green, 1990; Kliewer *et al.*, 1992).

The endogenous ligand of PPARγ is still unknown, but agonists of the transcription factor are known to cause weight gain. The thiazolidinediones (TZDs) are potent agonists of PPARγ that promote weight gain with an average increase of 3-4 kg in the first 6 months of treatment and continued weight gain at a reduced rate afterwards. Importantly this weight gain is dose dependent for each TZD that has been studied (Wilding, 2006). In patients with type 2 diabetes, a 1.6% gain in body fat mass was observed after 12 weeks of treatment with one of the most potent TZDs, rosiglitazone (BRL49653) (Miyazaki *et al.*, 2005). Weight gain is typically associated with insulin resistance and increased risk of cardiovascular disease, but what is unique about TZD-induced weight gain is that TZDs markedly improve insulin sensitivity and reduce fasting glucose levels. In fact, it has been shown that PPARγ activation in mature adipocytes alone is enough for whole-body insulin sensitization in adipose gain-of-function PPARγ mutant mice. Thus, TZDs are marketed as antidiabetic drugs (Nolan *et al.*, 2000; Wilding, 2006; Sugii *et al.*, 2009). Rosiglitazone binds to PPARγ with a K_D of 40nM (Lehmann *et al.*, 1995).

5.1.3 Mechanisms of Lithium-Induced Weight Gain

Though lithium has been linked to weight gain in many studies and meta-analyses, it has actually been shown to inhibit adipogenesis. Lithium activates Wnt signalling, thereby inhibiting the differentiation of preadipocytes and priming human mesenchymal stem cells toward osteogenesis rather than adipogenesis

(Baptista *et al.*, 1995; Ross *et al.*, 2000; Kang *et al.*, 2007; Satija *et al.*, 2013; Chi *et al.*, 2016; Volkmann, Bschor and Köhler, 2020). This inhibition of adipogenesis is associated with a reduction in C/EBPα and PPARγ expression (Ross *et al.*, 2000; Kang *et al.*, 2007).

Separate conflicting lines of evidence have found that lithium directly enhances C/EBPα and PPARγ activity. Shim and Smart found that lithium dose-dependently increases C/EBPα protein levels in BALB/MK2 keratinocytes and 20mM LiCl increases C/EBPα-dependent promoter activity by 10-fold. This increase in *in vitro* C/EBPα activity was independent of GSK-3-mediated phosphorylation of C/EBPα. Rather, it was driven by inhibition of proteasomal degradation of C/EBPα, thus increasing the half-life of the protein (Shim and Smart, 2003). Previous work from our lab has also shown that lithium activates C/EBPα in the murine MMHD3 cell line. An examination of transcription factors that are activated by lithium and inositol monophosphatase inhibitor L690,488 revealed the upregulation of C/EBPα-dependent genes. This activation of C/EBPα was found to play a role in lithium-driven circadian period and amplitude increase (Sanghani, 2018).

Another study linked lithium's therapeutic signature of gene expression to PPARγ activation. Rivera and Butt focused their study of lithium's actions on its alteration of the transcriptome in astrocytes. They performed *ex vivo* microarray analysis of astrocytes in organotypic cultures of optic nerves to examine lithium-induced transcriptional changes. They then performed a meta-analysis to determine which small molecules could mimic lithium's transcriptional alterations of genes in astrocytes that are related to BD. The top drugs that matched lithium's signature

were piogliatazone (a TZD) and betulinic acid, which are both linked to PPARγ activation (Rivera and Butt, 2019). These findings corroborate earlier results showing that lithium increases the accumulation of PPARγ and its target AP2 in human monocyte-derived immature dendritic cells (Liu *et al.*, 2011).

5.1.4 Aims

Lithium has been shown to inhibit adipogenesis, leading to some confusion as to how it could induce weight gain. It has been suggested that lithium could alter feeding behaviours or thyroid function, but no coherent picture has emerged. Lithium has, however, been shown to increase the activity of C/EBPα and PPARγ through post-translational modifications in the case of C/EBPα. Furthermore, it has been observed that lithium-induced weight gain is more prominent in obese patients. We therefore hypothesised that lithium could promote weight gain by increasing the activity of C/EBPα and PPARγ. Consequently, we predicted that overweight mice would gain weight under lithium administration given the larger volume of adipose tissue where these transcription factors promote lipid accumulation. If true, this would provide the first explanation for lithium-induced weight gain that explains weight gain in only certain patient populations.

5.2 Results

5.2.1 Lithium Increases PPARγ Transcriptional Activity

The literature suggests that lithium increases weight gain in pre-obese patients taking lithium relative to nonobese patients. We hypothesised that lithium

could exert these effects by increasing C/EBPα and PPARγ in adipose tissue, which is more abundant in overweight individuals. PPARγ is considered the master regulator of adipogenesis so we focused on this transcription factor. In order to establish that lithium is capable of increasing PPARγ-driven activity, we transiently transfected U2OS cells a reporter of PPARγ activity. For this a PPRE construct was selected consisting of three DR1 sites upstream of a luciferase reporter (PPRE::LUC) (Kim *et al.*, 1998). The U2OS cell line was chosen for ease of transfection and for the fact that these cells endogenously express PPARγ but not C/EBPα, thus making it possible to separate the contribution of these two transcription factors (Sjöstedt *et al.*, 2020; The Human Protein Atlas, 2022b, 2022a).

Progressively increasing concentrations of lithium were applied to the U2OS cells to measure a dose-response of lithium-driven PPRE activity (Fig. 5.2). As predicted, lithium dose-dependently increased PPRE activity. 10mM lithium chloride (LiCl) and 30mM LiCl significantly increased PPRE activity by 1.87 ± 0.38 and 2.67 ± 0.68 fold, respectively (Fig. 5.2B). The EC50 of LiCl-driven induction of PPRE activity was calculated as 10.78mM with an R of 0.80.

The next step was therefore to confirm that PPARγ was actually responsible for the lithium-driven increase in PPRE activity that we had observed. We therefore repeated the transient transfection of U2OS cells with PPRE::LUC, but this time we added non-targeting control (NTC) siRNA or PPARγ siRNA to knockdown expression of the transcription factor. After recording baseline luminescence, we applied 10mM LiCl (approximately the EC50 for lithium-driven PPRE induction). As a positive control the PPARγ agonist rosiglitazone was added at its EC50 of 50nM in

U2OS (liang Ye *et al.*, 2009). siRNA-mediated knockdown of PPARγ reduced lithium and rosiglitazone induction of PPRE activity back to basal levels, indicating that PPARγ is indeed largely responsible for lithium's induction of PPRE activity. Two main isoforms of PPARγ, PPARγ1 and PPARγ2, have been detected in humans – with PPARγ2 in much lower abundance (Fajas *et al.*, 1997). Validation of siRNA-mediated knockdown with real-time polymerase chain reaction (PCR) confirmed that PPARγ1 expression was knocked down to 0.43±0.10% and PPARγ2 was knocked down to 2.01±0.48% of the NTC condition (Fig. 5.3C).

Our transfections indicated that lithium is capable of increasing PPARγ activity. We next wanted to investigate whether lithium could increase lipid accumulation *in vitro*. 3T3-L1 preadipocytes fail to differentiate when differentiated in the presence of lithium (Ross *et al.*, 2000). However, PPARγ is expressed in the later stages of differentiation in mature adipocytes (Rosen and MacDougald, 2006). We therefore hypothesised that lithium would increase lipid accumulation in adipocytes if it was applied once the preadipocytes were already fully differentiated into mature adipocytes.

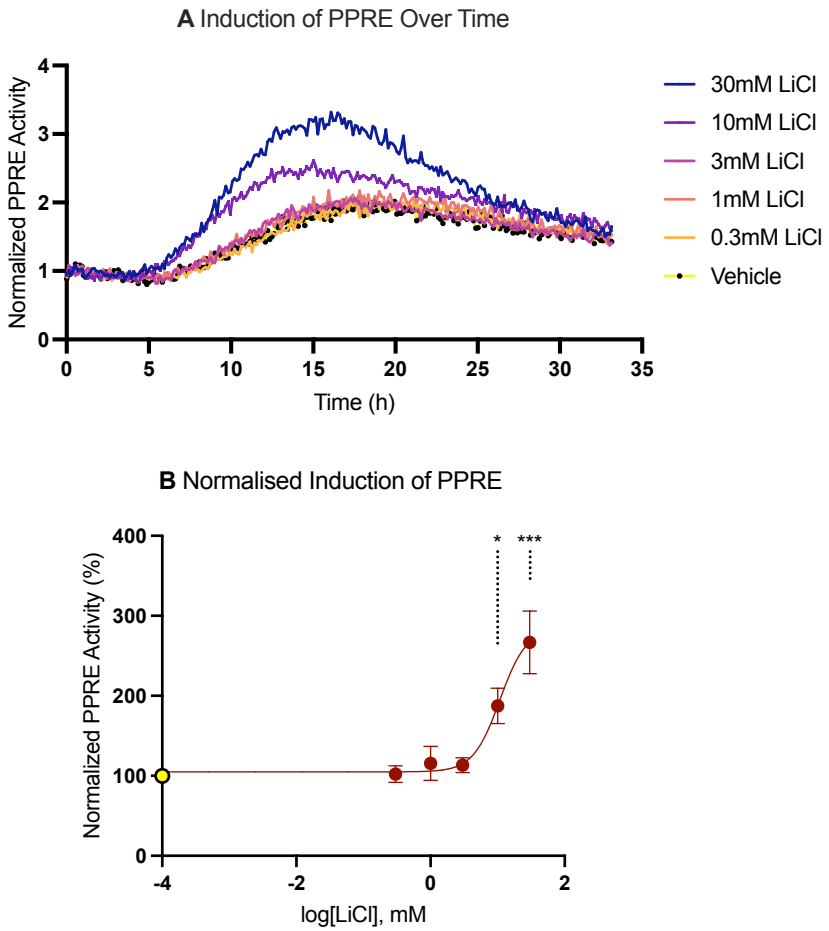

Figure 5.2 Lithium Increases PPRE Activity in U2OS Cells. U2OS cells were transiently transfected with a PPRE::LUC reporter of PPARγ activity. After two hours of baseline recording, LiCl was added and luminescence recorded for a further 30 hours. (A) Mean traces of luminescence driven by PPRE:LUC activity. (B) Peak induction of PPRE activity was calculated relative to baseline for each condition and normalised to vehicle. Significance calculated using a one-way ANOVA with a Dunnett's post hoc test. N=3.

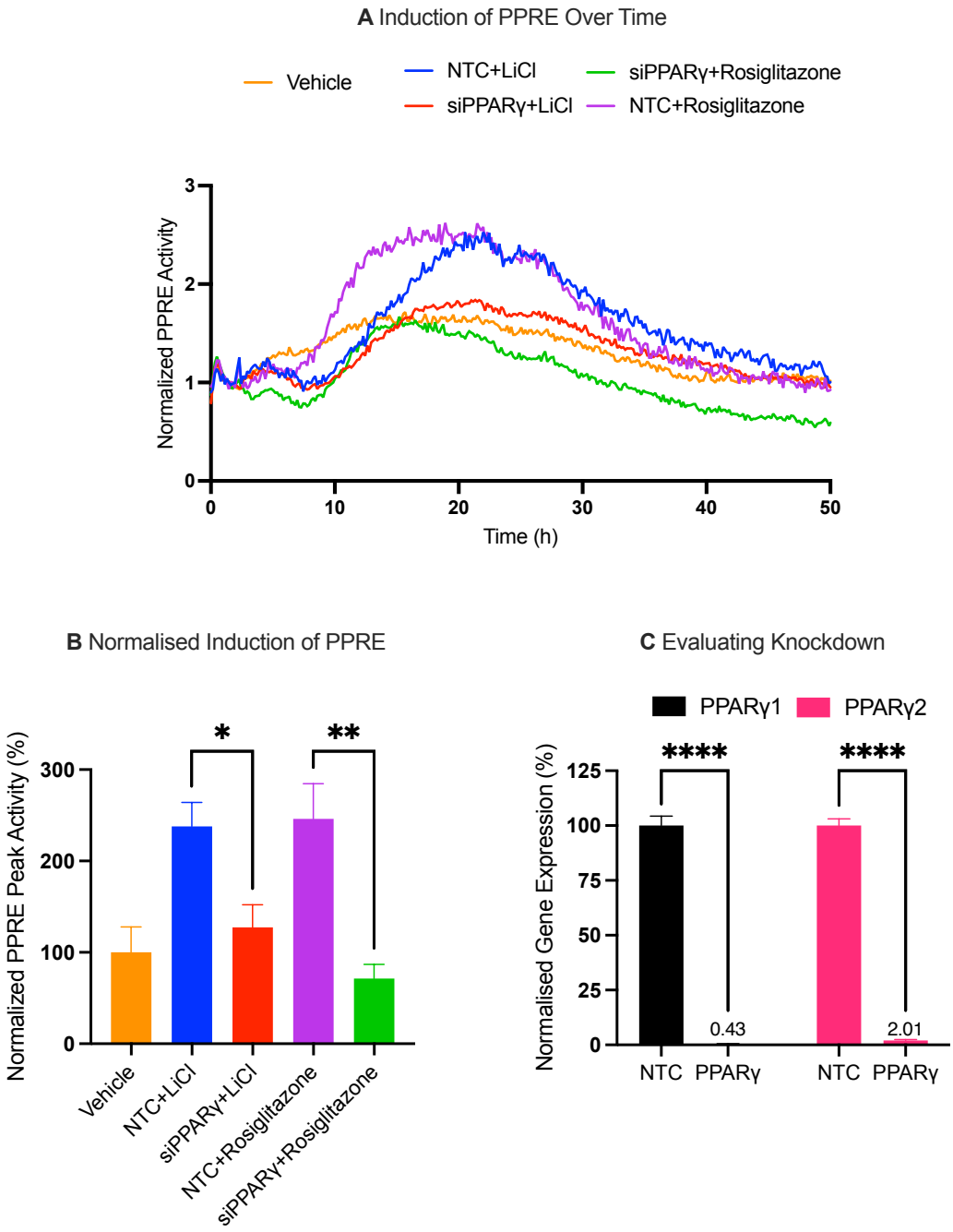

Figure 5.3 PPARγ Drives Lithium's Induction of PPRE Activity. U2OS cells were transiently transfected with a PPRE::LUC reporter and NTC or PPARγ siRNA. After baseline recording of two hours, 10mM LiCl or 50nM rosiglitazone were applied and luminescence was measured. (A) Mean traces of luminescence driven by PPRE:LUC activity. (B) Peak induction of PPRE was calculated relative to baseline for each condition and normalised to vehicle. Significance in (B) calculated using one-way ANOVA with a Dunnett's post hoc test. (C) Knockdown of PPARγ1 and PPARγ2 were validated normalised to Actin Beta and Beta-2-Microglobulin. Significance in (C) calculated using two-way ANOVA with a Šídák's post hoc test. N=4.

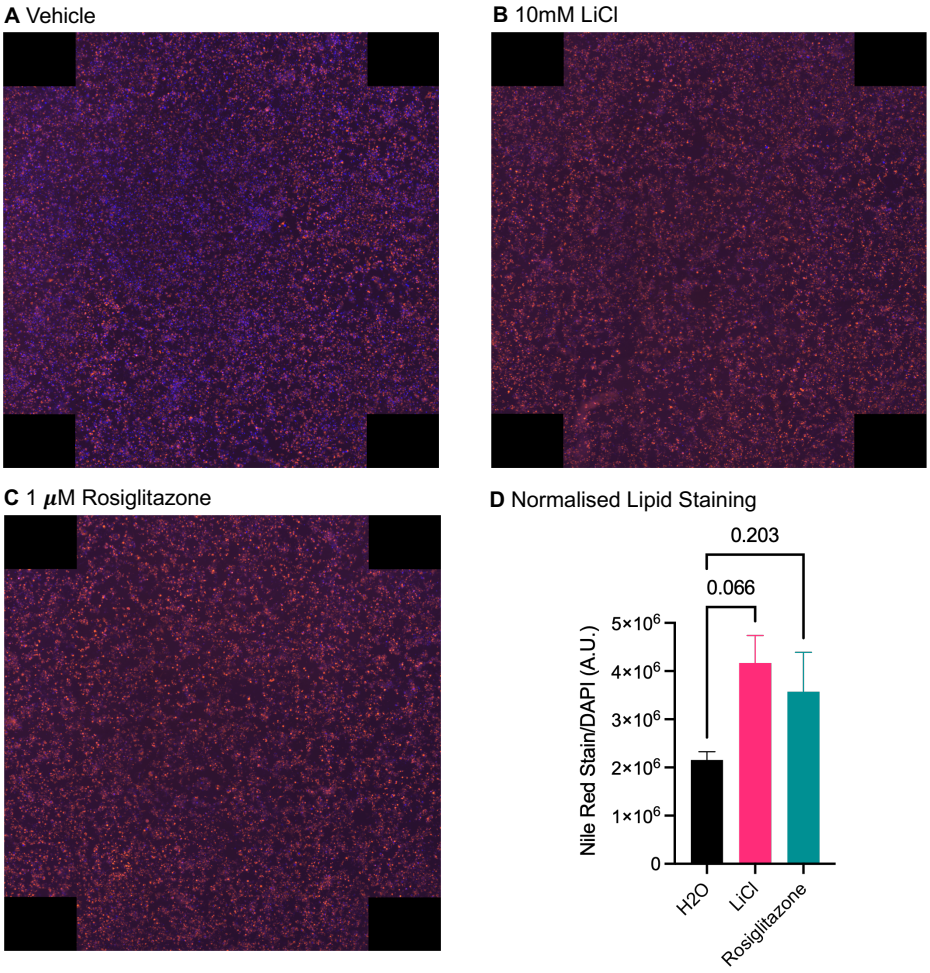

Figure 5.4 Lithium Trends Toward Increasing Lipid Accumulation in Mature Adipocytes. 3T3-L1 preadipocytes were differentiated and drug or vehicle treatment added for four days. Microscope images of (A) vehicle, (B) 10mM lithium, and (C) 1μM rosiglitazone treatments were taken at magnification 10X and image-stitched. (D) Lipid accumulation was assessed with nile red staining and normalised to cell number (DAPI staining). Significance calculated using one-way ANOVA with a Dunnett's post hoc test. N=4.

We differentiated 3T3-L1 preadipocytes and added lithium seven days after differentiation had been initiated (when adipocytes are considered mature). The PPARγ agonist rosiglitazone was used as a positive control. 10mM LiCl was applied at approximately the EC50 for lithium-driven PPARγ activity. Accordingly, rosiglitazone was applied at 1μM, half the maximal concentration for stimulating differentiation in human preadipocytes and half the concentration used in our differentiation cocktail (Wabitsch *et al.*, 2001). Each drug was applied to the mature

adipocytes for four days before the cells were stained for lipid accumulation and cell number (Fig. 5.4). Lithium increased lipid accumulation by 93.22±38.27% with a P value of 0.066. Rosiglitazone did not trend toward significantly increasing lipid accumulation relative to cell number. Thus at the concentrations chosen, lithium trended towards increasing lipid accumulation while rosiglitazone had no effect.

5.2.2 Lithium Increases Weight Gain in Obese Mice

Given that lithium increased PPARγ activity and trended towards increasing lipid accumulation *in vitro*, we wanted to examine whether it could increase weight gain *in vivo*. Our hypothesis was that lithium increases PPARγ, so we predicted that lithium would increase lipid accumulation in mice that had more adipose tissue (i.e. were already overweight). We therefore administered a high-fat diet (HFD, 60% Kcal fat) to four-week-old C57/BL6 male mice for 14 weeks in a diet-induced model of obesity (DIO). This resulted in significant weight gain of 11.79±1.03 grams relative to age-matched controls (Fig. 5.5A) (Jackson Laboratory, 2015). Next, we applied 7mM LiCl in the drinking water for one week followed by a full dose of 14mM LiCl in drinking water for 9 weeks. The lithium group was supplemented with a bottle of 0.9% sodium chloride (NaCl), as described in Chapter 4. All mice and their food were weighed every 3-5 days to monitor weight gain and food intake (Fig. 5.5B,D). In support of our hypothesis, lithium did indeed significantly increase weight gain relative to controls on a HFD (Fig. 5.5B). The weight gain was not due to increased caloric intake as both control HFD mice and the lithium cohort ate the same amount (Fig. 5.5D).

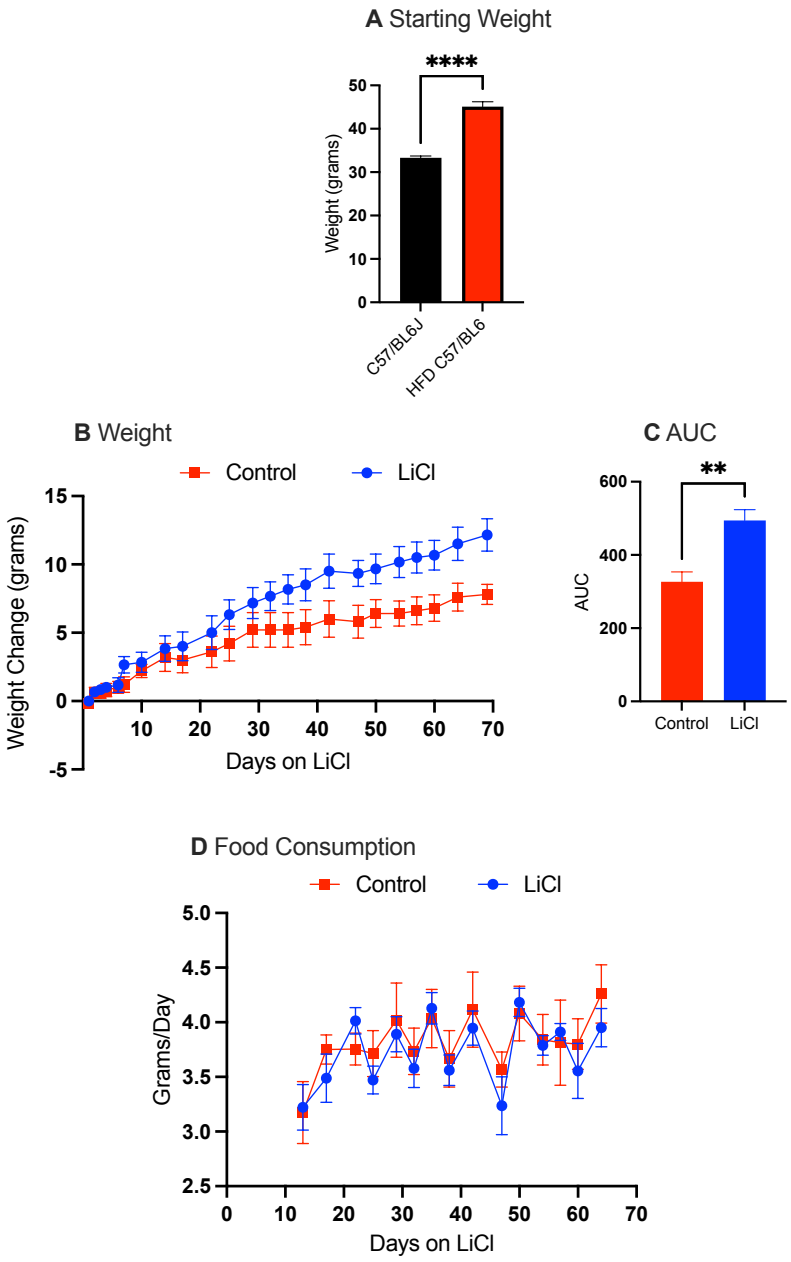

Figure 5.5 Lithium Increases Weight Gain in DIO Mouse Model. (A) Four-week-old mice were fed a HFD for 18 weeks, which caused significant weight gain relative to age-matched controls (Jackson Laboratory, 2015). N=11-40. (B) 7mM LiCl was administered in drinking water for a week followed by 14mM LiCl. Weight change relative to baseline was measured. N=5-6. (C) AUC for (B) was calculated. (D) Food intake was measured every 3-5 days. Significance in (A,C) determined using an unpaired one-tailed t-test. N=5-6.

To evaluate if our findings were specific to obese mice, we repeated our experiment in age-matched controls on normal chow. 16-week-old mice were administered 7mM LiCl in drinking water for a week and 14mM LiCl in drinking

water for 6 weeks while their weight was constantly monitored. The mice on normal chow did not gain weight following lithium administration. Indeed, they trended towards a decrease in weight upon the same dose (P=0.102, Fig. 5.6A,B) and this decrease in weight was not due to lower food consumption (Fig. 5.6C).

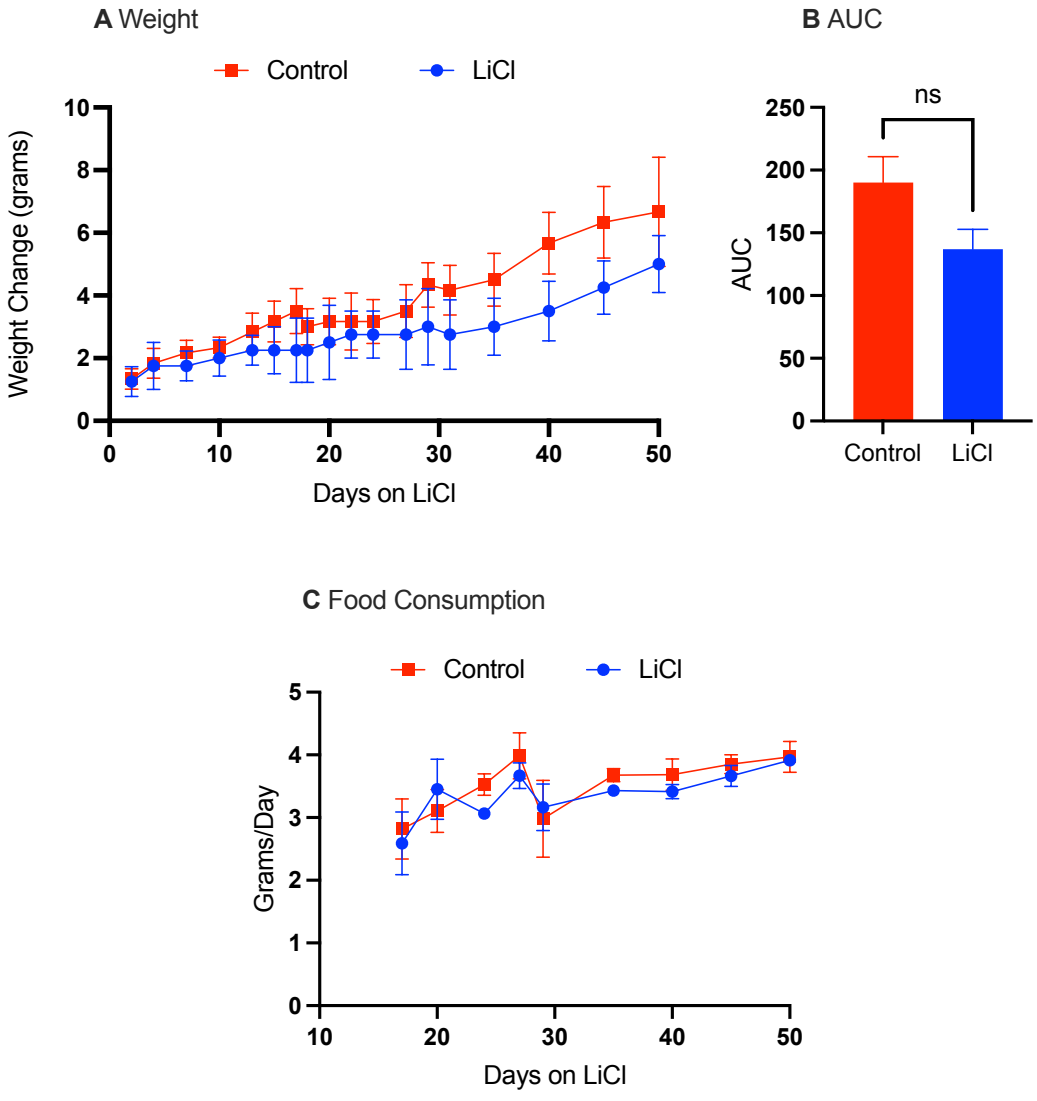

Figure 5.6 Lithium Does Not Increase Weight Gain in Mice on Standard Chow. (A) 16-week-old mice were administered lithium in their drinking water and their change in weight was measured every 3-6 days. (B) AUC was calculated for (A). (C) Food was weighed every 3-6 days to monitor food consumption. Significance determined using an unpaired two-tailed t-test. N=4-6.

5.2.3 Lithium Improves Insulin and Glucose Tolerance in Obese Mice

Generally, obesity is associated with insulin resistance. Insulin resistance is characterised by increased resistance to insulin-stimulated glucose uptake, which leads to type 2 diabetes (Kahn and Flier, 2000). Weight gain driven by PPARγ agonists (TZDs), however, is somewhat unique in that they actually improve insulin and glucose tolerance (Nolan *et al.*, 2000; Wilding, 2006). We therefore wanted to see if our lithium-treated HFD mice showed greater insulin and glucose tolerance than their controls, as this would be consistent with increased activation of PPARγ.

The oral glucose tolerance test (OGTT) was performed on the mice after 11 weeks of drug administration. Lithium-treated mice were significantly fatter than their controls at the time of the OGTT by 10.95±4.48%. Despite this weight difference, fasting levels of glucose were not significantly different between the two groups after 16 hours of fasting (Fig. 5.7A,B). For controls, weight was strongly positively correlated with fasting glucose levels, while no correlation existed between weight and glucose levels for lithium-treated HFD mice (Fig. 5.7C,D). As predicted, lithium significantly improved glucose tolerance in the lithium cohort (Fig. 5.7E,F). The insulin tolerance test (ITT) was performed on the same mice within one week of the OGTT. Analysis of the area under the curve (AUC) of the ITT revealed no significant differences in insulin tolerance between the cohorts, however, the lithium mice cleared significantly more glucose from their blood after an hour of the insulin intraperitoneal injection (Fig. 5.7G,H). Thus, our data supported our hypothesis that lithium increases glucose tolerance overall and improves insulin tolerance an hour after injection despite increasing weight in obese mice.

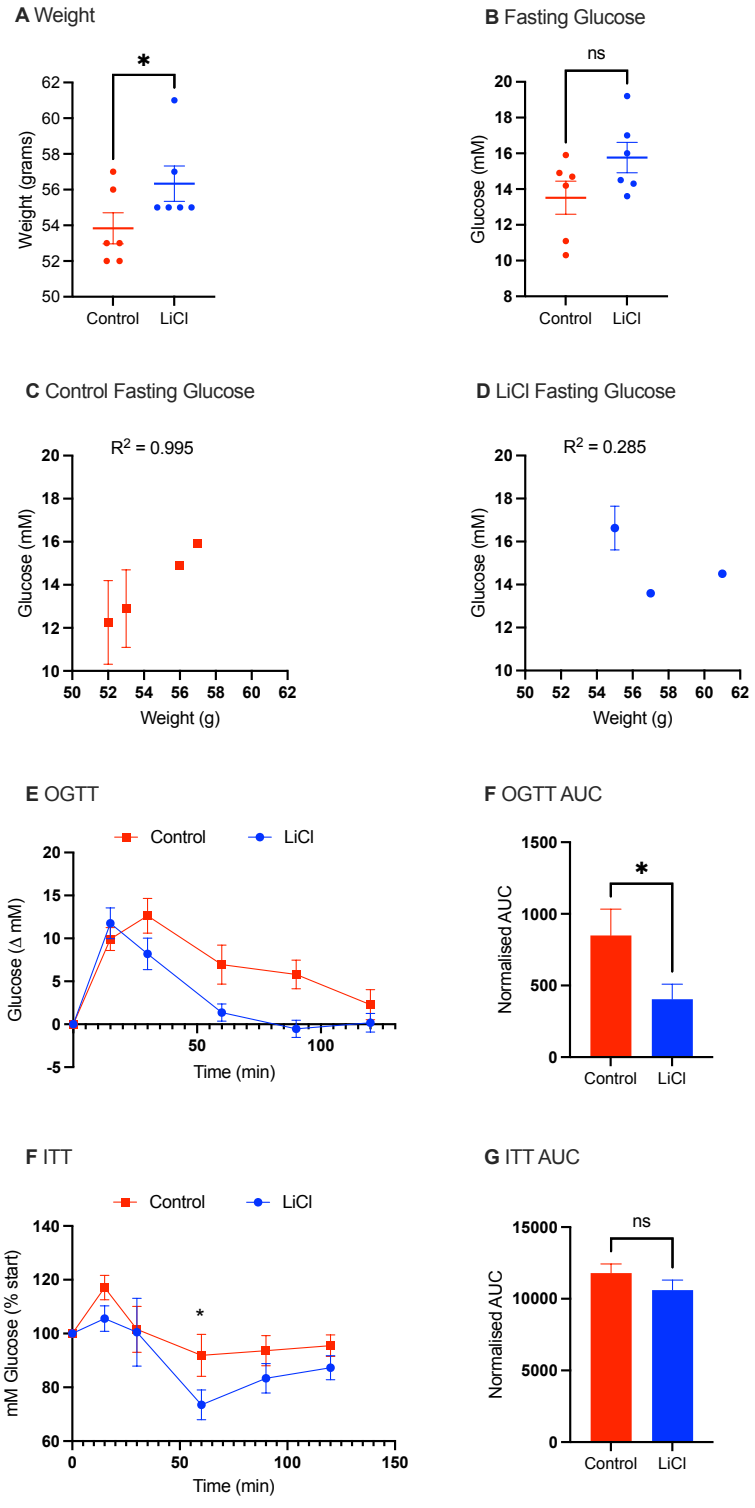

Figure 5.7 Lithium Improves Glucose and Insulin Tolerance in HFD Mice. The OGTT and ITT were performed on 29-week-old mice after 25 weeks of HFD administration and 11 weeks of drug administration. (A) Weight and (B) fasting glucose levels were measured. The correlation between weight and fasting glucose levels was calculated for (C) control and (D) lithium-treated mice using the Pearson correlation coefficient. An oral gavage of glucose was administered and (E) the OGTT performed. (F) AUC of (E) was calculated for lithium and control mice. (F) The ITT was performed. (G) AUC of (F) was calculated for lithium and control mice. Unpaired one-tailed t-tests were used to determine significance. N=6.

Given that lithium improved glucose sensitivity in HFD mice, we wanted to investigate if lithium would improve glucose and insulin tolerance in mice on standard lab chow. We predicted that there would be no difference between our lithium and control mice because there was less adipose tissue and therefore less PPARγ expression in these mice. Thus, we performed the OGTT and ITT after 8 weeks of 14mM LiCl administration in drinking water (Fig. 5.8). The fasting blood glucose levels of the lithium group trended towards being lower than the control cohort by 2.58±1.18mM despite there being no significant difference in weight (Fig. 5.8A,B). Weight and fasting glucose levels showed a moderate positive correlation in control mice, whereas in lithium-treated mice there was a moderate negative correlation (Fig. 5.8C,D). Overall lithium did not improve insulin or glucose tolerance in age-matched controls fed standard lab chow.

Finally, to fully investigate the relationship between weight and lithium-induced improvement in insulin and glucose tolerance, the data between lithium-treated HFD and standard chow mice was compared and the data between control mice on HFD and standard chow was also compared. Lithium improved glucose tolerance by 69.67±25.17% in HFD mice relative to mice on standard chow (Fig. 5.9A). Meanwhile the HFD treatment did not alter glucose tolerance in control mice (Fig. 5.9B). Lithium did not improve insulin tolerance in HFD mice relative to mice on standard chow, however, insulin tolerance was 39.20±9.95% worse in control HFD mice relative to mice on standard chow. Therefore, lithium improves insulin tolerance in HFD mice as lithium tolerance did not get worse with HFD like it did in control mice (Fid. 5.9C,D).

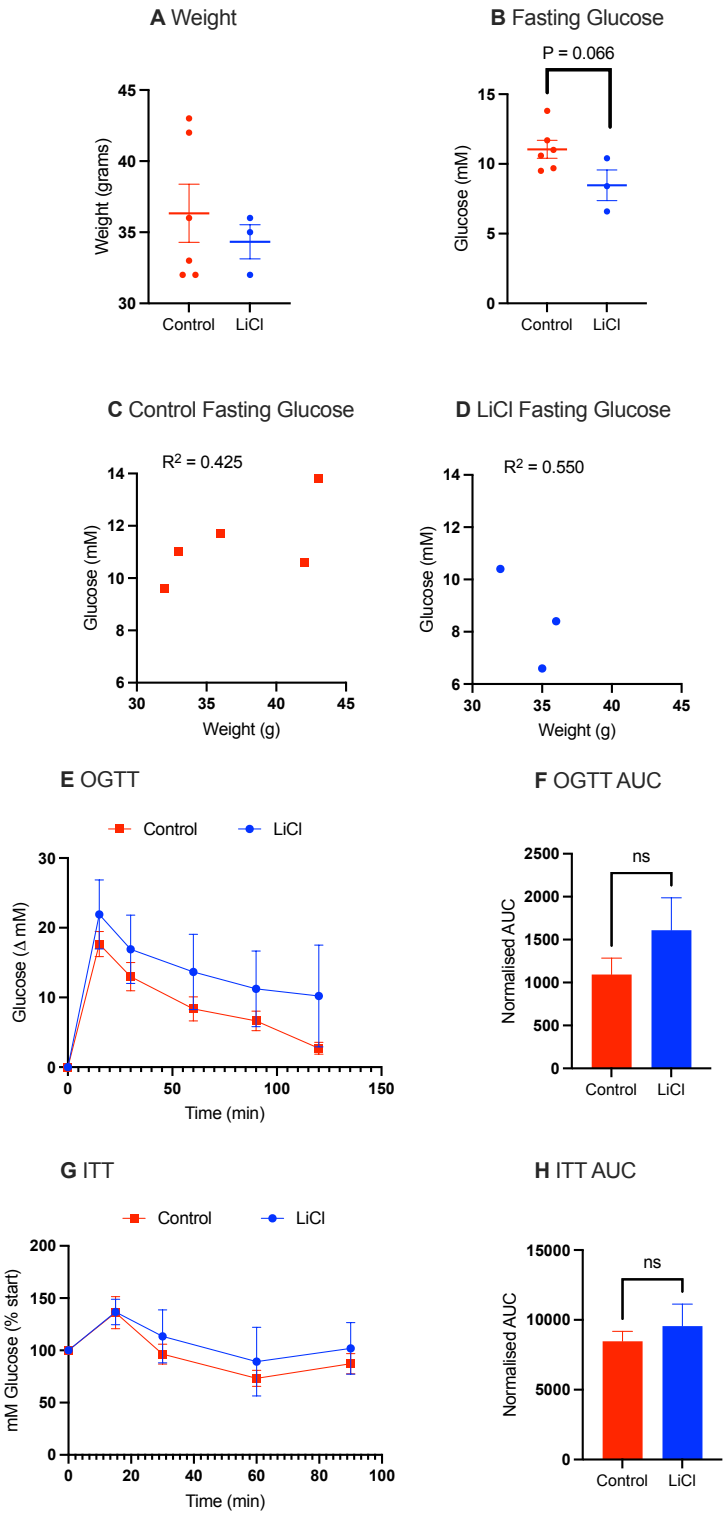

Figure 5.8 Lithium Does Not Improve Insulin or Glucose Tolerance in Mice on Standard Chow. The OGTT and ITT were performed on 25-week-old male mice fed standard chow and given access to 14mM LiCl in drinking water for eight weeks. (A) Weight and (B) fasting glucose levels were measured. The correlation between weight and fasting glucose levels was calculated for (C) control and (D) lithium-treated mice using the Pearson correlation coefficient. An oral gavage of glucose was administered and (E) the OGTT performed. (F) AUC of (E) was calculated for lithium and control mice. (G) The ITT was performed. (H) AUC of (G) was calculated for lithium and control mice. Unpaired two-tailed t-tests were used to determine significance. N=3-6.

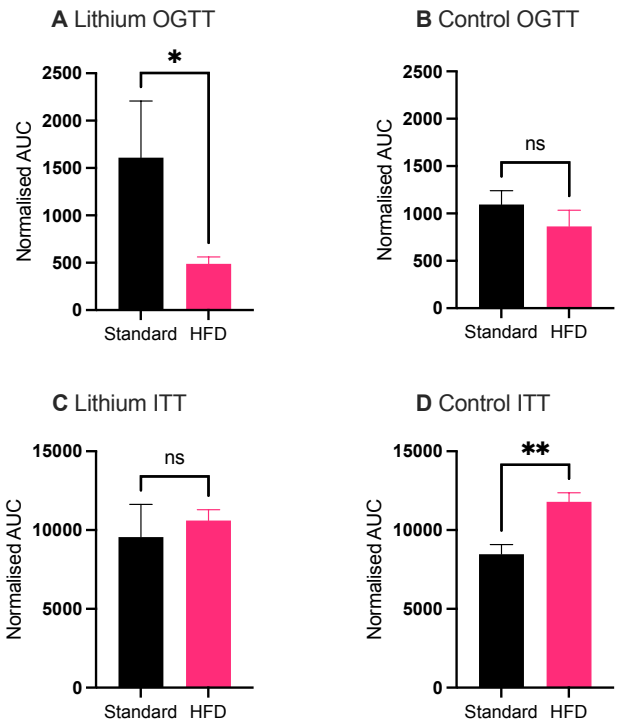

Figure 5.9 Lithium Improves Glucose and Insulin Tolerance Better in HFD Mice. The normalised AUC of the OGTT was compared for mice on a standard diet and mice on a HFD under (A) lithium treatment and (B) no treatment. The normalised AUC of the ITT was compared for mice on a standard diet and mice on a HFD under (C) lithium treatment and (D) no treatment. Significance in (A,C) calculated using unpaired one-tailed t-test. Significance in (B,D) calculated using unpaired two-tailed t-test. N=3-6.

5.2.4 Lithium Upregulates PPARγ-Driven Transcriptional Changes

The metabolic changes to glucose and insulin sensitivity and weight all pointed to lithium increasing PPARγ activity. We therefore wanted to see if lithium induced transcriptional changes that are characteristic of PPARγ engagement in the white adipose tissue (WAT) of our HFD mice. WAT was chosen as the tissue of interest because adipose tissue contains the highest level of expression of PPARγ1 and PPARγ2 in human and mouse (Vidal-Puig *et al.*, 1996, 1997). We performed RNA sequencing on WAT isolated from three lithium-treated and three control HFD mice.

23,545 genes were detected in our sample dataset. Gene expression analysis was performed with the DESeq2 package, which was set to drop genes with less than 10 counts in the dataset and then perform enrichment analysis (Love, Huber and Anders, 2014). This investigation revealed only one upregulated gene and 35 downregulated genes in response to lithium treatment with an adjusted P value of 0.05. A principal component analysis revealed that one control sample accounted for 81% of the variance on our dataset (Fig. 5.10). Given that we were primarily interested in the trends of pathways that were upregulated by lithium and not the specific genes themselves, we excluded the control outlier and reanalysed our data for differential gene expression using a non-adjusted P value of 0.05 as a cut-off for up- and downregulated genes. The new criteria for our gene expression analysis detected 23,065 genes total with 314 upregulated and 816 downregulated genes in response to lithium-treatment (Fig. 5.11).

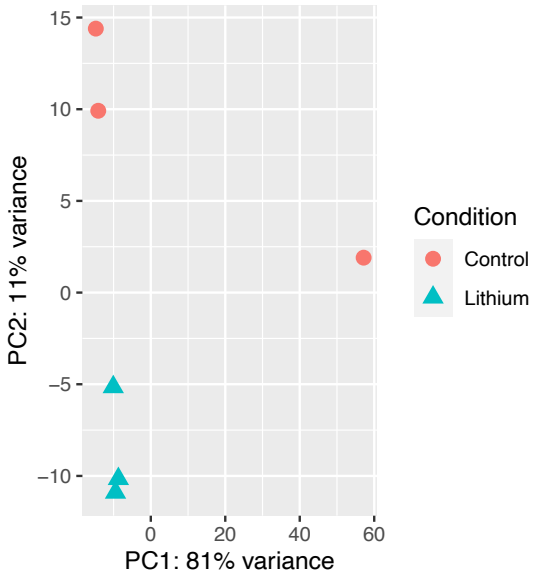

Figure 5.10 One Control Sample Skewed the RNA-Sequencing Results. A principal component (PC) analysis was performed on gene expression data from lithium-treated and control HFD mice. N=3.

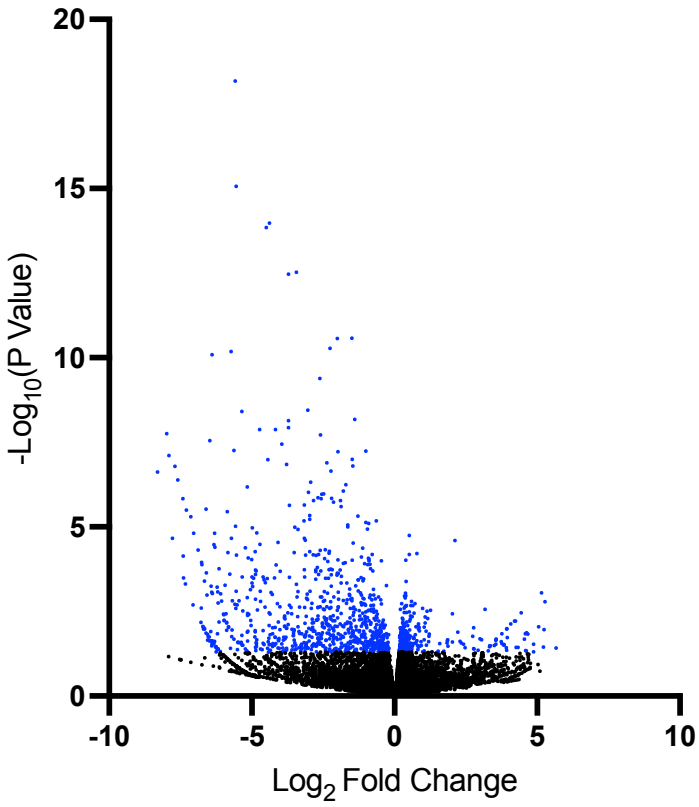

Figure 5.11 Differential Gene Expression Driven by Lithium Treatment. 23,065 genes were detected in the WAT of our HFD mice. 314 were upregulated and 816 were downregulated in response to lithium treatment. Genes that were differentially expressed with P<0.05 are represented in blue. N=2-3.

Our primary interest was identifying the kinds of pathways that lithium up- or downregulates in WAT. We therefore used G:Profiler to detect which KEGG pathways were significantly enriched in our up- and downregulated genes under lithium treatment. As expected, the most significantly upregulated KEGG pathway was the metabolism pathway itself with 56 upregulated genes. The other upregulated pathways were subsets of metabolism, including PPAR signalling and fatty acid biosynthesis (Fig. 5.12A). The KEGG pathways that lithium downregulated in WAT were largely processes involving the immune system (Fig. 5.12B). The

differential gene expression of a select number of genes known to be altered by lithium or rosiglitazone were examined in detail (Table 5.1).

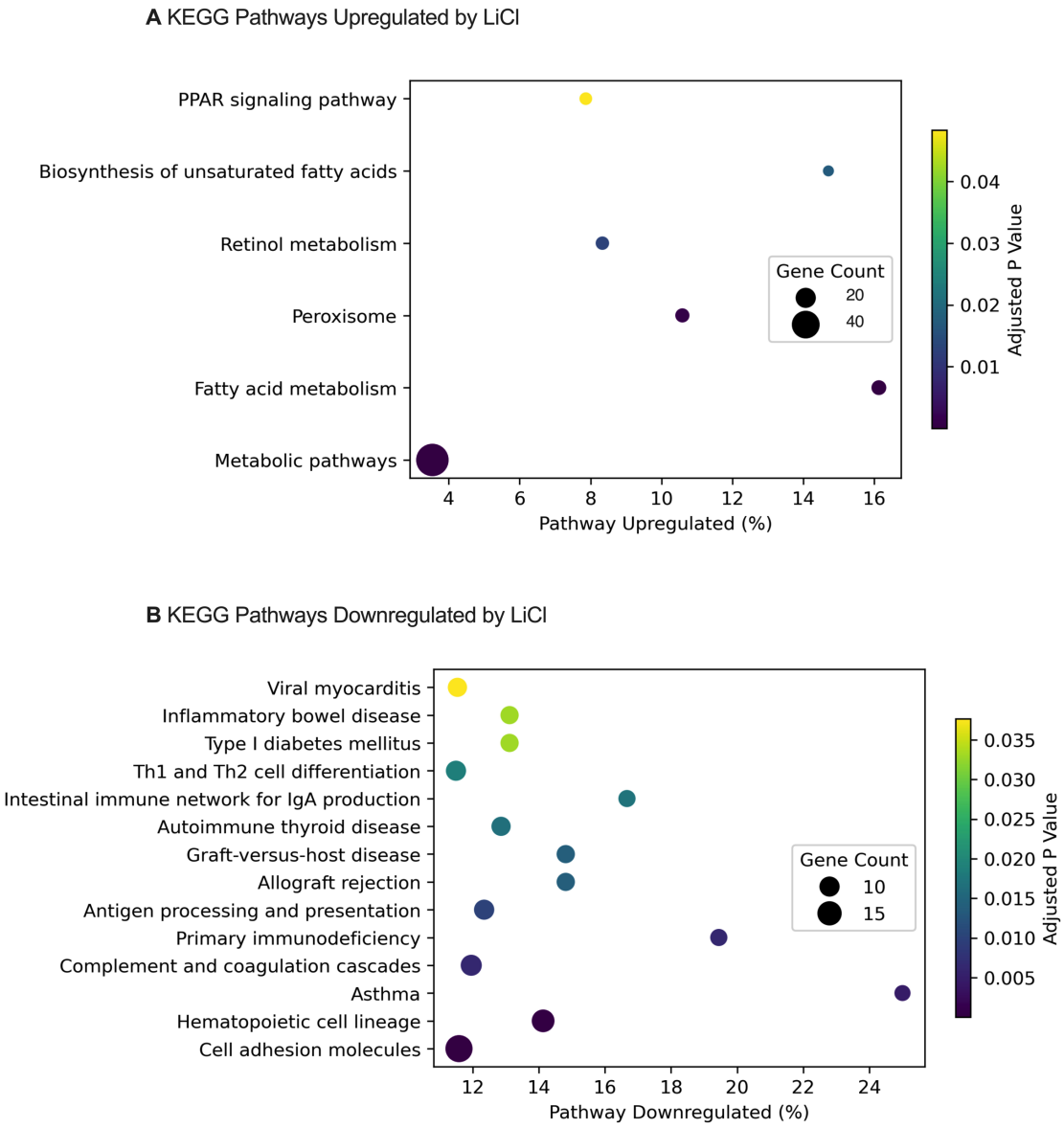

Figure 5.12 KEGG Pathways Altered by Lithium. Functional enrichment analysis for the KEGG pathways altered by lithium in the WAT of HFD mice was performed. (A) Pathways significantly upregulated by lithium and the extent (percent of genes in the pathway) to which they were upregulated. (B) Pathways significantly downregulated by lithium and the extent (percent of genes in the pathway) to which they were downregulated.

Gene Symbol	Ensembl Gene ID	P Value	Fold Change Induced by Lithium
Pck1	ENSMUSG00000027513	0.0021	1.3301
Dlat	ENSMUSG00000000168	0.0011	1.2789
Aco2	ENSMUSG00000022477	0.0484	1.1385
Acadm	ENSMUSG00000062908	0.0887	1.1341
Sdha	ENSMUSG00000021577	0.0371	1.1330
Traf3	ENSMUSG00000021277	0.5351	1.0901
Slc2a4	ENSMUSG00000018566	0.9425	1.0133
Tollip	ENSMUSG00000025139	0.5008	0.9623
Nfkb1	ENSMUSG00000028163	0.2859	0.9290
C2	ENSMUSG00000024371	0.0019	0.7252
Ptgs2	ENSMUSG00000032487	0.5719	0.6789
Clu	ENSMUSG00000022037	6.68E-06	0.6403
Kng2	ENSMUSG00000060459	0.0403	0.5802
Lck	ENSMUSG00000000409	0.0122	0.4703
Stat4	ENSMUSG00000062939	0.0181	0.2877
Gzmb	ENSMUSG00000015437	0.0024	0.2136

Table 5.1 Differential Gene Expression Induced by LiCl in the WAT of HFD Mice for Selected Genes.

The pathways that lithium upregulated were connected to PPARγ, but we wanted to more directly see if lithium increased PPARγ activity. For this we used the enrichment tool Enrichr to identify transcription factors whose target genes were enriched among the genes upregulated by lithium (Fig. 5.13) (Chen *et al.*, 2013). The ChIP-x Enrichment Analysis (ChEA) database of gene targets for transcription factors mined from published literature was used for this analysis. Gene targets in this database were obtained from large-scale experiments that reported the binding of transcription factors to DNA regions through chromatin immunoprecipitation (ChIP) (Lachmann *et al.*, 2010). ChIP detects the cross-linking of protein with DNA

via immunoprecipitation. Sequencing the bound DNA regions allows the detection of targets genes that are bound by the protein of interest (Rodríguez-Ubreva and Ballestar, 2014). This data was used to link transcription factors to gene expression changes by computing binding site over-representation. Additionally, an odds ratio was calculated for the association between lithium-enriched genes and the target genes of each transcription factor in separate ChIP experiments (Lachmann *et al.*, 2010; Chen *et al.*, 2013). PPARγ and its binding partner RXR were among the significantly enriched transcription factors. PPARγ had the highest odds ratio of 3.24. Other transcription factors related to PPARγ were enriched including liver X receptor (LXR) and PPARα. Estrogen receptor α (ESR1) was also significantly enriched among the lithium-upregulated genes. The same transcription factor enrichment analysis was performed using the ChEA database for genes downregulated by lithium, however, no transcription factor regulated gene sets were significantly enriched after correcting for multiple testing.

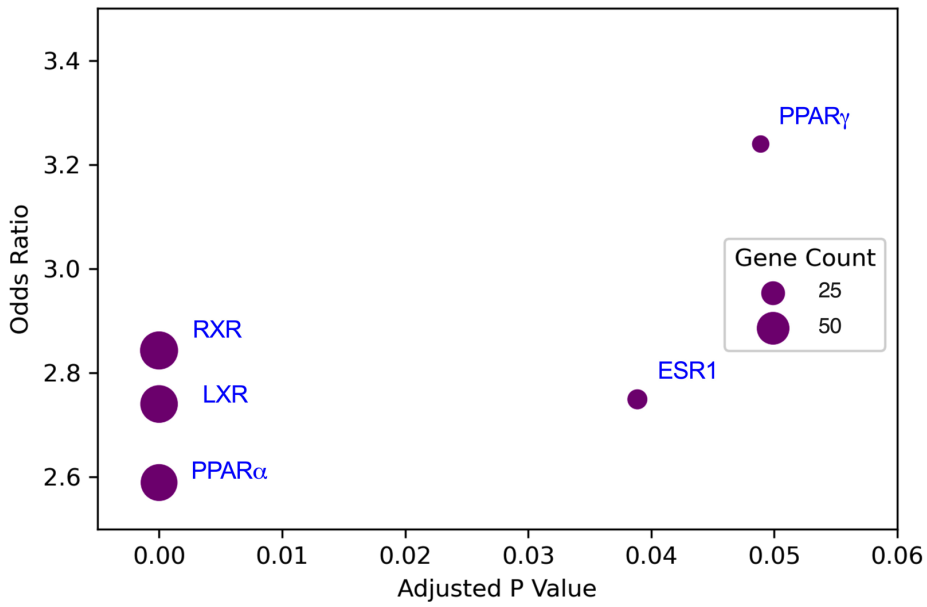

Figure 5.13 Lithium Upregulates Genes that are Under PPARγ Regulation. An enrichment analysis using the ChEA database in Enrichr was performed on the genes upregulated by lithium treatment in the WAT of HFD mice.

The RNA-sequencing data confirmed that lithium upregulated processes related to PPARγ activity and enrichment analysis even indicated that PPARγ could be driving the differentially upregulated genes in our lithium-treated WAT samples. To increase our confidence that lithium does indeed upregulate PPARγ-dependent genes, we compared our differentially expressed genes with a previously published gene enrichment study using the PPARγ agonist rosiglitazone. For this comparison a study by Soccio *et al.* was chosen as the researchers used C57BL/6 mice fed the same HFD as our mice. The researchers administered the HFD to six-week-old mice for 10 weeks followed by two weeks of rosiglitazone administration in the diet at 36 mg/kg of food. RNA-sequencing was then performed (Soccio *et al.*, 2017). In their analysis the authors differentiated between visceral (epididymal - eWAT) and subcutaneous (inguinal - iWAT) white adipose tissue. We collected eWAT and iWAT

from the gonads of our mice (gWAT) as we were not interested in the distinction for our analyses.

We reanalysed the authors' raw gene counts with the same differential expression analysis performed on our own samples. This analysis revealed an overlap of 32.48% between the genes upregulated by lithium in our study and the genes upregulated by rosiglitazone in iWAT in the study of Soccio *et al*. This overlap reduced to 10.48% in the eWAT rosiglitazone samples (Fig. 5.14A). The authors of the study, however, concluded that rosiglitazone did not substantially alter gene expression in eWAT and focused on iWAT for their own analysis. In addition, there was only one upregulated gene in eWAT that was not present in the overlap of our data with iWAT so we also focused on comparing our data to the iWAT rosiglitazone samples going forward (Soccio *et al.*, 2017). In terms of downregulated genes there was an overlap of 17.74% between our lithium-treated gWAT samples and rosiglitazone-treated iWAT samples (Fig. 5.14B).

G:Profiler was used to calculate the enrichment of KEGG pathways for the genes upregulated by LiCl in our gWAT and by rosiglitazone in the iWAT of Soccio *et al* (Fig. 5.15). The pathway with the largest proportion of genes upregulated by lithium and rosiglitazone was the fatty acid metabolism pathway with 16.13% of the genes in the pathway upregulated. The majority of pathways involved metabolism, energy production, and energy storage in the form of fatty acid biosynthesis and elongation. As expected, the PPAR signalling pathway was significantly upregulated by lithium and rosiglitazone. For the 33 genes that were downregulated by both lithium and rosiglitazone, the only significantly enriched KEGG pathway was the

complement and coagulation cascades pathway with three of 92 genes downregulated.

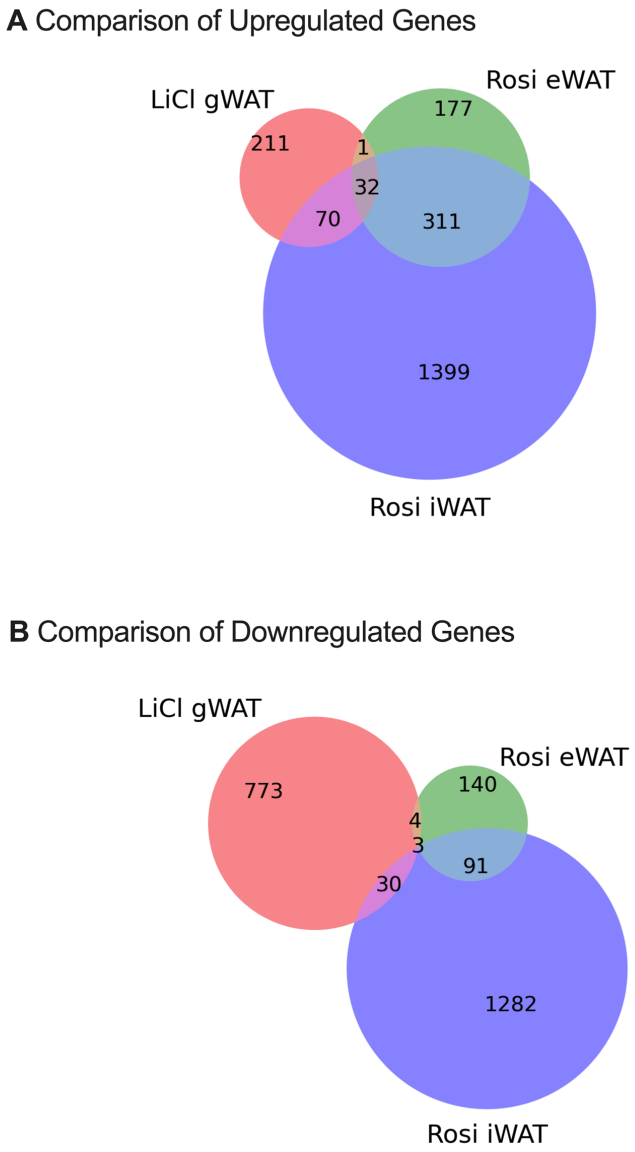

Figure 5.14 Overlap Between the Genes Differentially Regulated by Lithium and Rosiglitazone in the WAT of HFD Mice. Differential gene expression analysis was performed on WAT from HFD mice administered LiCl and rosiglitazone (rosi). A comparison of the genes (A) upregulated and (B) downregulated by lithium and rosi was performed (Soccio et al., 2017).

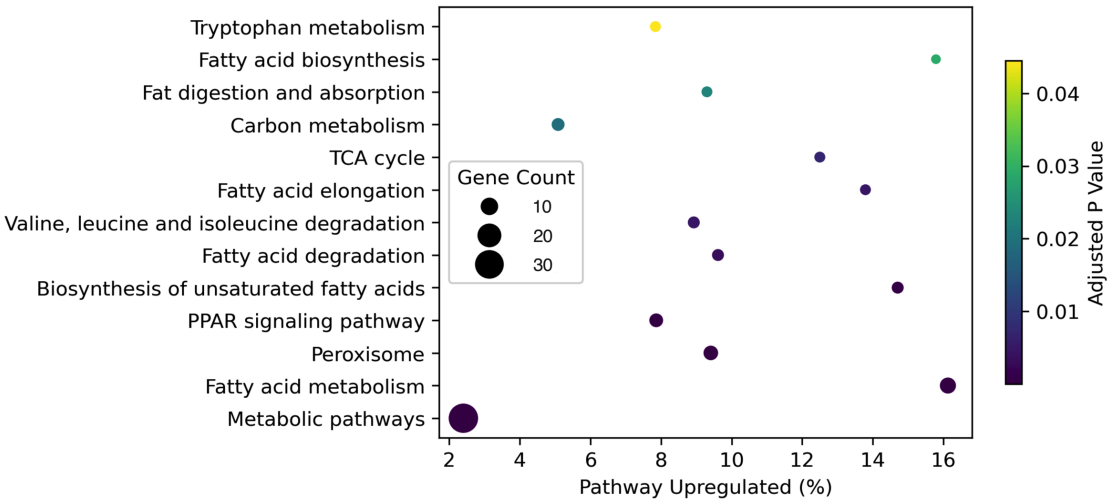

Figure 5.15 KEGG Pathways Upregulated by Lithium and Rosiglitazone Treatment in the WAT of HFD Mice. Functional enrichment analysis for the KEGG pathways upregulated by lithium and rosiglitazone in the WAT of HFD mice was performed.

To confirm that the pathways upregulated in WAT by lithium and rosiglitazone were in turn also regulated by PPARγ, the ChEA enrichment analysis was performed on the gene set upregulated by lithium and rosiglitazone (Fig. 5.16). In support of our hypothesis that rosiglitazone and lithium share the common target PPARγ, genes from three PPARγ ChIP sequencing datasets (PPARG 20887899, PPARG 20176806 and PPARG 19300518) using 3T3-L1 cells were significantly upregulated by lithium and rosiglitazone. Other transcription factors enriched included RXR and PPARα (with equal significance and odds ratios), LXR, Estrogen Related Receptor β (ESRRB), and TATA-Box Binding Protein Associated Factor 7 Like (TAF7L). No transcription factors were significantly enriched in the 33 common downregulated genes of lithium and rosiglitazone.

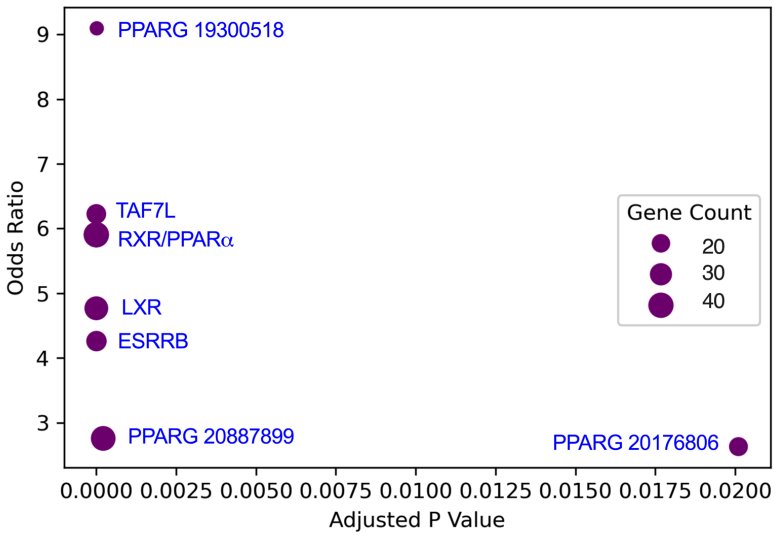

Figure 5.16 Lithium and Rosiglitazone Upregulate Common Genes that are Under PPARγ Regulation. An enrichment analysis using the ChEA database in Enrichr was performed on the genes upregulated by lithium and rosiglitazone treatment in the WAT of HFD mice.

To further confirm our overlap with the dataset of Soccio *et al.*, we compared our upregulated genes to a dataset from the same researchers in which they administered rosiglitazone to mice on a low-fat diet and performed ChIP-sequencing (Soccio *et al.*, 2015). Under these conditions, 28.34% of our lithium upregulated genes were also upregulated in their dataset, while 6.00% of our downregulated genes overlapped with theirs. The slight decrease of overlap in upregulated genes is to be expected as PPARγ expression is increased in mice given a HFD (Vidal-Puig *et al.*, 1996). Thus overall, this agreement with another dataset increased our confidence in our dataset.

5.3 Discussions

5.3.1 *In Vitro* and *In Vivo* Data Support a Role for PPARγ in Lithium-Induced Weight Gain

It has been shown that lithium increases weight gain in obese bipolar patients 5.5-fold relative to nonobese bipolar patients over one year, however the mechanism of this selective weight gain has not been discovered (Bowden *et al.*, 2006). Previous work from our lab found that lithium is capable of increasing C/EBPα activity in murine MMHD3 cells (Sanghani, 2018). C/EBPα is a key player in the process of adipogenesis and lipid accumulation. PPARγ and C/EBPα form a positive feedback loop and both bind 60% of the genes upregulated during 3T3-L1 differentiation (Lefterova *et al.*, 2008). PPARγ, however, is the master regulator as it can drive adipogenesis in C/EBPα-deficient cells (Rosen *et al.*, 2002). We therefore hypothesised that lithium could promote weight gain in obese individuals by increasing PPARγ activity.

Our *in vitro* transfections of the PPRE::LUC reporter indicated that lithium is indeed capable of increasing PPARγ activity. Our siRNA knockdown confirmed that this increase in activity was largely driven by PPARγ1 and/or PPARγ2. Previous research has indirectly linked lithium to PPARγ. As discussed previously, lithium's signature of gene expression in astrocytes was matched to the signature of PPARγ agonists (Rivera and Butt, 2019). PPARγ has even been linked to mood improvements in BD patients. A study examining BD patients with insulin resistance found that pioglitazone (a TZD) improved insulin resistance and simultaneously decreased depressive symptomatology (Kemp *et al.*, 2014). To the best of our

knowledge, our study, however, is the first to show that lithium is capable of directly inducing PPARγ activity.

PPARγ is a highly relevant transcription factor for metabolism and its induction is tied to several metabolic phenotypes, including weight gain. Our incubation of mature adipocytes with lithium indicated that lithium trends toward increasing lipid accumulation *in vitro*. We did not see a statistically significant increase in lipid accumulation when 1μM of the positive control rosiglitazone was applied for four days after full differentiation. Previous work has shown that differentiation and maintenance of adipocytes in the presence of 2μM rosiglitazone for seven days after full differentiation increases lipid accumulation by four-fold (Szychowski *et al.*, 2021). Our results are similar to those of Wang *et al.* who found no statistically significant difference in lipid accumulation after applying 0.5μM rosiglitazone to 3T3-L1 mature adipocytes for two days, so it is likely we would have seen a significant increase if we had extended the length of rosiglitazone treatment (Wang *et al.*, 2007). We did try to extend the incubation period, but the adipocytes accumulated lipids to the extent that they detached during media changes, which is indicative that rosiglitazone did increase lipid accumulation. However, four days was sufficient to show a trend in lipid increase with lithium treatment. Overall, our *in vitro* results supported the notion that lithium only increases lipid accumulation in mature adipose tissue, as it actually inhibits differentiation of preadipocytes (Ross *et al.*, 2000).

We were able to link this *in vitro* lipid increase to *in vivo* weight gain. In control mice with less adipose tissue, lithium did not alter weight, but lithium

administration in a DIO mouse model led to significant weight gain. These are the first results to experimentally support the observations that lithium tends to increase weight gain in obese patients (Bowden *et al.*, 2006). Previous work in BTBR-*ob/ob* mice found that lithium did not induce weight gain. However, the serum levels of lithium in these mice were on the lower end of the therapeutic spectrum at 0.48±0.04 mM. In addition, BTBR-*ob/ob* mice are model of type 2 diabetes which have a homozygous *ob/ob* mutation inducing leptin deficiency and causing the animals to overeat (Groot *et al.*, 2017). The leptin expression in our mice was not genetically altered and their feeding activity was the same as control mice. Therefore, it is possible that the increased intake of fat in our HFD mice was necessary to reveal lithium-increased lipid storage.

One previous study measured the effect of rosiglitazone on weight gain in control and obese mice for 14 days. The researchers administered rosiglitazone at 80 mg/kg in the diet of male control and leptin receptor-deficient obese-diabetic *db/db* mice and found that it barely increased body weight by 5.51±4.43% in control mice but increased weight by 15.91±3.01% in *db/db* mice. In addition, they found that rosiglitazone increased fluid retention and as a result, control mice exhibited increased urine volume and sodium excretion. The *db/db* mice, however, did not exhibit polyurea or natriuresis (Zhou *et al.*, 2013). We made similar observations in our lithium-treated mice fed standard chow and our lithium-treated mice fed HFD, as standard chow mice exhibited polyurea while none of the HFD animals did.

The majority of clinical trials involving TZDs have used moderately to severely obese individuals with diabetes mellitus (Fonseca, 2003). One study using clinical data from U.K. Clinical Practice Research Datalink found that BMI influenced the reduction of glycated haemoglobin levels (HbA_{1c}, correlated to blood glucose levels) after one year of TZD treatment with pioglitazone or rosiglitazone. Patients with a BMI greater than 30 showed a greater glycaemic response to TZD treatment (Dennis *et al.*, 2018). We replicated this finding in our own data by showing that lithium significantly improves glucose tolerance in obese mice on a HFD, but not mice fed standard chow.

TZDs have been shown to increase insulin sensitivity, but primarily only in insulin resistant individuals (Lee *et al.*, 2008). Our results match these findings as our control HFD mice had significantly worse insulin tolerance compared to control mice fed standard chow (indicating insulin resistance) and we saw an improvement in insulin tolerance at 60 minutes of the ITT in our lithium-treated HFD mice - not in our lithium-treated mice on standard chow. In addition, the fact that overall insulin tolerance was not different between our lithium-treated HFD mice and lithium-treated standard chow mice does indicate an overall improvement in insulin tolerance in HFD mice.

One perhaps perplexing finding from our *in vivo* study is that glucose tolerance was not worse in our control HFD mice compared to our control mice on standard chow. Mice on a HFD have been found to exhibit worsened glucose tolerance as soon as three days after commencement of the HFD, though the glucose tolerance test in this case was performed after only 5 hours of fasting (Williams *et*

al., 2014). Another study using two independent cohorts of high-fat, high-sucrose-fed (HFHS) C57Bl/6 mice at two different institutions found no difference in glucose tolerance despite hyperinsulemia. Using stable isotope-labelled glucose, they found that though overall glucose disposal rate was the same between HFHS and control mice, HFHS mice showed enhanced rates of hepatic futile glucose cycling. They concluded that glucose taken up by hepatocytes was dephosphorylated and released back into circulation (Kowalski *et al.*, 2017). It is possible that our own HFD control mice were exhibiting futile glucose cycling; their glucose tolerance (or disposal) was unchanged, but their fasting glucose levels were significantly higher than the fasting glucose levels of mice on standard chow.

In both HFD mice and mice on standard chow, lithium removed the positive correlation between weight and fasting glucose levels. TZD treatment has been shown to increase glycogen synthesis and improve insulin-stimulated glucose uptake in patients with type 2 diabetes, which reduce fasting glucose levels and increase glucose and insulin sensitivity as we observed (Boden *et al.*, 2003). A study of rosiglitazone administration in *db/db* mice found that rosiglitazone decreases gluconeogenesis (a process that is enhanced in type 2 diabetes and results in high blood glucose levels) in the liver. Furthermore, it increased mitochondrial energy production in inguinal adipose tissue (a mixture of white and brown adipose tissue) via the tricarboxylic acid (TCA) cycle and β-oxidation (Galizzi *et al.*, 2010).

Our findings that lithium improves insulin sensitivity matches previous research in pancreatectomized diabetic rats, which proposed lithium could be a treatment for non-insulin-dependent diabetes mellitus due to its restoration of

insulin sensitivity (Rossetti, 1989). Lithium has also been found to decrease fasting glucose levels and improve glucose sensitivity as measured by the glucose tolerance test in the *ob/ob* mouse model of obesity (Groot *et al.*, 2017). In addition, lithium increases glucose uptake in skeletal muscle. However, it primarily does so by augmenting the action of insulin; it does not increase glucose uptake on its own (Tabata *et al.*, 1994; Jung *et al.*, 2017). Like rosiglitazone, this insulin sensitization is linked to an increase in glucose transporter 4 (GLUT4) in the plasma membrane (Jung *et al.*, 2017). This improvement in glucose and insulin tolerance has not previously been tied to PPARγ activity until now.

5.3.2 Lithium's Alteration of the White Adipose Tissue Transcriptome Indicates Engagement of PPARγ

TZDs are widely accepted agonists of PPARγ that improve insulin and glucose tolerance, but cause weight gain. One mechanism that has been proposed to explain these metabolic changes is a TZD-driven PPARγ-dependent increase in glyceroneogenesis (Tordjman *et al.*, 2003). Glyceroneogenesis allows adipocytes to re-esterify FFAs and store them as triacylglycerol (also known as triglycerides) (Reshef *et al.*, 2003). FFAs contribute to insulin resistance in type 2 diabetes by inhibiting insulin-stimulated peripheral glucose uptake (Boden, 1997). Therefore, an increase in glyceroneogenesis reduces FFAs and improves insulin sensitivity. Rosiglitazone-driven increase in glyceroneogenesis has been linked to increased expression of the rate-limiting enzyme phosphoenolpyruvate carboxykinase (PCK1) in adipocytes (Glorian *et al.*, 2001; Tordjman *et al.*, 2003). Likewise we found that lithium also significantly augmented PCK1 expression by 33.01% relative to our

HFD controls. In addition, the significantly enriched KEGG pathways by both rosiglitazone and lithium treatment in WAT supported an upregulation of lipid storage through pathways such as fatty acid biosynthesis, biosynthesis of unsaturated fatty acids, fatty acid elongation, and fat digestion and absorption. Fatty acid degradation can also reduce FFAs and this pathway was upregulated by lithium and rosiglitazone. TZDs increase fatty acid oxidation in the plasma of diabetic patients as measured by indirect calorimetry and this has been correlated to increased expression of acyl-CoA dehydrogenase (ACADM) and acetyl-CoA acetyltransferase (Boden, 1997; Boden *et al.*, 2003). Lithium trended towards significantly increasing ACADM expression by 13.41% in the WAT of our HFD mice.

It has been suggested that TZDs also sensitize tissues to insulin by increasing the presence and expression of GLUT4 – an insulin-sensitive glucose cellular transporter. One study found that rosiglitazone increased GLUT4 in the plasma membrane by increasing endosomal recycling, while another found that rosiglitazone also increases the expression of GLUT4 in the adipose tissue of *db/db* mice (Young *et al.*, 1995; Martinez *et al.*, 2010). Lithium slightly increased GLUT4 expression by 1.33% in the WAT of our HFD mice, but this was not significant. This could be due to a lack of statistical power as we only had two control samples, but it has been suggested that lithium increases GLUT4 activity rather than GLUT4 expression and this would be consistent with our results (Jung *et al.*, 2017). In our cross comparison, lithium and rosiglitazone both significantly upregulated four genes (PCK1, mitochondrial aconitate hydratase, succinate dehydrogenase complex flavoprotein subunit A, and dihydrolipoamide s-acetyltransferase) in the TCA cycle. The TCA cycle feeds into fatty acid biosynthesis and elongation via acetyl-CoA,

providing multiple levels at which TZDs and lithium promote lipid storage and reduce FFAs. Thus, our data suggests that lithium mostly exerts its metabolic effects by altering lipid metabolism rather than glucose metabolism as primarily lipid pathways were upregulated by lithium.

Intriguingly, lithium significantly upregulated genes involved in retinol metabolism and these were not upregulated by rosiglitazone treatment. Retinoid metabolism has been tied to improved insulin sensitivity (Rhee and Plutzky, 2012). TZDs and rexinoids are believed to exert their insulin-sensitising effects through differential regulation of gene expression in WAT so this provides a potentially PPARγ-independent mechanism through which lithium could increase insulin sensitisation as well (Ahuja *et al.*, 2001)

The pathways that were downregulated in lithium-treated WAT were pathways of the immune system. This is consistent with the literature showing that lithium has anti-inflammatory properties. Lithium has primarily been tied to reduced secretion of interleukin-6 and tumor necrosis factor-α, but its alteration of inflammatory gene expression is still being explored (Nassar and Azab, 2014). Lithium has been tied to reduced lipopolysaccharide-induced expression of cyclooxygenase-2 (*Ptgs2*) (Nassar and Azab, 2014). In our WAT tissues, lithium reduced *Ptgs2* expression by 32.11%, but this was not significant likely due to our lack of statistical power. Lithium has also been shown to reduce NF-κB-driven inflammation by increasing the expression of inhibitors TNF Receptor Associated Factor 3, Tollip, and NF-κB1/p50 in macrophages (Makola *et al.*, 2020). None of these genes were significantly altered in our lithium-treated WAT. We do, however,

present new evidence of further inflammatory genes that lithium significantly reduces the expression of, including signal transducer and activator of transcription 4 (STAT4), proto-oncogene Lck, and Granzyme B.

Intriguingly, TZDs have also been shown to reduce inflammation in adipose tissue. They reduce NF-κB activation and reduce expression of its target genes (Pascual *et al.*, 2005). When the differential gene expression driven by lithium and rosiglitazone were compared, three genes from the complement and coagulation cascades were significantly downregulated. These genes included complement C2, clusterin, and kininogen 2, which have been implicated in the inflammation and insulin resistance tied to obesity, but not to TZD or lithium action (Morais *et al.*, 2015; Köhler *et al.*, 2020; Shim *et al.*, 2020; Wittwer and Bradley, 2021).

5.3.3 Enrichment Analysis Points to Lithium-Induced Activation of PPARγ

While lithium's pathway changes pointed to metabolic alterations that mimic TZD action, perhaps the most convincing evidence of lithium-driven PPARγ engagement came from the ChEA enrichment analyses which showed that lithium's upregulation of gene expression mimicked the upregulation driven by PPARγ in ChIP experiments. While PPARγ genes in 3T3-L1 cells had the highest odds ratio, the most significantly enriched transcription factor for lithium-upregulated genes was PPARγ's heterodimeric binding partner RXR. When rosiglitazone and lithium upregulated genes were combined, three PPARγ gene sets were significantly enriched. These results support our hypothesis that lithium increases PPARγ activity in adipose tissue.

In the liver, PPARα activates mitochondrial and peroxisomal fatty acid β-oxidation (Varga, Czimmerer and Nagy, 2011). Accordingly, the PPARα ChIP-sequencing data that lithium upregulated came from the liver. Given that lithium upregulated genes involved in peroxisomal β-oxidation from the fatty acid degradation pathway in our WAT samples, this connection is unsurprising and points to additional points of regulation for lithium in the liver. In addition to PPARγ and PPARα, RXR also heterodimerises with LXR to induce lipogenic genes in the liver. Accordingly, genes from an LXR ChIP-sequencing experiment in liver were also significantly enriched in the lithium upregulated genes. It has been noted that PPARα binds 71 to 88% of the sites bound by the RXR:LXR heterodimer which partially explains why all three transcription factors were hits (Boergesen *et al.*, 2012). Indeed 27 lithium upregulated genes were common among the three ChIP-sequencing datasets. This amounts to 39.13% of the lithium upregulated genes for PPARα, 37.5% of the lithium upregulated genes for LXR, and 36.49% of the lithium upregulated genes for RXR. LXR is also known to regulate cholesterol levels and triglyceride and glucose homeostasis, tying it into the metabolic changes driven by lithium (Laurencikiene and Rydén, 2012). It is therefore possible that lithium could also induce transcriptomic changes in the liver via LXR.

Perhaps the most surprising transcription factor hits in our ChEA analyses was the enrichment of ESR1 target genes in lithium upregulated genes and the enrichment of ESRRB target genes in the intersection of rosiglitazone and lithium upregulated genes. Oestrogen has been shown to promote adipogenesis in canine adipose-derived stem cells (Kim *et al.*, 2021). Oestrogen has even been tied to

glucose homeostasis. Specifically, ESR1 increases the expression of GLUT4 in adipocytes, improving glucose sensitivity (Gregorio, Laurindo and Machado, 2021). ESRRB is activated by ESR1, suggesting that it too could improve glucose sensitivity (Krishna *et al.*, 2018). Therefore, while lithium's metabolic and transcriptomic actions suggest it is a PPARγ agonist, our data also suggests that it upregulates other transcription factors which contribute to improvement of glucose and insulin sensitivity.

5.3.4 Conclusion

In this chapter we investigated whether lithium increases PPARγ activation and additionally if this activation could explain lithium's metabolic effects. We presented *in vitro* evidence that lithium increases PPARγ-dependent activity. Furthermore, we found that it mimicked the phenotype of PPARγ agonists by trending toward increasing lipid accumulation in mature adipocytes *in vitro*. These results were confirmed *in vivo*, as lithium increased weight gain in a DIO mouse model, but not in healthy control mice. Lithium's improvement of glucose sensitivity and partial improvement of insulin sensitivity along with its alteration of the adipose transcriptome strongly suggest that lithium alters metabolism in a PPARγ-dependent fashion. The mechanisms of how lithium increases PPARγ activity remain unclear from our data. Lithium does not seem to increase the rate of PPARγ degradation (Fig. 5.2A), however, a cycloheximide chase assay would be needed to fully rule that possibility out. It seems likely that lithium increases PPARγ activity though a phosphorylation-dependent mechanism as PPARγ activity is dramatically

altered by phosphorylation and as we demonstrated in Chapter 3, lithium is capable of inhibiting widespread kinase activity (Yin, Dong and Li, 2006; Burgermeister and Seger, 2007). The revelation that lithium is capable of increasing PPARγ activity provides an explanation for lithium-induced weight gain in certain (overweight) patient populations. Given that weight gain is a major deterrent to lithium treatment, this understanding of lithium's metabolic actions can be used to estimate the risk of weight gain and to implement measures to mitigate it.

5.4 References

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6. GENERAL DISCUSSION

Bipolar disorder (BD) is a chronic mood disorder that affects 1-5% of the world's population (Akiskal *et al.*, 2000). In 2018-2019 the economic burden of BD was estimated as £6.43 billion in the UK mainly due to productivity losses and informal care (Simon *et al.*, 2021). 20-60% of BD subjects attempt suicide and the rate of completed suicides is 10-30 times higher than the general population, making treatment of BD of vital importance (Dome, Rihmer and Gonda, 2019). Lithium is the gold standard treatment for BD. It is a true mood stabiliser as it is effective in treating both mania and depression (Goodwin *et al.*, 2016). However, lithium's mechanism(s) of action remain unknown. Recent studies have focused on lithium's modulation of circadian rhythms. Circadian rhythms modulate 24-hour rhythms in behavioural and physiological processes ranging from metabolism and temperature regulation to sleep and hormonal rhythms (Jagannath *et al.*, 2017). It has therefore been proposed that lithium could exert its mood stabilising effects by modulating circadian rhythms. Thus, recent research has focused on identifying the mechanisms by which lithium alters these rhythms (Yin *et al.*, 2006; Sanghani, 2018).

Though effective, lithium pharmacotherapy is mired by difficult dosing regimens and toxic side effects that reduce patient compliance and the duration for which the treatment can be prescribed. These side effects range from weight gain to diabetes insipidus and nephrotoxicity (McKnight *et al.*, 2012; Azab *et al.*, 2015). For the particular side effect of weight gain, the mechanisms are poorly understood. In addition, it is not clear the extent to which lithium can alter metabolism. Research into the molecular processes underpinning these side effects would enable the

development of additional treatments to mitigate them for patients who rely on lithium.

6.1 Key Findings

Kinases are powerful chronomodulators and play key roles in the regulation of sleep and circadian rhythms (Wang *et al.*, 2018; Brüning *et al.*, 2019; Taylor *et al.*, 2021). Lithium's alteration of kinase activity was examined as a potential explanation for its impact in widespread signalling pathways. It was found to be capable of inhibiting kinases across genetic families *in vitro* – a property consistent with the hypothesis that lithium could inhibit these enzymes by competing with magnesium. Furthermore, many of these lithium-sensitive kinases were shown to play a role in regulating circadian period and/or amplitude. Thus an additional mechanism of chronomodulation for lithium was proposed in which it inhibits rhythmic kinase activity. Investigations of lithium's chronomodulation *in vivo* yielded the surprising discovery that lithium tolerance is different between male and female mice. Though lithium alters the master and peripheral clocks of male and female mice with equal efficacy, *in vivo* tolerance for lithium dose appeared to be higher in females and possibly explained why females were more sensitive to lithium's behavioural period elongation. Finally, lithium's alteration of metabolic pathways was investigated and a new mechanism of lithium-induced weight gain was proposed in which lithium increases the activity of the transcription factor peroxisome proliferator activated receptor γ (PPARγ). This augmentation of PPARγ

activity was used to explain lithium's induction of weight gain and improvement of insulin and glucose tolerance in overweight patients.

6.2 The Implications of Lithium's Inhibition of Kinase Activity

The examination of lithium's inhibition of kinase activity revealed that lithium inhibits kinases at multiple steps in various cellular pathways. This widespread kinase inhibition could have far reaching consequences and impact many systems beyond the circadian rhythm. Most notable among the pathways impacted by lithium's kinase inhibition was the the MAPK signalling pathway. Activation of the MAPK pathway has been linked to antimanic and neuroprotective effects – both of which have been attributed to lithium. These effects have previously primarily been attributed to activation of mitogen-activated protein kinase kinase 1/2 (MAP2K1/2), extracellular signal-regulated kinase (ERK1/2) and Ribosomal S6 kinase 1 (RSK1) (Einat *et al.*, 2003; Haupt *et al.*, 2020). Our study revealed up to 26 kinases in the MAPK pathway can be inhibited by lithium *in vitro* and the inhibition or knockdown of 15 of these alters circadian rhythms. Thus additional lithium-sensitive targets in the MAPK pathway were revealed and a functional consequence for this inhibition discovered. The impact of the inhibition of these MAPK kinases is likely to be highly cell type specific (as discussed in Chapter 3) as to whether it activates or inhibits the MAPK pathway (or subpathways).

Lithium shares some of its previously established MAPK targets with other prescribed bipolar medications, such as valproic acid (VPA) (Einat *et al.*, 2003). The mechanism for VPA's inhibition of these select kinases is unknown, but given the

short chain fatty acid structure of VPA, it is unlikely to compete with magnesium as we have proposed for lithium. Indeed, VPA does not consistently inhibit glycogen synthase kinase 3β (GSK-3β) in the same cell lines as lithium (Ryves *et al.*, 2005). Lithium remains the gold standard treatment over other mood stabilisers, and this improved efficacy could be attributed to the multiple levels at which lithium is capable of altering pathway activity through inhibition of kinases. It has previously been shown that inhibition of the MAPK pathway with a blood brain barrier permeable MAP2K1 inhibitor reduces time spent immobile in the forced swim test (used as an indication of reduced depression) but increases activity in the open field test – a trait reminiscent of amphetamine-induced mania (Einat *et al.*, 2003). Thus, the cell type specific expression of kinases may explain lithium's mood alterations and how it reduces time spent immobile, but does not alter basal activity in the open field test (Sanghani *et al.*, 2021).

The impact of lithium's inhibition of components of the MAPK pathway even ties into the regulation of the metabolic transcription factor PPARγ whose actions were explored in Chapter 5. The ERK/c-jun N-terminal kinase (JNK) MAPK pathway inhibits PPARγ activity though phosphorylation and nuclear shutting (Yin, Dong and Li, 2006; Burgermeister and Seger, 2007). Thus, lithium could potentially activate PPARγ through MAPK kinases. Furthermore, lithium-sensitive kinases that altered circadian rhythms were found to play a significant role in the insulin signalling pathway and the mammalian/mechanistic target of rapamycin (mTOR) signalling pathway (which is downstream of insulin signalling). We found that lithium protected against worsened insulin tolerance due to weight gain in diet-induced obese mice, so it is possible that lithium improved insulin tolerance by inhibiting

kinase activity in the insulin and mTOR signalling pathways, which could alter PPARγ activity.

Lithium's well-known neuroprotective and anti-inflammatory effects were further explained by its inhibition of a number of kinases in the neurotrophin and the T cell receptor signalling pathways which overlap with MAPK signalling. Structural alterations and decrease in brain volume and cognitive decline have been found in BD patients (Ochoa, 2022). Lithium has been shown to increase expression of the neuroprotective B-cell lymphoma 2 and brain-derived neurotrophic factor proteins, and this occurs through MAPK-driven activation of the neurotrophin signalling pathway (Quiroz *et al.*, 2010). Our study reveals additional MAPK kinase targets through which lithium could exert a cell type dependent protective or apoptotic effect. BD is further characterised by increased levels of neuroinflammation and lithium pharmacotherapy alters the ratio of T cell subtypes in the blood (Wahlin, Knorring and Roos, 1984; Panaccione, Spalletta and Sani, 2015). Our study proposes kinase targets that could induce alternate effects on T cell survival which warrant further investigation.

All of our *in vitro* results regarding kinases were shown in MMHD3 prehepatocytes and U2OS epithelial cells which contain circadian circuitry and are regularly used as circadian models (Baggs *et al.*, 2009; Ramanathan *et al.*, 2014). They are therefore useful for determining the role of lithium-impacted kinases in core circadian circuitry. They are not, however, used as models for BD. It would therefore be of great interest to repeat these screens in cells derived from bipolar

patients and transfected with a circadian reporter. Such experiments would show the relevance of lithium's kinase inhibition for BD specifically.

6.3 Sex-Based Differences in Lithium Tolerance

Sex-based differences in lithium administration are relatively unexplored. To the best of our knowledge, there are no differences in the recommended methods of prescribing lithium to male and female patients unless the female patients are pregnant (Goodwin *et al.*, 2016; Tondo *et al.*, 2019). There are, however, noted differences between male and female circadian rhythms, with women generally having shorter rhythms and higher sleep pressure (Paul, Turek and Kryger, 2008; Duffy *et al.*, 2011). Therefore, if there are sex-based differences in basal circadian phenotype, it stands to reason that there could be differences in lithium's chronomodulation. We established that lithium's alteration of the molecular clock is similar in male and female mice both at the level of the master clock and at the level of the peripheral liver clock. Lithium, however, did not lengthen the behavioural circadian clock in male mice in our experimental paradigm and we propose that this is due to an underlying difference in lithium pharmacokinetics and/or taste aversion.

Our data revealed that female mice drank more lithium dissolved in water than males. In addition, the females did not compensate for this increased lithium intake via increased sodium intake. Previous studies have examined sex-based differences in lithium accumulation and tolerance when animals are administered a controlled dose of lithium via injection or infusion. Such studies point to increased

lithium reabsorption in females, but higher accumulation of lithium in the tissue of males (García *et al.*, 1999; Uwai, Yamaguchi and Nabekura, 2021). Our data supported the finding that lithium reabsorption may be higher in females, as polyurea was observed at higher rates in our male mice despite the fact that they drank less lithium and liquid volume on average. To the best of our knowledge, ours is the first study suggesting that the lithium tolerance of female mice is higher than their male counterparts and this accounts for their overrepresentation in circadian studies of lithium's action.

6.4 *Cry* Genotype Does Not Determine Lithium's Chronomodulation

Previous research has shown that BD patients with longer rhythms in their molecular circadian clocks display a muted response to lithium's chronomodulation (Sanghani *et al.*, 2021). We tried to extend this research further by examining whether mice with a longer circadian rhythm also display a muted response to lithium's behavioural period lengthening. For our model of long rhythm mice, we chose mice lacking cryptochrome 2 (Cry2KO mice) and our short rhythm mice lacked cryptochrome 1 (Cry1KO mice). All female mice lengthened in response to lithium treatment regardless of *Cry* genotype. Thus, we established that *Cry* genotype does not play a role in lithium's behavioural period lengthening, but we do not rule out the possibility that other genetic mouse models with long rhythms may not respond to lithium's behavioural period elongation. Future studies could use other circadian knockout mice with short periods (such as the $CK1\varepsilon^{tau/tau}$ and $Per1^{Brdm1}$ mouse lines) and long periods (such as the $CK1\varepsilon^{-/-}$ and after-hours mutant

mouse lines) (Zheng *et al.*, 2001; Godinho *et al.*, 2007; Meng *et al.*, 2008; Ripperger, Jud and Albrecht, 2011). *Cry1* knockout has been shown to alter the circadian profile of GSK-3β phosphorylation under lithium treatment (Schnell *et al.*, 2015). Therefore our finding that CRY1 does not play a role in lithium's chronomodulation reinforces our suggestion from Chapter 3 that lithium's widespread inhibition of kinase signalling is more therapeutically relevant than its inhibition of GSK-3β alone. We do, however, suggest that CRY1 plays a protective role in lithium tolerance.

6.5 Lithium Increases PPARγ Activity

Though effective, lithium's administration is accompanied by side effects that lead to discontinuation of treatment. It has been found that up to 7% of patients on lithium discontinue treatment due to weight gain (Öhlund *et al.*, 2018). The odds of gaining more than 7% body weight upon lithium treatment is 89% above controls (Volkmann, Bschor and Köhler, 2020). Hyperparathyroidism and hypothyroidism have both been reported with lithium treatment, and it has been speculated that these side effects could contribute to lithium-induced weight gain (McKnight *et al.*, 2012). Indeed, lithium-sensitive circadian kinases were enriched in the thyroid signalling pathway. However, all but one of the hits in the thyroid signalling pathway were also involved in other hit pathways, including insulin and mTOR signalling. We therefore propose a new additional mechanism to explain the observation that obese patients are significantly more likely to gain weight under lithium pharmacotherapy (Bowden *et al.*, 2006).

We propose a mechanism of lithium-induced weight gain in which lithium increases the activity of the transcription factor PPARγ. Our studies demonstrated that lithium's increase of PPARγ-driven transcriptional activity mimics that of known PPARγ agonist rosiglitazone. This was shown through a trending increase in lipid accumulation in mature adipocytes *in vitro* and *in vivo* through significant weight gain in obese mice fed a high-fat diet. Lithium's augmentation of PPARγ activity is a plausible mechanism for lipid-dependent weight gain in which only overweight mice gain weight during lithium pharmacotherapy. It further explains the improved insulin and glucose tolerance in obese mice relative to controls. PPARγ agonists are often prescribed for the treatment of type 2 diabetes mellitus to improve glycaemic control (Nolan *et al.*, 2000; Wilding, 2006). Lithium treatment has been tied to nephrogenic diabetes insipidus but not to diabetes mellitus despite increasing weight gain (McKnight *et al.*, 2012). We present the first transcriptomic evidence for lithium-driven PPARγ engagement in the white adipose tissue of male obese mice.

An interesting follow up to this work would be to extend our sex-based studies of lithium tolerance in Chapter 4 to these metabolic studies of lithium's activation of PPARγ. There is research to suggest that the relative expression of PPARγ1 and PPARγ2 isoforms varies in different adipose depots between male and female rats (Kadowaki *et al.*, 2007). Furthermore, our own transcriptomic analyses revealed that lithium and rosiglitazone upregulate transcription factors involved in oestrogen signalling, suggesting a potential point for sex-based differences in lithium's metabolic actions. These investigations are needed as it has been shown

that women are three times more likely than males to discontinue lithium treatment because of weight gain (Öhlund *et al.*, 2018).

A role for PPARγ has recently been found not just in metabolic pathways, but in mood regulation as well. Indeed, it has been suggested that insulin sensitivity is linked to a decrease in depressive symptoms using the PPARγ agonist pioglitazone as an example (Kemp *et al.*, 2014). PPARγ agonism could explain lithium's anti-inflammatory properties given that pioglitazone (among other PPARγ agonists) reduces some of the same inflammatory markers as lithium (Nassar and Azab, 2014; Zhao *et al.*, 2016). In addition, bioinformatic analysis of lithium-driven alterations in the astrocyte transcriptome suggested lithium mimics PPARγ agonists (Rivera and Butt, 2019).

To the best of our knowledge, we are the first to directly show a lithium-driven increase in PPARγ activity and to explore the metabolic ramifications of this agonism. We demonstrated this augmentation of PPARγ activity in U2OS cells. These cells were chosen as they do not express CCAAT-enhancer-binding protein α (C/EBPα) and thus it was possible to isolate activity specifically driven by PPARγ (The Human Protein Atlas, 2022). However, these bone tissue cells are not a good model for lipid accumulation and metabolism and thus our results should be verified in hepatocyte and adipocyte cell lines. Future studies should also verify that PPARγ is involved in lithium's significant improvement of glucose tolerance. This could be done with a glucose uptake assay in PPARγ knockout cells. PPARγ knockout adipocytes could also be used to confirm that lithium's alteration of signalling pathways in the white adipose tissue of high-fat diet mice are driven by PPARγ.

6.6 Final Conclusions

Stated briefly, the original aims of this thesis were to investigate lithium's modulation of kinase activity, examine lithium's alteration of the behavioural clock, and explore the mechanisms underlying lithium-induced weight gain. We successfully established that lithium is capable of altering kinases across genetic families. Furthermore, we confirmed a functional consequence of this inhibition by demonstrating that inhibition of lithium-sensitive kinases alters molecular circadian rhythms. Our investigations of lithium's alterations of the behavioural clock revealed that *Cry* genotype does not play a role in lithium-induced behavioural period lengthening. We do, however, present the surprising outcome that female mice exhibit greater lithium tolerance than male mice. Finally, we propose a PPARγ-driven mechanism of lithium-induced weight gain that explains why lithium increases weight gain in obese patients while also improving glucose tolerance and preventing worsened insulin tolerance due to weight gain.

These findings provide insight into the molecular interactions of lithium and pave the way for the development of new bipolar treatments. Kinase inhibitors are quickly becoming a focus of the clinical world as over 70 drugs have been clinically approved since the approval of the first kinase inhibitor in 2001 (Cohen, Cross and Jänne, 2021). From our results, we hypothesise that lithium's mood stabilisation is not driven by action at a single kinase, but by its alteration of the overall pattern of kinase activity. Therefore, future studies will need to focus on a particular pathway or aspect of lithium pharmacotherapy which is of interest and use this narrow lens to identify targets of interest for specific phenotypes. The next focus would be to develop inhibitors for these target kinases (if they have not been developed for

another disorder already). Special attention should be made during these investigations to identify any sex-based differences in treatments. Future research should also confirm that lithium is a PPARγ agonist and examine whether this agonism is essential for lithium's mood stabilising properties. If it is not, PPARγ antagonists could be used to mitigate the risk of weight gain associated with lithium treatment. Therefore, by investigating the molecular actions of lithium in detail, we have used the gold standard mood stabiliser itself to guide future investigations into the development of alternative bipolar treatments and/or treatments for some of lithium's side effects.

6.7 References

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