Circadian rhythms in the neurobiology of bipolar disorder

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

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Circadian rhythms in the neurobiology of bipolar disorder
Joseph Timothy, PhD (Neuroscience), University of Manchester 2014

Daily rhythms of physiology and behaviour in mammals are orchestrated by a hierarchical network of cellular oscillators. The master pacemaker that defines local and systemic timing across the brain and body are the suprachiasmatic nuclei of the hypothalamus (SCN). Disruption to the timing of sleep and daily behavioural activity can manifest in a range of pathologies including neuropsychiatric disorders. Bipolar disorder (BPD) is once such neurological condition that exhibits profound associations with altered circadian rhythm generation and whose toolkit of pharmacological interventions impact upon circadian rhythm generation. Currently it is unclear exactly how changes to rhythmic physiology contribute to the aetiology and pathology of BPD.

In recent years, rodent models possessing lesions within genes that make up the basic cellular oscillator are widely reported to exhibit concomitant changes in affective behaviours, namely mania-like phenotypes. Recently a mouse model possessing a mutation within the neuron-specific Na⁺/K⁺-ATPase (NKA) alpha3 subunit, known as Myshkin, was described as a model of the manic phase of BPD. The NKA alpha3 is not reported as a critical element of the circadian oscillator and we used this opportunity to characterise the behavioural and physiological circadian system of these animals.

Under wheel-running paradigms Myk/+ animals exhibited a broad array of behavioural deficits including lengthened, low amplitude and labile free-running rhythms, altered phase re-setting and elevated metabolic activity. Physiological characterisation of the SCN revealed deficits in amplitude of electrical output and changes to post-synaptic signalling although the ex vivo molecular pacemaking of the SCN remained intact. Myshkin animals therefore represent a novel model in which changes to central output arise independently of changes to basic molecular pacemaking. Despite this seemingly distinct mechanism Myshkin animals share many mood and circadian phenotypes with other clock gene models of affective behaviours highlighting that changes to pacemaking output of the SCN may be a critical factor across animal models exhibiting circadian and mood deficits.

In addition, the impact of the mood stabiliser lithium, commonly prescribed in BPD, on cellular pathways within the SCN was investigated. Lithium consistently lengthens the period of cellular and behavioural rhythms in mammals although the mechanism of this action is yet undefined. Glycogen synthase kinase 3β (GSK3β) and inositol monophosphatase (IMPase) are the major biochemical targets of lithium at therapeutic concentrations. GSK3β is known to shorten rhythms and this study targeted IMPase and inositol phosphate turnover in the period lengthening effects of lithium. We reveal that although inhibition of IMPase dampens SCN molecular rhythms, the period of oscillations remains unchanged and therefore lithium acts upon distinct cellular pathways within the SCN to exert effects on period.
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Finally, this thesis, and all the work I have put in and around it, is dedicated to the memory of my two grandparents who passed away during the past three years,

Mr. Robert Wootton and Ms. Jacqueline Wootton
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AMPA</td>
<td>α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>AVP</td>
<td>Arginine Vasopressin</td>
</tr>
<tr>
<td>BPD</td>
<td>Bipolar Disorder</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CT</td>
<td>Circadian Time</td>
</tr>
<tr>
<td>DAG</td>
<td>Diaglycerol</td>
</tr>
<tr>
<td>DD</td>
<td>Constant Dark</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma aminobutyric acid</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3β</td>
</tr>
<tr>
<td>Hb</td>
<td>Habenula</td>
</tr>
<tr>
<td>IGL</td>
<td>Intergeniculate Leaflet</td>
</tr>
<tr>
<td>IMPase</td>
<td>Inositol Monophosphatase</td>
</tr>
<tr>
<td>InsP</td>
<td>Inositol Phosphate (generic)</td>
</tr>
<tr>
<td>IPPase</td>
<td>Inositol Polyphosphatase-1 Phosphatase</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol Trisphosphate</td>
</tr>
<tr>
<td>LD</td>
<td>Light-Dark</td>
</tr>
<tr>
<td>MDD</td>
<td>Major Depressive Disorder</td>
</tr>
<tr>
<td>Myk/+</td>
<td>Myshkin transgenic mouse</td>
</tr>
<tr>
<td>NKA</td>
<td>Sodium/Potassium-ATPase</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>PER2:LUC</td>
<td>Period2 Luciferase Fusion Protein</td>
</tr>
<tr>
<td>PK2</td>
<td>Prokineticin 2</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PtdIns</td>
<td>Phosphatidylinositol (generic)</td>
</tr>
<tr>
<td>PIP</td>
<td>Phosphatidylinositol phosphate (generic)</td>
</tr>
<tr>
<td>RHT</td>
<td>Retinohypothalamic Tract</td>
</tr>
<tr>
<td>RMP</td>
<td>Resting Membrane Potential</td>
</tr>
<tr>
<td>SCN</td>
<td>Suprachiasmatic Nuclei</td>
</tr>
<tr>
<td>SCZ</td>
<td>Schizophrenia</td>
</tr>
<tr>
<td>SFR</td>
<td>Spontaneous Firing Rate</td>
</tr>
<tr>
<td>SMIT</td>
<td>Sodium/myo-inositol co-transporter</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive Intestinal Polypeptide</td>
</tr>
<tr>
<td>VIP2R</td>
<td>Vasoactive Intestinal Polypeptide Receptor 2</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral Tegmental Area</td>
</tr>
<tr>
<td>ZT</td>
<td>Zeitgeber Time</td>
</tr>
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Declaration

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Introduction
Introduction

Daily rhythms in physiology and behaviour are an almost ubiquitous feature of biological organisation and manifest in the majority of species on earth. Aside from the need to compartmentalise cellular processes in space, selective pressures have also bestowed an advantage on organisms to further segregate physiological functions in time. The pressure on this temporal regulation is apparent in the divergent internal time-keeping mechanisms across different phylogenetic kingdoms and classes (Dunlap, 1999). A living organism’s ability to represent time internally alongside the biological machinery that underlies this capacity, are collectively known as circadian rhythms.

Over recent decades, the specific molecular and physiological processes that give rise to these intrinsic rhythms have been rapidly uncovered in prokaryotes, eukaryotes and fungi. One of the major challenges that have arisen in light of these developments is an attempt to understand how changes to this rhythm-generating machinery may impact on human health.

From these considerations two key questions have become apparent. Firstly, is how humans are able to adapt to life in a 21st century society in which a synthetic representation of time is continually imposed through the manipulation of our external environment (Roenneberg et al., 2012). The second main challenge is an attempt to understand how disruption to intrinsic circadian time-keeping mechanisms can have pathological consequences in man (Takahashi et al., 2008). As circadian rhythms by their nature are responsive to external stimuli, systemic changes could be a result of abnormal interactions with the environment, or instead may be rooted more fundamentally in one’s genetic or physiological disposition.

Numerous lines of evidence have emerged that directly implicate genetic and physiological changes to the circadian system in diseases as varied as metabolic syndromes, cancer and neuropsychiatric disorders (Foster et al., 2013; Fu et al., 2002; Turek et al., 2005). Although many associations are apparent between clocks and disease, the mechanisms through which the circadian system underpins aetiology and pathophysiology, particularly in relation to neurological disease, remain unclear. The aim
of this thesis is to utilise both in vivo and in vitro laboratory models to investigate the neurophysiological basis of circadian dysfunction in bipolar disorder (BPD), a neuropsychiatric condition with profound associations with sleep and circadian disruption.

Using an animal model of the manic phase of BPD and an ex vivo tissue culture system, two aspects relating to the circadian association with BPD are addressed. Firstly, how is the circadian, diurnal and metabolic behaviour altered in this disease model and are these changes accompanied by changes to central pacemaking within the CNS? The second point of focus is upon the biochemical interactions of the mood stabiliser lithium, commonly prescribed in BPD and known to have a profound effect on circadian rhythms. In this section attempts are made to elucidate the molecular targets of lithium that result in its profound in vitro and in vivo effects on biological timekeeping.

The discovery and emergence of circadian biology

The ability of an organism to generate internal rhythms irrespective of external cues and influence were first recorded by Jean-Jacques d’Ortous de Mairan in 1792. Observations of diurnal leaf movements in the Mimosa pudica were maintained even in constant dark conditions. This study revealed for the first time that organisms are able to exert temporal control of their physiology outside the influence of light-dark cycles (De Mairan, 1792). Despite this early demonstration of circadian pacemaking, the notion that daily rhythms were truly intrinsic and not the result of an unknown zeitgeber, at the time denoted factor X, persisted into the 20th century. Following the demonstration of free-running rhythms in mammals, the theoretical groundwork that defined the properties of the mammalian circadian system were laid down by the pioneers of chronobiology Jürgen Aschoff and Colin Pittendrigh from the 1950s (Aschoff, 1960; Pittendrigh and Daan, 1976a, 1976b). Using human studies alongside wheel-running paradigms in rodents, still widely in use today, the groups of Aschoff and Pittendrigh described in detail the intrinsic nature of the mammalian circadian oscillator. The key findings included the in-depth description of the nature of behavioural rhythms in different lighting conditions and the influence of external stimuli, known as zeitgebers, on setting the phase of rhythms. Based upon these principles the field of circadian rhythms has rapidly expanded and through
harnessing technical innovation has dissected the anatomical, molecular and physiological mechanisms of circadian systems across species. The major challenge that is now presented to the field is attempting to understand the emerging role circadian biology in human disease and potential therapeutic interventions.

**Mammalian circadian architecture**

The anatomical basis of circadian rhythm generation in mammals was first described in 1972 with the simultaneous publication of two papers, revealing that lesions to the suprachiasmatic nuclei of the hypothalamus (SCN) ablated behavioural, drinking and corticosterone rhythms in rats (Moore and Eichler, 1972; Stephan and Zucker, 1972). Confirmation of the role of the SCN as the structures that defined pacemaking properties of an entire organism was provided in a classic study in which transplanted SCN tissue restored behavioural rhythms in SCN-lesioned hamsters with the original periodic properties of the donor (Ralph et al., 1990). Since the publication of these papers, the dominant co-ordination of circadian rhythms in mammals is now accepted as being orchestrated by this singular central location.

In addition to the maintenance of stable rhythms that project time across the brain and body, the SCN also respond and entrain to external zeitgebers. This critical property allows alignment of internal time generated by the SCN with extrinsic conditions with light acting as the predominant entraining stimulus. The SCN maintain this phase-relationship through a range of afferent connections that possess a diverse array of neurochemical inputs to relay this information (Morin and Allen, 2006). Exposure to light pulses of even short duration during the subjective night is sufficient to phase shift behavioural rhythms in rodents for several hours at a time (Johnson, 1999). The direction of the shift, either advancing or delaying the subsequent behavioural onset, depends on the phase of the night at which the animal is exposed. Due to the strong photic influence on behavioural rhythms, when animals are exposed to light-dark cycles (LD) as seen in the natural environment, circadian rhythms are continually adjusted and animals entrain to the rhythm of the diurnal cycle (Golombek and Rosenstein, 2010). Light input to the SCN arrives from a direct retinal projection known as the retinohypothalamic tract (RHT) and is relayed by a sub-type of melanopsin containing intrinsically photosensitive retinal
ganglion (ipRGC) cells known as M1 iPRGCs (Panda et al., 2002a; Schmidt et al., 2011). These cells respond to specific wavelengths of light, predominantly around 480nm, and transmit this signal principally to the ventral SCN through glutamatergic gray type I synapses (Brown et al., 2013; Castel et al., 1993). The role of glutamate in the phase shifting response of the SCN can be observed in vitro where the application of glutamate agonists to acute or organotypic SCN cultures can phase shift circadian rhythms in a similar phase-dependent pattern (Shirakawa and Moore, 1994). The SCN also receives a secondary photic input from a γ-aminobutyric acid (GABA) and neuropeptide Y (NPY) containing projection from the intergeniculate leaflet (IGL) that modulates phase shifting and entraining properties of the circadian pacemaker (Edelstein et al., 1999; Morin and Allen, 2006; Pickard et al., 1987).

Although light acts as the major circadian zeitgeber, arousal stimuli such as novel-environment exposure or exercise transmit to the SCN and are able to phase shift rhythms (Mrosovsky, 1996). The window of sensitivity of the SCN to these zeitgebers occurs during the subjective day of the circadian cycle. The major projections from which these stimuli originate are the intergeniculate leaflet (IGL) and also the median raphe nucleus (MRN; Morin and Allen, 2006). The neurochemical signals that underlie this behaviour are NPY and GABAergic IGL projections and serotonin that signal to a specific subset of 5-HT receptors within the SCN with an emerging role for 5-HT7 in these effects (Besing et al., 2012; Takeuchi et al., 2014). As in the case of glutamate, in vitro rhythms of the SCN can also be phase shifted by NPY and serotonin/5HT receptor agonists (Belle et al., 2014; Besing et al., 2012; Prosser, 2003).

The photic projection from the RHT and arousal encoding IGL and MRN connections delineate the major anatomical pathways that provide environmental information to the SCN. As an intact network these structures define circadian rhythm generation and entrainment in mammals. A broad literature in which targeted molecular lesions ablate behavioural rhythms, phase-shifting and entrainment, demonstrates that this anatomical network is fundamentally dependent on single cells and their genetic and physiological makeup.
Molecular circadian rhythms

Circadian rhythms within cells and tissues arise from a well-described interlocked molecular transcription-translation feedback loop (TTFL). The mammalian TTFL oscillates not just within the neurons of the SCN, but across cells throughout the brain and body (Balsalobre et al., 2000; Panda et al., 2002b). Upon physical isolation of cells of peripheral tissues, extra-SCN brain regions and neurons of the CNS maintain independent molecular rhythms (Balsalobre et al., 2000; Liu et al., 2007; Webb et al., 2009). Due to a lack of intercellular communication in these models however, overt rhythms are rapidly lost. The role of the SCN is to provide local and systemic signals as timing cues to which individual oscillators can adapt and entrain in order to maintain a coherent phase relationship.

The TTFL that defines cellular-level pacemaking is driven by an inter-dependent network of transcription factors that promote and inhibit the rhythmic expression of clock controlled targets, providing a tissue-specific temporal profile of circadian controlled transcripts (Partch et al., 2014). The near 24-hour period that defines molecular rhythmicity arises through the interaction of the principal transcription factors of the TTFL with an array of molecular and enzymatic elements that modulate the timing of protein localization and degradation through phosphorylation, sumoylation and ubiquitination. Further temporal control of expression is also imparted through chromatin re-modelling via histone acetylation and in peripheral tissues a role is also emerging for post-transcriptional mRNA stabilisation of clock and clock-controlled transcripts.

The discovery of the first putative clock gene named Period was made in Drosophila in the 1970s, but it was not until 1994 that the first mammalian clock gene, Circadian Locomotor Output Cycle Kaput (CLOCK) was described (Vitaterna et al., 1994). An inactivating mutation of this gene resulted in lengthened behavioural rhythms that gradually degenerated to arrhythmicity in free-running conditions. Many other genetic lesions have revealed further clock genes that translate directly to altered free-running behaviour and light interactions.

Continuing work on the function of CLOCK revealed its role as the main positive transcription factor of the TTFL by forming a PAS-dependent heterodimer with Bmal1
(Bunger et al., 2000; Gekakis et al., 1998). The CLOCK-BMAL1 complex promotes the expression of clock-controlled genes (CCG) through the interaction at E-Box elements including the negative elements of the TTFL the genes Period1,2,3 and Cryptochrome1 and 2 (Bae et al., 2001; Ko et al., 2010b; Vitaterna et al., 1999; Zheng et al., 1999). Upon dimerization PER-CRY complexes re-enter the nucleus to inhibit the actions of CLOCK-BMAL1. This activity inhibits the expression of Period and Cry genes themselves and results in the release of CLOCK-BMAL1 heterodimers to begin the TTFL and promotion of PER-CRY and other CCGs once more (Ko and Takahashi, 2006).

This interlocked cycle results in near 24-hour periodicity of CLOCK-BMAL1 and PER-CRY levels and their CCG targets. The precise timing of this oscillation however relies on further transcriptional and post-translational interactions with other TTFL elements that are also essential to circadian pacemaking. Major elements include the nuclear receptors Rev-erbα/β and Rorα/β that play antagonistic roles on the expression of Bmal1 (Guillaumond et al., 2005; Preitner et al., 2002). Loss of both Rev-erb genes independently results in small changes to behavioural rhythms, yet conditional loss of both genes simultaneously leads to severe disruption in free-running rhythms and metabolism (Cho et al., 2012). This recent discovery of the redundancy between Rev-erbα/β has revealed that subsidiary transcriptional loops are essential to circadian rhythm generation and do not simply modulate timing of the clock.

As nuclear receptors, Rev-erbα/β and Rorα/β also regulate metabolic processes such as lipid and bile acid synthesis, creating molecular links between circadian and metabolic pathways (Duez and Staels, 2009). Another element of TTFL control that provides a link to metabolic cycles is the histone acetylation-dependent re-modelling of chromatin states. The interaction of the intrinsic acetylase transferase activity CLOCK with the histone deacetylase SIRT1 regulates chromatin states in a temporal fashion and provides a broad chromatin-wide regulation of clock-gene expression (Koike et al., 2012; Nakahata et al., 2009; Ramsey et al., 2009). An interacting loop of SIRT1 and co-factors PGC1-α and NAMPT interact to increase the amplitude of the molecular output of the TTFL (Chang and Guarente, 2013). SIRT1 activity is dependent on the cellular levels of the product of redox cycles nicotinamide adenine dinucleotide (NAD⁺) and in turn the rate-limiting enzyme in the production of NAD⁺, NAMPT, is under rhythmic control by CLOCK-BMAL1 (Ramsey et
In addition to their critical roles within the TTFL, SIRT1 and Rev-erba/β provide bi-directional links between circadian and metabolic processes. A diverse range of metabolic pathways – including glucose and lipid metabolism - exhibit circadian rhythms and thus these molecular elements act as integrators of these systems within cells (Asher and Schibler, 2011).

**Post-translational mechanisms**

Outside of transcriptional regulation, phosphokinases act within the TTFL in determining the transition of clock proteins across cellular compartments and their eventual degradation. Perhaps the most important kinases in this process are casein kinase1δ/ε that differntially regulate circadian period and phase re-setting through phosphorylation of PER2 (Etchegaray et al., 2009; Meng et al., 2008). Loss or gain of function in casein kinase1ε/δ results in longer or shorter free-running rhythms respectively and CK1ε also plays a critical role in phase shifting and re-entrainment light (Pilorz et al., 2014).

Other kinases are involved in the localisation of clock proteins including glycogen synthase kinase 3β (GSK3β), regulating the amplitude of PER2 as well as nuclear nucleus (Iitaka et al., 2005; Li et al., 2012). GSK3β represents an interesting potential integrator of molecular circadian rhythms and BPD pathophysiology. As an identified target of the mood stabiliser lithium and known to play an important role within the CNS in the expression of affective behaviours, GSK3β has the potential to act as an regulatory factor between circadian and mood behaviours (O’Brien and Klein, 2009; O’Brien et al., 2004).

In addition to the movement of clock proteins throughout cellular compartments, the degradation of clock proteins is critical in the timing of molecular rhythms. The circadian TTFL utilises ubiquitin-dependent pathways for proteasomal degradation of clock proteins from the cytosol. Mutations that affect this process have a profound effect on molecular and behavioural rhythms. A mutation in the F-box protein Fbxl3 results in greatly lengthened circadian period and phase re-setting to light due to the delayed degradation of CRY1 and CRY2 (Godinho et al., 2007; Guilding et al., 2013).

The fundamental basis of circadian rhythms, critical to tissue and organism-level biological timing, depend on a molecular TTFL that via an array of cellular processes
generate near-24 hour cellular oscillations of central transcription factors and their targets. The result of these interactions is the temporal control of cellular transcriptomes that drive tissue-specific physiology and behaviour (Koike et al., 2012; Panda et al., 2002b). The molecular revolution has facilitated the rapid elucidation of the TTFL and increasing levels of detail are continually added to this current model. As described above however, physically isolated cells cannot maintain accurate rhythms across large populations. Increasing complexity instead relies upon coupling factors that maintain coherent phase relationships between cellular oscillators in tissues (Balsalobre et al., 2000; Webb et al., 2009). This is most apparent and well-defined within the SCN whose unique network properties result in a tightly-coupled and high amplitude population-level rhythmic output that possesses powerful resistance to environmental, pharmacological and even genetic disruption (Welsh et al., 2010).

Pacemaking properties of SCN neurons

In addition to possessing autonomous molecular rhythms, neurons of the SCN also maintain rhythms in spontaneous electrical activity (Welsh et al., 1995). In isolation SCN neurons exhibit much greater noise and variability in their expression of both molecular and electrical rhythms and fail to maintain population rhythmicity (Webb et al., 2009). By increasing the density of SCN neurons, cellular and population-level rhythms emerge to highlight the powerful effect of the coupling factors of the SCN in promoting rhythms (Freeman et al., 2013). This property is also evident from studies into the cellular and network properties of clock-lesioned tissues. In dissociated neurons of the SCN, mutant and knockout strains, such as Cry1\(^{-}\) or ClockΔ19, the rhythmic expression of molecular or electrical rhythms in dissociated neurons is absent. When whole SCN explants are monitored however, tissue-level rhythms in emerge to further demonstrate the powerful capacity the SCN network can have on even very poor oscillators (Herzog et al., 1998; Liu et al., 2007; Nakamura et al., 2002).

The unique properties of the SCN are further emphasized by comparing differences in long-term rhythm generation in extra-SCN brain oscillators (Guilding and Piggins, 2007). Various brain nuclei outside the SCN express clock genes and are capable of maintaining molecular rhythms in culture including the dorsomedial hypothalamus, arcuate nucleus
and the habenula (Abe et al., 2002; Guilding et al., 2009, 2010). Despite possessing the same basic capacity for intercellular communication – electrical activity and synaptic transmission - rhythmic oscillations within these regions diminish far more rapidly than those in the SCN. Outside of the SCN, the only region that displays an long-term rhythmicity in the absence of SCN output is the olfactory bulb (Abraham et al., 2005). The SCN therefore possess network properties that are distinct from other regions of the CNS that allow the maintenance of long-term oscillations. The physiological mechanisms that underpin propensity lie within single SCN neurons and across the entire network and have become gradually elucidated to the point at where we now have a basic although incomplete model of how the SCN achieve this feat.

**Electrical rhythms of the SCN**

Within the SCN the expression of circadian rhythms in firing rates is a critical feature that defines the synchrony and output of the network. At the level of both single neurons and across the SCN population, there is a daily progression in electrical activity that peaks during the day and reaches a nadir at night (Belle et al., 2009; Groos and Hendriks, 1982; Kuhlman and McMahon, 2004; Rohling et al., 2011). The SCN was first shown to possess a day-night rhythm through multi-unit *in vivo* recordings in rats that persisted even when the SCN was synaptically isolated (Inouye and Kawamura, 1979). The importance of firing activity in the SCN is observed through studies utilising tetrodotoxin (TTX) that block the propagation of action potentials. *In vivo* the application of TTX within the SCN abolishes behavioural rhythms although behavioural activity re-emerges in-phase with wheel-running activity prior to treatment (Schwartz et al., 1987). *In vitro* the effect of TTX on the SCN appears more pronounced with reductions in the amplitude of single cell and tissue-level molecular rhythms as well as alterations to the spatiotemporal organisation of the SCN network (Baba et al., 2008; Enoki et al., 2012; Yamaguchi et al., 2003). These studies of electrical activity within the SCN highlight the importance that rhythms in electrical output play in defining both behavioural output network pacemaking of the SCN.

The excitability of individual SCN neurons is driven through a complex array of circadian and non-circadian ionic conductances (Colwell, 2011). The interaction of these electrochemical mechanisms provide an excitatory drive within single neurons that result
in higher spontaneous firing rates (SFR) and resting membrane potential (RMP) during the day and lower SFR and RMP at night (Belle et al., 2009; Kuhlman and McMahon, 2004; Wang et al., 2012a). Currents that exhibit circadian expression within SCN neurons include an excitatory L-type Ca\(^{2+}\) conductance (CACNA1C, CACNA1S) that peaks during the day in rats (Pennartz et al., 2002), two K\(^+\) rectifier channels that modulate action potential generation A-type (KCND1,KCND2; Granados-Fuentes et al., 2012; Itri et al., 2010) and fast-delayed rectifier (FDR; KCNC, KCNC2; Kudo et al., 2011), large conductance Ca\(^{2+}\)-dependent K\(^+\) channel (BK; KCNMA1; Meredith et al., 2006; Pitts et al., 2006) and an unidentified TEA-sensitive K\(^+\) conductance (Kuhlman and McMahon, 2004). A diurnal rhythm in the activity of the electrogenic Na\(^+\)/K\(^+\)-ATPase is also observed in the rat SCN (Wang and Huang, 2004). As can be observed in rodent models expressing lesions to core clock genes, mice lacking the genes encoding A-type and BK channels exhibit altered free-running behaviour and phase-shifting responses to light (Granados-Fuentes et al., 2012; Meredith et al., 2006).

Other currents that are important in SCN function yet are not reported as circadian in nature include a persistent Na\(^+\) current (SCN8A, SCN9A; Jackson et al., 2004) that provides excitatory drives on SCN neurons, T-type Ca\(^{2+}\) current (CACNA1G; Huang, 1993) and small-conductance Ca\(^{2+}\)-activated K\(^+\)-currents (KCNN2/3; Belle et al., 2009). In addition to the day-night rhythms expressed in RMP and SFR, the result of these interacting conductances is the expression of specific electrical behaviours of SCN neurons. SCN neurons are generally characterised by higher frequency regular firing and lower frequency irregular firing behaviours (Kononenko and Dudek, 2004; Pennartz et al., 1998). Some variation exists in the literature on the details neuronal with further difficulty added by species differences between mice and rats. In rats SFR is generally higher than in mice, peaking at around 8Hz during the day in regular firing neurons (Jackson et al., 2004; Kononenko and Dudek, 2004; Pennartz et al., 2002). Irregular firing neurons with higher coefficient variation are generally reported to fire at frequencies less than 3.5Hz. RMP peaks between -43mV and -55mV during the day and reaches a night-time nadir between -55 and -61mV (Pennartz et al., 2002; Wang et al., 2012a).

In mice firing rates are reported between 3 and 6Hz during daytime peaks and decrease to less than 2Hz at night although most reports suggest frequencies below 1Hz (Belle et
RMP in mice is widely reported as more depolarised than in rats, with peak firing neurons resting between -36 and -41mV with night-time nadirs between -47 and -68mV. In both species SCN neurons exhibit hyperpolarised states that arise mainly during the night characterised by lower firing rates and in some cases the cessation of spontaneous firing activity (Belle et al., 2009; Pennartz et al., 2002). In a recent study in mice, Per1 expressing neurons exhibited unique electrophysiological properties that result in a transition to highly depolarised potentials (-35 to -25mV) in which cells achieve depolarisation block and display sub-threshold membrane oscillations. These electrical states define the pacemaking activity of SCN neurons at the plasma membrane and ultimately inter-cellular communication across the network. There remains a further critical factor that defines the unique properties of SCN neurons; the expression of Ca\(^{2+}\) rhythms, which are believed to act as a critical interface between electrical states and molecular oscillations.

**Calcium Rhythms**

Similar to the expression of electrical rhythms, both single neurons and the SCN population exhibit a day-night flux in Ca\(^{2+}\) levels that peaks during the day and reaches a nadir at night (Brancaccio et al., 2013; Colwell, 2000; Enoki et al., 2012; Ikeda et al., 2003; Irwin and Allen, 2009). This oscillation is dependent on entry from voltage-sensitive Ca\(^{2+}\) channels and intracellular stores mediated by ryanodine receptors (Colwell, 2000; Ikeda et al., 2003). Loss of a day-night rhythm in Ca\(^{2+}\) results in the loss of molecular rhythms in both Per1::LUC and PER2::LUC models (Lundkvist et al., 2005). When molecular and Ca\(^{2+}\) rhythms are visualised across the entire SCN network via high-resolution single-cell imaging, a clear spatiotemporal pattern is apparent with a dorsal to ventral wave of peak activity that mimics similar patterns of clock gene peaks in activity (Brancaccio et al., 2013; Enoki et al., 2012; Evans et al., 2011; Yamaguchi et al., 2003). In the mouse SCN the daily peak in Ca\(^{2+}\) levels occurs prior to that of clock gene mRNA expression and also precedes rhythmic Ca\(^{2+}\)/cAMP responsive elements (CRE) expression in the SCN (Brancaccio et al., 2013; Enoki et al., 2012). This suggests that the Ca\(^{2+}\) oscillation within the SCN may act as a driving force to relay signals from the membrane to the molecular clock.
Many core clock genes of the SCN possess CRE-elements, such as \textit{Per1} and \textit{Per2}, providing a mechanism through which these changes to Ca\textsuperscript{2+} and Ca\textsuperscript{2+}-dependent intracellular cascades can interact directly with the TTFL (Travnickova-bendova et al., 2002). Cyclic AMP (cAMP) is also a critical element of the circadian clock that possesses a circadian rhythm peaking in the day and is likely driven by adenylyl cyclase 1 (Fukuhara et al., 2004; O’Neill et al., 2008). Loss of cAMP rhythms within SCN tissue results in the loss of PER2::LUC expression highlighting the critical role of this pathway. There are various Ca\textsuperscript{2+}-dependent adenylyl-cyclases expressed within the SCN including adenylyl cyclase 1, providing a mechanism through which Ca\textsuperscript{2+} levels can influence the expression and phase-lead the induction of clock gene transcription within the SCN (Fukuhara et al., 2004). Roles for other Ca\textsuperscript{2+}-dependent elements exist within the SCN including protein kinase C (PKC) and receptor of activated kinase 1 (RACK1) (Robles et al., 2010).

Changes in Ca\textsuperscript{2+} levels within the SCN are not restricted to long-term oscillations with intercellular signals on short time-scales capable of inducing large rises and falls in concentration. Electrical stimulation of SCN neurons through current application that increases action potential firing rates result in rapid increases of intracellular Ca\textsuperscript{2+} (Irwin and Allen, 2009). Stimulation of SCN neurons that mimic the effects of light, through glutamatic receptor agonists such as NMDA or via RHT-stimulation also induce post-synaptic increases in Ca\textsuperscript{2+} with the latter dependent on entry through L-type voltage-gated Ca\textsuperscript{2+} channels (Irwin and Allen, 2007). In addition many of the signalling cascades that are induced by photic and glutamatergic transmission to induce expression of core clock genes such as \textit{Per1}, \textit{Per2} are dependent on Ca\textsuperscript{2+} entry (Shearman et al., 1997). These pathways include MAP kinases (Coogan and Piggins, 2004; Obrietan et al., 1998), calmodulin (Yokota et al., 2001) and PKC (Jakubcakova et al., 2007). Other major neurotransmitters and neuropeptides of the SCN including GABA, VIP and AVP also induce rapid changes in Ca\textsuperscript{2+} providing a further link between post-synaptic signalling and intracellular Ca\textsuperscript{2+} dynamics (Irwin and Allen, 2009, 2010). These studies demonstrate the importance of Ca\textsuperscript{2+} and that its role can be visualised across all temporal and spatial scales of the SCN. Changes in Ca\textsuperscript{2+} are crucial in responses to various extracellular stimuli and mediating communication between these signals, electrical activity and molecular oscillations.
Network properties of the SCN

The current understanding of the cellular physiology of SCN neurons is based upon a model of the tripartite interactions of molecular, Ca^{2+} and electrical rhythms that result in self-sustaining oscillations. Neurons of the SCN must also utilise a range of paracrine and synaptic signals to establish communication and maintain synchronous phase relationships of individual rhythms.

Transynaptic signalling is critical in mediating coupling signals within the SCN as described in studies on TTX-sensitive communication discussed above (Baba et al., 2008; Enoki et al., 2012; Schwartz et al., 1987; Yamaguchi et al., 2003). Gap junctions have received less attention than TTX-dependent mechanisms, although Connexin36 mice show fragmentation of behavioural rhythms (Long et al., 2005). Despite this observation, in vitro application of the gap junction blocker carbenoxolone appears to have little effect upon the spatiotemporal architecture of the SCN, unlike TTX (Enoki et al., 2012). TTX can have several effects on neuronal function including blockade of action potentials, alterations to RMP and loss of synaptic vesicle release (Itri et al., 2004; Jackson et al., 2004; Pennartz et al., 2002). The mechanism through which TTX exerts its effects remains unclear, but disruptions to major neurochemical signals of the SCN have profound effects on circadian pacemaking and reveal that synaptic mechanisms play a critical role (Granados-Fuentes and Herzog, 2013).

Over the last decade work on the array of neurochemicals expressed within the SCN has revealed a prominent role for the neuropeptide vasoactive intestinal polypeptide (VIP) and its receptor VPAC2 (Vip2r^{-/-}) and the ubiquitously expressed GABA in the maintenance of pacemaking within the SCN. Loss of either VIP or VPAC2 signalling results in profound behavioural deficits in mice including low amplitude and arrhythmic wheel-running activity, abnormal entrainment to LD cycles and altered phase shifting responses to light (Aton et al., 2005; Hughes and Piggins, 2008). The SCN of Vip2r^{-/-} knockout animals display profound changes in both molecular and electrical output, with daily peak in firing activity absent, low amplitude PER2::LUC rhythms and increased variability in phase and period of SCN neurons (Aton et al., 2005; Cutler et al., 2003; Maywood et al., 2006). Tissues from Vip^{-/-} mice also demonstrate similar deficits including dampened electrical rhythms and a
decrease in the appearance of rhythmically firing neurons in vitro (Brown et al., 2007). A recent study utilising DREADDs in organotypic mouse SCN tissue revealed that Gq-dependent activation of VIP expressing neurons is able to re-programme the typical spatiotemporal progression of PER2::LUC rhythms across the SCN thus interrupting normal network architecture (Brancaccio et al., 2013). The importance of VIP within the SCN can be further observed following environmental disruption again in PER2::LUC mice. Exposure to increasing day-lengths decouples the SCN into separate dorsal and ventral populations that express divergent phases in peak accumulation of PER2. Following excision of SCN tissue, the gradual re-synchronisation of these subpopulations is greatly attenuated in the absence of VIP signalling, highlighting a critical role of VIP in population synchrony (Evans et al., 2013). This collection of work alongside the profound behavioural deficits observed in knockout animals in vivo demonstrate a critical role in the network properties of the SCN for VIP and its receptor VPAC2.

GABAergic neurotransmission perhaps represents the only other coupling factor that exists on par with VIP signalling in the hierarchy of intercellular signals that maintain the SCN network. GABA is the most ubiquitous neurotransmitter within the SCN and is expressed in nearly all constituent neurons (Moore and Speh, 1993). Inhibitory post-synaptic currents evoked by GABA exhibit a circadian rhythm within the dorsal SCN with the expression of this rhythmic profile also dependent on VIP signalling (Itri et al., 2004). The inhibitory actions of GABA are important in defining the action potential frequency and resting membrane potential of spontaneously active SCN neurons (Jackson et al., 2004; Kononenko and Dudek, 2004). A distinct property of GABA within the SCN is that post-synaptic GABA responses of SCN neurons can be excitatory as well as inhibitory. This property is observed post-synaptically both in terms of EPSP direction and relative changes in Ca\(^{2+}\) concentration, and is thought to be regulated through alterations in the activity of the Na\(^{+}\)-K\(^{+}\)-Cl\(^{-}\)-co-transporter (NKCC1; Choi et al., 2008; Irwin and Allen, 2009).

Initial studies on the effects of GABAergic transmission on the SCN network suggested that loss of GABA\(_A\) and GABA\(_B\) signalling did not have major impact on overt pacemaking. Instead pharmacological blockade of these GABA pathways failed to attenuate persistence of molecular rhythms and also sharpened the peak of daily electrical activity (Aton et al., 2006). Recent studies have however revealed that GABA plays a critical role...
in the SCN with its actions determined by the current state of the network. In this model GABA functions to de-stabilise and reduce intercellular synchrony when neurons are in a synchronised and high amplitude state. When the SCN is subsequently de-synchronised, GABA acts to promote coupling and re-align the phase of temporally disparate oscillators (Evans et al., 2013). This property of GABA persists in high density culture of SCN neurons, acting through GABA_\textsubscript{A} receptors to reduce period stability of firing SCN neurons (Freeman et al., 2013). Antagonism of GABA_\textsubscript{A} receptors using gabazine, therefore reducing Cl\textsuperscript{-} dependent GABAergic transmission, can increase amplitude and reduce damping rates in VIP-deficient SCN tissues (Freeman et al., 2013). The mechanism through which GABA may impose these opposing effects is apparent under altered day-lengths. Long photoperiods that de-synchronise the SCN result in increases to both post-synaptic frequency and excitatory tone of GABA responses. These changes are regulated by NKCC1 activity and reveal how plastic post-synaptic responses to GABA underpin the divergent roles of GABA within the SCN (Farajnia et al., 2014). GABA signalling therefore plays a critical state-dependent role within the network organisation of the SCN in which there is also a consistent and inter-dependent interaction with VIP and VPAC2 signalling.

Although the role of VIP and GABA appear particularly dominant within the SCN, other neuropeptides are also implicated in maintaining SCN rhythmicity both in vitro and in vivo. One such subsidiary coupling factor is gastrin releasing peptide (GRP). GRP can act to phase shift circadian rhythms when applied in vivo through the recruitment of glutamatergic and serotonergic pathways (Kallingal and Mintz, 2006, 2014; Piggins et al., 1995). The downstream effects of GRP activity can induce both c-FOS and Per1 gene expression through recruitment of cAMP (Gamble et al., 2007; Piggins et al., 2005). GRP can therefore impact directly on both molecular and behavioural rhythms. In the absence of VIP signalling, GRP is able to compensate and maintain circadian output in both electrical and molecular rhythms in vitro (Brown et al., 2005; Maywood et al., 2011).

Other key neuropeptides expressed within the SCN include arginine vasopressin, expressed principally in the dorsal shell of the nucleus and acting through the receptors V1a, V1b (Abrahamson and Moore, 2001; Yamaguchi et al., 2013). At a behavioural level V1a\textsuperscript{-/-} deficient mice exhibit alterations in tau and low amplitude wheel-running rhythms alongside altered paracrine output (Li et al., 2009). Similar to observations made with
GRP, the loss of molecular rhythms due to deficient VIP signalling can be restored through AVP-mediated paracrine signalling through both V1a and V1b receptors (Maywood et al., 2011). Perhaps the most pertinent role of AVP signalling is its actions in mediating re-entrainment to rapidly shifting light-dark cycles that mimic trans-meridian travel. Genetic deletion of both V1a and V1b receptors in mice permits rapid re-entrainment of behavioural and molecular rhythms to 8-hour phase shifts of the LD cycle (Yamaguchi et al., 2013). This definitive study suggests that AVP has a critical role in preventing the SCN from abrupt re-alignment of phase to transient external signals.

A final SCN-derived neuropeptide with a distinct role in circadian function is prokineticin2 (PK2) which determines the output of the SCN in the maintenance of behavioural rhythms. In mice lacking the receptor for PK2 the amplitude of wheel-running rhythms is severely attenuated alongside alterations in behavioural consolidation (Prosser et al., 2007). The role of PK2 is interesting as despite these behavioural abnormalities the in vitro pacemaking of SCN tissues is not altered. This reveals that PK2 mediates the output of the SCN and highlights the role of SCN neuropeptides on downstream central targets.

The architecture of the SCN is complex with specific roles played by synaptic, paracrine and gap junctions that re-enforce cellular oscillations into a coherent and robust network. Work on the diverse array of neurochemicals expressed within neurons of the SCN has begun to reveal the specific and intricate role that each plays within the circuitry of the SCN with various levels of redundancy apparent between these coupling factors. It is through these diverse mechanisms that the SCN is able to re-enforce cellular rhythms and maintain robust, long-term pacemaking even in the face of genetic and neurochemical deficiencies.

**Circadian rhythms in clinical and pre-clinical pathology**

The use of animal models has been a powerful tool in the path towards understanding the genetic and physiological basis of circadian rhythms. From the widespread use of these models, there has been a realisation that inducing changes to clock function can have severe pathological consequences. This has added a new branch to the field of circadian biology; understanding the role of central and peripheral clocks in disease.
Some of the major laboratory examples include Bmal1-/- knockout animals that exhibit diverse early-ageing phenotypes and increased mortality rates (Kondratov et al., 2006). Per2bdrm-/- mutant mice display increased mortality and tumorigenesis in response to gamma radiation while ClockΔ19 animals exhibit obesity and metabolic syndrome (Fu et al., 2002; Turek et al., 2005). Altered circadian physiology or environmentally-induced disruption is also associated with pathophysiology in humans that is observed in laboratory models. There is strong clinical evidence for the role of the circadian system in disparate conditions including cardiovascular disease, metabolic syndrome and cancer (Maury et al., 2010; Takeda and Maemura, 2011). The role of circadian disruption through rhythm misalignment is also increasingly recognised as a risk-factor in occupations that involve chronic shift work or jet lag with increased prevalence of these clock-associated conditions (Knutsson, 2003; Roenneberg et al., 2012). In both clinical and laboratory settings however, one of the strongest pathological associations of altered circadian rhythmicity exists with neuropsychiatric conditions (Foster et al., 2013; Schnell et al., 2014).

**Neuropsychiatric disorders: a clock controlled phenomena?**

Changes to sleep-wake rhythms have been recognised within the symptomatology of neuropsychiatric and affective disorders for decades. Recent developments have seen a large increase in research trying to establish the role of sleep and circadian physiology in the aetiology and pathology of these conditions. The strongest associations with sleep and circadian rhythm disruption exist between major depression (MD), bipolar disorder (BPD) and schizophrenia (SZ). In all three examples sleep and rhythm disturbance are common and changes to sleep-wake cycles are stipulated as part of the diagnostic criteria of clinicians (Harvey, 2008; Phillips and Kupfer, 2013; Wulff et al., 2010). MD, BPD and SZ are conditions with a heritable and therefore genetic element and there exists strong SNP and allelic associations of certain clock genes within populations and as risk factors of these conditions (Etain et al., 2011; Vacic et al., 2011). Animal models have also reinforced these associations; many of the models that mimic the behavioural phenotypes of BPD, MDD or SZ consistently exhibit sleep and circadian rhythm disturbance as part of their phenotypic traits (Etain et al., 2011; Landgraf et al., 2014; Oliver et al., 2012). In this
study focus is specifically placed upon the associations between circadian rhythms and BPD. BPD demonstrates a particularly strong clinical, genetic and behavioural relationship with sleep and circadian rhythm abnormalities, although little is currently known of the role the circadian system may play in disease aetiology or pathology (Gonzalez, 2014; Landgraf et al., 2014).

**Bipolar disorder**

BPD is a condition that affects between 0.5-1.5% of the global population (Craddock and Sklar, 2013; Wittchn et al., 2011. Epidemiological studies also place BPD within the top 10 non-communicable diseases exhibiting the highest global socio-economic burden (Lozano et al., 2012). BPD can be a debilitating condition for sufferers and family members and also carries an increased mortality rate due to suicide (6-10% of patients), highlighting the need for effective short and long-term management (Harris and Barraclough, 1997; Nordentoft et al., 2011). The clinical manifestation of BPD is characterised by cycling episodes of mania and depression that last from days to months and are interspersed with episodes of euthymia, a clinically normal mood state (Phillips and Kupfer, 2013). During episodes of depression, patients experience reduced motivational and mood behaviours; anhedonia, low energy, increased anxiety, suicidal thoughts and altered sleep-wake behaviour (ICD-10, 2014; DSM-V, 2013). Manic episodes appear as an opposing manifestation of these mood states including; excessive energy, increased irritability, pressured speech, increased risk-taking, heightened sex-drive and reduced anxiety. Across both episodes the psychological deterioration of patients can result in psychosis, delusions and auditory, visual or somatic hallucinations and often requires hospitalisation (Baethge et al., 2005; Keck et al., 2003).

The International Classification of Disease fails to discriminate between BPD sub-types, with the appearance of both single a manic and depressive episode sufficient for a diagnosis of BPD (ICD-10, 2014). The Diagnostic and Statistical Manual of Mental Disorders (DSM), the most widely cited diagnostic text, categorises BPD into type I, type II, cyclothymia or BPD not otherwise specified (DSM-V, 2013; Phillips and Kupfer, 2013). These categories define BPD based on the severity and frequency of manic episodes, with
BPD type I requiring a single appearance of a full-scale episode of mania for diagnosis and BPD type II referring to depression with cycling bouts of less severe hypomania.

**Genetics of bipolar disorder**

Despite well-defined behavioural traits associate with BPD, the disease is difficult to both diagnose and manage therapeutically. A major reason for this is a lack of understanding into the neurobiology of BPD and the lack of diagnostic markers for the disease. Strong lines of evidence however do show that BPD has a highly heritable component, with genetic pre-disposition in families of BPD sufferers (Craddock and Sklar, 2013). Concordance rates of BPD in monozygotic twins is approximately 50-60% with diagnosis occurring in 7% of first-degree relatives (Craddock and Jones, 1999; Merikangas, 2007). Despite this clear heritable basis, genetic studies using linkage and population-level methods have provided inconsistencies in results and replicability. More recent genome-wide association studies (GWAS) have begun to elucidate some common interactions including strong evidence for the involvement of CACNA1C (L-type Ca\(^{2+}\) channel subunit), ANK3 (Ankyrin3) NCAN (Neurocan) and ODZ4 (cell surface protein teneurin-4; Ferreira et al., 2008; Note, 2011). The general heterogeneity that is apparent from these studies has led to the hypothesis that BPD is a polygenic condition, defined by the accumulation of small risk-factors that increase susceptibility to the disease (Craddock and Sklar, 2013). The state of understanding into the neurobiology of BPD is also at a similar stage to the genetics field. Despite certain associations between cellular, physiological and specific neurological circuits within BPD pathology, there remains a broad spectrum of diversity and heterogeneity in the aetiology of BPD.

**Neurobiology of bipolar disorder and affective disorders**

Within the spectrum of affective disorders, a number of brain circuits and physiological pathways exhibit evidence of altered function. Interestingly many of these pathways also display mechanistic associations with the circadian system. One of the major causes of pathophysiology across BPD, MDD and SZ is dysregulation of reward processing circuits of the CNS. Alterations in monoaminergic and more specifically mesolimbic dopaminergic systems are commonly reported across the spectrum of affective disorders and BPD.
specifically (Cousins et al., 2009; Russo and Nestler, 2013). The most convincing evidence of the role of dopaminergic pathways in BPD is the widespread use of antipsychotics in the management of mania (Cipriani et al., 2011). Antipsychotics are antagonists of dopamine systems, such as haloperidol that block dopamine D2 receptors, and are fast-acting and potent anti-manics (Cousins et al., 2009). Concomitantly the use of psychostimulants to innervate dopamine systems can induce mania and methamphetamine can be applied as pharmacological model of mania-like behaviour (Cousins et al., 2009; Kato et al., 2007).

Brain-imaging studies have recently begun to demonstrate brain regions that are part of or are highly connected to reward pathways exhibit changes in activity, size and density, particularly in cases of depression. This work has revealed general reductions in brain size and grey matter and heightened activity in the nucleus accumbens (NAcc), hippocampus, basolateral amygdala and prefrontal cortex (PFC; Kato, 2008; Russo and Nestler, 2013). In patients that suffer from neurodegenerative conditions, specific loss of dopaminergic neurons within the major hub of mesolimbic dopamine production, the ventral tegmental area (VTA), results in depression (Torack and Morris, 1988). In animal models genetic deficits targeting dopamine systems have profound consequences on affective behaviours such as mice with whole-brain dopamine-deficiency mice that result in depression and depression-like behaviours (Zhou and Palmiter, 1995).

The recent advances made in optogenetic and deep-brain stimulation (DBS) tools have also allowed for fine spatiotemporal manipulation of reward circuits and provided some insight into their effects on behaviour on short time-scales. Direct stimulation or inhibition of dopamine neurons within the VTA projecting to the mPFC, NAcc or lateral habenula (LHb) can induce aversive and anti-depressant-like behaviour (Chaudhury et al., 2013; Stamatakis and Stuber, 2012). Deep-brain stimulation specifically within neurons of the VTA in rats also has a distinct anti-depressant effect on behaviour (Friedman et al., 2009). These methodological advances have augmented the knowledge basis of the mesolimbic circuitry in mood disorders particularly highlighting the speed with which activation or inhibition of these specific pathways can alter affective behaviours.
Dopamine systems are also fast-becoming critical to the specific association of circadian systems with mood disorders, principally due to recent developments that implicate the molecular clock in the temporal regulation of dopamine synthesis in the mesolimbic system (Mendoza and Challet, 2014). The SCN indirectly projects to the VTA via the medial preoptic nucleus (Luo and Aston-Jones, 2009). In addition there is an in situ circadian rhythm in dopamine release within the striatum, tyrosine-hydroxylase (TH) protein and TH positive c-FOS neuronal activity (Baltazar et al., 2013; Chung et al., 2014). In vivo electrophysiological recordings demonstrate a diurnal rhythm in a sub-type of fast-spiking neuron within the VTA, however ex vivo imaging of Per2 within the VTA, fails to detect autonomous rhythmicity when this structure is isolated from the rest of the CNS (Abe et al., 2002). These data suggest that although the VTA exhibits rhythmicity in its activity and output, it is not intrinsically generated and is instead dependent on input from the SCN.

The direct interaction of circadian and dopamine systems has been reinforced through studies on the VTA of the clock gene mutant and knockout strains, ClockΔ19 and Rev-erba−/−. These transgenic models were initially characterised based upon their circadian behavioural characteristics, revealing a role of these two genes within the TTFL, yet both mouse lines also exhibit mania-like behaviours (Chung et al., 2014; Roybal et al., 2007). In addition, these behavioural deficits are both at least partly attributable to changes in dopamine regulation and the activity of dopamine neurons within the VTA.

The behavioural phenotype of ClockΔ19 mice can be partially rescued through VTA-specific transfection of a functional Clock gene, and +/+ animals can be induced to display mania and depression-like phenotypes through knockdown of Clock within the VTA (Mukherjee et al., 2010). ClockΔ19 animals show direct physiological changes in the profile of VTA neurons and their target regions with increased burst-firing, dopamine levels and elevate D2 expression in the striatum alongside altered VTA-NAcc interactions (Coque et al., 2011; Dzirasa et al., 2011; Spencer et al., 2012). Rev-erba−/− mice show similar changes to dopaminergic systems with elevated dopamine release within the striatum (Chung et al., 2014). This study also revealed a circadian rhythm in TH protein expression that was dependent on the actions of Rev-erba and that phase-specific pharmacological inhibition of Rev-erba is sufficient to induce mania-like behaviours. The
overall role of dopamine pathways within the behavioural alterations in affective disorders is supported by a broad array of evidence and this recent work has implicated circadian regulation as a physiological mechanism through which elements of the circadian system can influence mood and affective behaviours.

**Serotonin**

Although dopaminergic systems have a particularly strong association with affective disorders, other monoamine pathways also play a role within mood pathology. Serotonin is generally associated within the scope of depression and depression-like behaviour. Clinical studies in patients with anxiety disorders and depression demonstrate altered levels of serotonin, receptor and transporter levels (Holmes et al., 2003). Animal models with altered serotonin and 5-HT receptor expression also exhibit behavioural depression and depression resistant behaviours (Domínguez-López et al., 2012; Perona et al., 2008). Within the spectrum of BPD serotonergic abnormalities also appear, with downregulation of serotonin reported during episodes of depression and even euthymia (Mahmood and Silverstone, 2001). The powerful effects of selective serotonin re-uptake inhibitors (SSRI) in the treatment of depression highlights the beneficial role that upregulation of serotonergic signalling can have upon mood (Ressler and Nemeroff, 2000).

Circadian associations with serotonergic pathways as observed with dopaminergic circuits are also widely reported in the literature. Unlike the case of the VTA however, serotonin release directly influences circadian rhythms rather than itself being under circadian control. The SCN receive one of the densest serotonergic innervations of the entire CNS with a direct input from the median raphe nuclei (MRN) as well as an indirect projection arriving from the dorsal raphe nuclei (DRN) via the IGL (Morin and Allen, 2006; Pontes et al., 2010). Serotonin is believed to be important in mediating photic phase shifts of behavioural rhythms as well as providing arousal feedback on to the SCN. In order to translate afferent serotonergic information, the SCN express an array of 5-HT receptors and 5-HT agonists can phase shift rodent behavioural rhythms as well as molecular rhythms *in vitro* (Horikawa and Shibata, 2004; Prosser, 2003; Takeuchi et al., 2014). If normal serotonin function is indeed compromised in affective disorders then this has the potential to impact circadian pacemaking. Current evidence however has not revealed a
mechanism by which the circadian system influences serotonin function bi-directionally. Without this evidence it appears difficult to suggest a role of the circadian system in the aetiology of serotonergic-based mood alterations.

**Hypothalamic-pituitary-adrenal axis**

Outside of monoaminergic circuits, other central pathways are implicated within the neurobiological mechanisms of mood dysregulation. One major region that can influence affective behaviours is the hypothalamic-pituitary-adrenal axis (HPA). The HPA axis regulates the daily release of corticosterone. This steroid hormone is important in stress responses and anxiety-like behaviour. Hypercortisolism is observed across both MDD and BPD and therefore appears to be involved in the emergence of anxiety phenotypes across both behavioural states (Watson and Mackin, 2006). Within a laboratory setting, mice with a specific glucocorticoid receptor knockout within the prefrontal cortex exhibit anxiety and depression-like phenotypes as well as a high sensitivity to tricyclic antidepressants (Wei et al., 2004). As with monoaminergic circuits, the HPA system exhibits strong interactions with the circadian system, with daily levels of cortisol driven by the SCN. Corticosterone also exhibits a profound effect on peripheral clocks, functioning as a powerful synchronising agent of cellular rhythms (Balsalobre et al., 2000; Kalsbeek et al., 2012). In the circadian knockout animal Bmal1−/−, behavioural deficits in this model associated with the manic phase of BPD appear alongside hypocortisolism suggesting a major role for the HPA axis in clock-associated affective deficits (Leliavski et al., 2014). The HPA axis therefore represents a further central pathway that exhibits evidence of altered function in affective disorders while possessing mechanistic associations with the circadian system.

**Neurogenesis and neurotrophy**

A further physiological mechanism that is consistently observed within the neurobiology of mood disorders and is influenced by the circadian system is the role of neurotrophy and neurogenesis. Stress and associated stress hormones, particularly corticosterone are known to impact on neurogenesis within the hippocampus (McEwen, 1999). BPD patients also exhibit increased non-specific sub-cortical hyperintensity (SCH) lesions and a general
reduction in neuronal density in various brain nuclei including the NAcc and hypothalamus as well as the hippocampus (Kato, 2008). Mood stabilisers such as lithium are known to have a neuroprotective effect specifically within the hippocampus while tricyclic anti-depressants such as SSRIs depend on neurogenesis within the dentate gyrus (DG) within their mechanism of action (Can et al., 2014; Samuels and Hen, 2011). The circadian influence upon this pathway is exhibited locally with a diurnal rhythm in neurogenesis and Per1 expression within the DG that is dampened by interactions with corticosterone (Gilhooley et al., 2011). The number of neuronal progenitor cells within the DG is also seemingly dependent upon corticosterone and suggests that circadian, HPA and neurogenesis may exhibit inter-dependent interactions within the hippocampus.

**Circadian rhythms in bipolar and affective disorders**

It is clear that a number of brain regions and physiological pathways implicated in affective disorders are influenced by the circadian system. Until recent work highlighting the role of clock regulation of dopamine production and release from the VTA referred to above, these relationships remained associative without substantial evidence that directly implicated the role of clock regulation to function (Mendoza and Challet, 2014). Over the past 50 years however, strong clinical and pre-clinical evidence has distinguished a consistent role of the role of sleep and circadian rhythm disruption in affective disorders (Gonzalez, 2014; McCarthy and Welsh, 2012). Below is a summary of the accumulated clinical evidence that demonstrates a consistent role for sleep and circadian abnormalities in mood disorders.

Sleep-wake alterations are a common facet of SCZ, MDD and BPD with various abnormalities in the architecture of sleep-wake cycles associated with these conditions (Boivin, 2000). BPD patients describe sleep-wake disturbance across episodes of both mania and depression. Epidemiological studies demonstrate that mania and depression are characterised by distinct alterations to sleep-wake patterns. Episodes of depression are associated with either hypersomnia, reported in 20-78% of patients, or insomnia, which is reported in up to 100% of patients depending on the study in question (Harvey, 2008). Sleep-wake alterations in mania are characterised by an overall reduction in the need for sleep and this symptom is a very common facet of manic episodes, reported in
69-99% of patients. A study of polysomnographic characterisation of sleep in young bipolar patients provided a greater level of detail into the sleep architecture of mania. Specific disruption included increased awakenings, reduced REM latency, reduced sleep time and decreased sleep efficiency (Hudson et al., 1993). Widely reported changes to sleep-wake patterns in BPD are not simply a symptom arising during episodes but instead are often reported prior to the appearance of full episodes. Long-term monitoring of BPD patients behaviour describes changes to sleep and daily rhythms that appear as part of a characteristic prodrome of BPD (Brietzke et al., 2012; Zeschel et al., 2013). This strengthens suggestions that sleep-wake disruption may be involved in the aetiology and development of mood episodes.

When interpreting studies that focus on patients’ sleep-wake rhythms, certain caution must be taken as sleep and circadian processes, although inter-dependent, are separate and controlled by distinct brain regions (Saper et al., 2005). Evidence of circadian rhythm disruption, and not altered sleep in itself, is necessary to determine an underlying role for circadian rhythms in mood disorders. The monitoring and manipulation of circadian rhythms can prove more difficult and invasive than sleep-wake cycles, yet clinical studies have had some towards identifying altered circadian rhythms in BPD.

Actigraphic assessment of BPD patients highlights altered phasing and reduced rhythm stability, with dampened rhythmicity exhibited during euthymia (Jones et al., 2005; Rock et al., 2014). The persistence of low amplitude rhythms into euthymia suggests again that circadian instability may be an enduring facet in BPD patients outside clinical episodes. Monitoring of circadian output factors including corticosterone, melatonin and body temperature also point towards both altered phase and amplitude in MDD and BPD patients (Etain et al., 2011; McClung, 2007; Souqtre et al., 1989; Srinivasan et al., 2009; Wong et al., 2000). A person’s phase of daily activity can also provide a marker for the alignment of their circadian system. When MDD and BPD patients daily phase of activity are monitored, both populations exhibit alterations in phases of behavioural activity relative to non-affected controls (Emens et al., 2009; Mansour et al., 2005). In the example of MDD specifically, evening preference even exhibits correlations with severity of symptoms. These findings show that circadian rhythm disruption, not just alterations to sleep architecture, appears to play a role in the pathophysiology of mood disorders.
Clinical evidence of changes to circadian rhythms in BPD and MDD is supported by genetic studies that demonstrate associations of various clock genes within patient populations. Clock gene associations with BPD have fallen short of stringent thresholds set within GWAS studies, although very few targets have shown consistent associations in the current GWAS literature on BPD (Craddock and Sklar, 2013). The seemingly polygenetic nature of these conditions instead gives tract to the idea that population-level associations, where clock genes do come to the fore, may be represented within the broad genetic spectrum of risk-factors that pre-dispose groups or families to the development of affective disorders. The core circadian genes Clock, Npas2, Arntl1, Per3 and NR1D1 all exhibit strong associations to BPD, with other clock genes also showing links to MDD and schizophrenia (Etain et al., 2011; Nievergelt et al., 2006; Shi et al., 2008). A recent study utilised post-mortem brains from MDD patients to determine changes in the cycling of clock genes across various brain regions (Li et al., 2013a). Tissue from control patients demonstrated clear rhythms of clock genes across an array of brain regions, yet in brains from MDD patients these oscillations were dampened and mis-aligned. This powerful data-set suggests that there may be significant alterations to central circadian pacemaking across the whole brain in MDD.

**Chronotherapeutics and mood stabilisers in affective disorders**

Another significant clinical association between circadian rhythms and mood disorders is the interactions of therapeutic tools used in the management of BPD and MDD with circadian systems. One class of treatment strategies are known as chronotherapeutics that utilise the manipulation of both light and circadian zeitgebers to effectively manage mood episodes. Particularly during episodes of depression, transient or daily alterations in light exposure, sleep patterns or exposure to regular zeitgebers can function as powerful tool to stabilise altered mood (Benedetti, 2012). Sleep deprivation exhibits a particularly profound effect on depression-like states, causing a rapid although transient improvement of mood (Wu et al., 2009). The speed of these effects can result in the reversal from an episode of depression into euthymia by the first morning after a single night’s loss of sleep. Typically effects manifest within 24-48 hours following intervention but can also rapidly dissipate following return to normal sleeping conditions.
The use of bright light to phase advance and delay rhythms as well as shifting sleep cycles are also used to treat episodes of depression and MDD as well as their widespread application in seasonal affective disorder (SAD; Benedetti, 2012; Lewy et al., 1998; Wehr et al., 1998). In contrast to these beneficial effects on depression, exposure to bright light or sleep deprivation can induce manic episodes in patients (Colombo et al., 1999; Noguchi et al., 2012; Schwitzer et al., 1990). In some clinical-based studies, there are even reports of the switch from depression into mania following sleep deprivation strategies that again serve to highlight the powerful influence of these strategies on affective behaviours (Colombo et al., 1999).

In addition to the management of sleep and light, pharmacological interventions used in the treatment of BPD impact on both molecular and behavioural circadian output. This begs the question whether therapeutic benefits of these drugs may at least be in part due to interactions with circadian physiology. Valproic acid is one such example and is an anti-convulsant also used as a mood-stabiliser in the management of BPD. The in vitro effects of VPA result in phase shifts of molecular rhythms in SCN explants that are mediated by actions on histone deacetylase activity (Johansson et al., 2011). Although this recent demonstration of the effects of VTA on SCN pacemaking in vitro is intriguing, the most profound modulations of circadian rhythms are observed with the use of lithium. Lithium exhibits a range of effects on cellular pacemaking in central and peripheral tissues but the role of lithium is particularly fascinating as cellular level effects translate all the way up to changes in behaviour.

Lithium has been recognised as a mood stabiliser for over 60 years and possesses a range of unique characteristics in the management and prophylaxis of BPD (Grof, 2010). Notably, lithium is prescribed almost exclusively in the management of BPD and not in across other affective disorders like many other pharmaceuticals used in the treatment of abnormal mood (Can et al., 2014). A further characteristic that sets lithium apart from its contemporaries is the longevity of its effects, representing the most effective tool in the prevention of relapses and reducing rates of suicide that are common in BPD (Cipriani et al., 2013). Lithium is also efficacious in the stabilisation across episode of mood both mania and depression (Baldessarini, 2013). Although more potent in the case of the former, this bi-modal effect highlights a common mechanistic target of lithium that
manifests across both mood states and is part of the reason why understanding the biochemical interactions of lithium is of great importance.

The profound impact of lithium on the circadian system is why this fascinating ion attracts the attention of pure circadian biologists as well as those focusing on the role of rhythms in mood disorders. Across a diverse array of species lithium consistently lengthens the period of free-running rhythms, with these effects even appearing in studies on humans (Klemfuss, 1992; Johnsson et al., 1983; Li et al., 2012; Welsh and Moore-Ede, 1990). In vitro investigations on central and peripheral tissues demonstrate that lithium interacts directly with the molecular clock and electrical activity within the SCN. Lithium application can lengthen the period of firing rate rhythms in isolated SCN neurons and transiently reduces action potential firing in SCN slice preparations (Abe et al., 2000; Mason and Biello, 1992). The period of molecular clock rhythms in both peripheral tissues and intact SCN explants can be lengthened by lithium application (Li et al., 2012). Under in vitro conditions lithium also enhances the amplitude of PER2::LUC rhythms although this property has yet to be reported behaviourally in rodents or humans. Interestingly, the ability of lithium to enhance the amplitude of molecular rhythms appears reduced in skin fibroblasts from BPD patients relative to healthy samples (McCarthy et al., 2013). This finding suggests a potential difference in the response of the molecular clock to lithium in BPD patients. Given that lithium responders represent a distinct group in BPD, plus an emerging genetic association with circadian clock genes including Rev-erba and Bmal1, such findings open up questions as to whether lithium’s therapeutic effects may act via clock mechanisms (Campos de Sousa et al., 2012; Grof, 2010; Rybakowski et al., 2014).

In order to understand the genetic basis of lithium’s effects on circadian systems, knowledge of the biochemical targets of lithium’s action is required. Despite efforts the cellular mechanisms through which lithium exerts its effects on period are currently unclear. A recent study was successful in exhibiting that the amplitude enhancing effects of lithium occur through direct interactions with Per2 expression and acts through inhibition of GSK3β, a phosphokinase involved in both circadian and mood regulation (Li et al., 2012; O’Brien and Klein, 2009). Although no direct causality has been demonstrated, the interactions of lithium across various levels of the circadian system suggest that part of its therapeutic efficacy may arise within these influences. Coupled
with observations on the effects of valproic acid and chronotherapy these therapeutic interactions further implicate the circadian system within affective disorder pathology. Whether targeting the circadian system represents a long-term strategy in the management of mood disorders however remains to be seen.

**Circadian control of mood**

The clinical picture of affective disorders highlights a strong connection to circadian rhythm disruption alongside potential genetic and neurobiological mechanisms that may contribute to the pathophysiology and aetiology of this group of diseases. In order to validate this interaction evidence is required that demonstrates a causal link between changes to rhythms and altered mood. There exist numerous human-based studies that reveal a circadian variation in subjective mood states across the day. These rhythms appear not just in pathological examples but also in healthy subjects, suggesting a circadian influence of the presentation of mood across the day (Wirz-Justice, 2008). Daily variations in mood states are also recognised in both MDD and BPD, with a morning nadir in subjective mood a distinct characteristic in MDD. The mis-alignment of circadian rhythms through sleep deprivation, shift work and jet lag can also have a negative impact on mood, with increased risk of depression and low mood states (Acheson et al., 2007; Katz et al., 2001; Levandovski et al., 2011; Selvi et al., 2007; Srinivasan et al., 2009).

The circadian regulation of mood appears feasible through the pathways discussed previously; HPA axis, dopaminergic systems, neurogenesis. Anatomical projections of the SCN exhibit further indirect connections to many brain regions involved in mood regulation. In addition to the VTA another key are is the lateral habenula (LHb), with both of these regions strongly associated with MDD and BPD (Chaudhury et al., 2013; Lecca et al., 2014; Li et al., 2013b). Both the LHb and VTA have recently been shown to possess rhythmic activity, the former of which is intrinsic to the LHb (Cho et al., 2012; Chung et al., 2014; Guilding et al., 2010; Sakhi et al., 2014). Various other brain regions that are associated with affective behaviours also show expression of clock gene activity. These areas include the retina, amygdala and prefrontal cortex (PFC) and reveals further mood circuits that may be influenced by local changes to clock activity (Menet and Rosbash, 2011). In addition to its rhythmic capacity the retina may be critical in its influence on the
circadian regulation of mood as the SCN is critically dependent on photic input via the RHT.

**Light, rhythmicity and affect**

The influence of light on mood is not restricted to the circadian system as non-image forming projections from the retina exhibit a growing association with changes in affective behaviours (LeGates et al., 2014). The role of light is perhaps most apparent in SAD, a condition in which sufferers exhibit seasonally restricted mood episodes, which appear with greater frequency during the shortened days of winter (Magnusson and Boivin, 2003). A powerful tool used in therapeutic interventions of SAD is the timed exposure to bright-light (Lewy et al., 1998). There is a widely-held assertion therefore that the pathological consequences of SAD are due to the effects of light upon mood.

Rodent models support this idea as many examples exist in which exposure to altered photoperiods result in changes to mood phenotypes (Ashkenazy et al., 2009; Dulcis et al., 2013; LeGates et al., 2012). The effect of light on mood regulation could be explained by an interaction with the circadian system. In *vitro* studies consistently demonstrate the effect altered day-length can have on the amplitude and network dynamics of molecular and electrical rhythms within the SCN (Brown and Piggins, 2009; Evans et al., 2013; Meijer et al., 2007; Mrugala et al., 2000; Schwartz et al., 2011). A decrease in central output represents a feasible mechanism through which photoperiodic changes light may influence affective behaviour via the circadian system. Light can alter mood-like behaviours independently of independently of the SCN. Mice exposed to a forced desynchrony protocol in which the light-dark cycle is restricted to 7 hours light and 7 hours dark, induces changes in both mood and learning behaviour despite an intact SCN and HPA axis. These influences arise through direct projections of intrinsically photosensitive retinal ganglion cells to regions across the CNS (iPRGC; Hattar et al., 2006). The iPRGCs that innervate and provide photic information to the SCN, and other iPRGC sub-classes, project to brain areas that are regions involved in mood behaviours, including a polysynaptic innervation of the LHb (Sakhi et al., 2014; Schmidt et al., 2011). The role of non-visual light pathways in the regulation of mood remains a growing area of great interest. Due to the natural interactions of light and circadian systems it appears a pre-
requisite to consider the potential confounding effects that each could play upon another when considering their roles in mood regulation.

**Pre-clinical associations of circadian rhythms and the regulation of mood**

Up to this point the clinical, genetic and therapeutic interactions of the circadian system with mood that have been discussed describe the strong case for the role of rhythms in affective disorder aetiology and pathology. The main issues in the field now lie in identifying and understanding common changes that arise within the circadian system and whether these changes impact upon other regions of the brain involved in mood disorders. How can central or regionally constrained rhythm alterations influence mood? What are the underlying genetic, cellular and network mechanisms in the circadian system and do these changes correlate with specific mood phenotypes? Many of these questions unfortunately cannot be addressed with tools that are currently available within the clinic. For example the use of forced de-synchrony, altered light cycles or constant conditions to manipulate circadian systems cannot be applied to patients with mood disorders due to the obvious ethical considerations. What is available to researchers however is an array of laboratory models with clock gene mutations that exhibit alterations in affective behaviours. These models can be used to study the neural mechanisms and correlates of circadian-mood association in much finer detail than is possible in humans.

The consistent appearance of abnormal affective behaviours in animal models with lesions in core clock genes has provided further evidence that altered rhythm generation translates to altered mood (Foster et al., 2013; Landgraf et al., 2014). Currently there are eight genetically distinct models that possess lesions within critical TTFL elements that concomitantly exhibit changes to mood behaviours, with a general trend towards mania-like phenotypes (Foster et al., 2013; Landgraf et al., 2014; Schnell et al., 2014). These laboratory models represent powerful tools to dissect mechanisms through which clock genes and circadian physiology influence higher-level behavioural outputs. The data from these models is now reaching a stage where correlations and associations between circadian phenotype, physiology and mood may begin to be made (Landgraf et al., 2014).
This may begin towards answering some the outstanding questions raised above. A summary follows of clock gene models that display abnormal mood-states, the various experimental paradigms used to define these behaviours and specific circadian deficits in each example.

**Circadian models of affective behaviours**

Animal models of complex psychological diseases such as BPD and SZ can never be fully validated owing to the impossible task of defining mood or psychological states directly. Instead indirect measures of behavioural and psychological states can be used to create a phenotypic profile of the model in question and how these behaviours associate with the disorder in question. Laboratory models are expected to possess face, construct and predictive validity of the disease that they represent to become accepted as an accurate model (Willner, 1986). Face validity requires the animal to possess specific behavioural phenotypes of the condition in question. In models of BPD this would ideally be manifested in the cycling of mania and depression over long time courses although currently no model has fully satisfied these criteria (Kato et al., 2007). Construct validity necessitates that the model shares common mechanisms that underpin the disease in question, such as genetic or physiological processes. Due to the association of circadian rhythms with affective disorders, many of the models discussed are defining a specific element within the mechanistic spectrum of affective disorders. This cohort may therefore be considered a sub-group that are modelling the role of the circadian system in these conditions. Predictive validity is the final criteria used to determine the strength of a model and assesses the response to therapeutic interventions prescribed in the clinical treatment of the disease in question.

**ClockΔ19**

The animal that has provided the greatest insight into the role of the circadian system in mood regulation is the ClockΔ19 model discussed above. The ClockΔ19 mouse satisfies face, construct and predictive validity of the manic phase of BPD (Roybal et al., 2007). In behavioural tests, these animals exhibit hyperactivity, reduced sleep, heightened reward preference and reduced helplessness, all phenotypes associated with mania. The specific
circadian phenotype of these animals is a long tau (~27h) that degenerates into arrhythmicity in DD and altered phase re-setting (Vitaterna et al., 1994). The central circadian pacemaker in this model is disrupted with a reduced molecular output observed in these animals, highlighting a deficit within the central pacemaking structure (Vitaterna et al., 2006).

The most interesting facet of this model has been the demonstration of altered dopaminergic signalling within the VTA as a major contributor to the ClockΔ19 behavioural phenotype. The VTA of ClockΔ19 animals exhibit increased burst firing of constituent neurons, increased dopamine release, increased D2 receptor expression within the striatum and altered coupling between the VTA and the NAcc (Coque et al., 2011; Dzirasa et al., 2011; Spencer et al., 2012). Restoration of certain behaviours in the ClockΔ19 is possible by transfection with functional Clock and targeted knockdown of Clock can induce mania-like behaviour in +/+ animals (Mukherjee et al., 2010). The work on the ClockΔ19 mouse has been enlightening in its demonstration of the physiological role of this major transcription factor of the TTFL within the VTA. Although this work suggests that there is a circadian regulation of VTA function, this has yet to be confirmed. To do so would require a demonstration that mood changes of ClockΔ19 are a result of altered rhythmicity within the VTA. The experiments described to date although elegant fail to delineate between a restoration of Clock-dependent rhythms and other transcriptional-dependent interactions that may be associated with Clock in the VTA.

Rev-erba⁻/−

An investigation into the Rev-erba⁻/− model of mania, has provided further evidence into a circadian regulation of mood via dopamine production within the VTA. This study supports deductions made from ClockΔ19 and has perhaps gone the furthest of any published work to date in providing evidence for a specific mechanism that provides rhythmic regulation of affective behaviour. Rev-erba⁻/− animals exhibit a lack of diurnal variation in mood phenotypes such as anxiety and immobility that are observed in +/+ cohorts with increased aggression and fear responses under baseline conditions (Chung et al., 2014). Dissection of mid-brain physiology revealed heightened dopamine release within the striatum alongside a loss of TH rhythmicity in the VTA and substantia nigra (SN)
highlighting a large dysfunction in dopaminergic systems. These phenotypes were inducible in +/+ animals in a time-of-day specific manner, under both diurnal and circadian conditions, by pharmacological antagonism of Rev-erba. Genetic dissection revealed that this activity manifest though direct regulation of TH by the transcriptional target of Rev-erbs, ROR elements, within the upstream promoter region of TH. This experiment demonstrates a regional and temporally restricted modulation of mood behaviours by targeted inhibition of clock gene function and represents the clearest example of a direct circadian control of affective behaviour.

Together with the work on ClockΔ19 circadian physiology is strongly implicated within normal regulation of dopamine production and control of mood. Interestingly however the circadian phenotype of Rev-erba−/− animals bares little similarity to that of ClockΔ19 animals. Under free-running conditions Rev-erba−/− animals exhibit a shortened tau and with little effects on free-running amplitude versus long tau and arrhythmic behaviour in ClockΔ19 (Preitner et al., 2002; Vitaterna et al., 1994). Although no effects on amplitude are reported in vivo or within the SCN, Rev-erba−/− and targeted deletion of Rev-erba results in blunted clock output in liver tissues (Cho et al., 2012). Both ClockΔ19 and Rev-erba−/− lines however do exhibit heightened phase re-setting responses to light and changes to metabolic physiology (Cho et al., 2012; Duez et al., 2008; Preitner et al., 2002; Raspe, 2002; Vitaterna et al., 2006). These behavioural differences present an interesting question into the role of specific circadian phenotype in the control of mood. These two models exhibit divergent circadian phenotypes yet both result in mania-like behaviours imparted through similar physiological alterations to the VTA. This suggests that general circadian disruption rather than a specific directional change to period or amplitude may be more relevant in the circadian clock controlled alterations to mood. Targeted manipulation of VTA circuits through pharmacological alterations to circadian phase and period may help resolve this question further.

*Per2Brdm*

Although perhaps lacking the mechanistic insight of the above examples, there are six further models with TTFL lesions that exhibit altered mood behaviours. Per2 is a critical element of the clock and there are two described mouse strains that exhibit different
mutations within the Per2 gene. Of these two models the Per2<sup>Brdm</sup> strain that lack a functional region of the PAS domain, required for heterodimerisation, exhibit mania-like phenotypes defined by reduced immobility in a forced-swim paradigm (Hampp et al., 2008; Zheng et al., 1999). Although limited in its exploration of behavioural phenotypes, this study revealed a reduction in amplitude of monoamine oxidase A within the VTA and NAcc, a rate-limiting enzyme in the production of dopamine. The rhythmic expression of MAOA was also revealed to be dependent on elements of the TTFL including Per2. The circadian phenotype of Per2<sup>Brdm</sup> animals is characterised by short period in DD conditions, reductions in rhythm amplitude and altered phase delay responses to light (Feillet et al., 2006; Zheng et al., 1999). These behavioural changes also manifest within the SCN with dampened or absent oscillations of core clock genes and output factors such as AVP and DBP <i>in situ</i>. The Per2<sup>Brdm</sup> model therefore shares low amplitude behavioural and SCN output rhythm phenotype with ClockΔ19 and a short tau with Rev-erba<sup>−/−</sup> models, a phenotypic chimera of the two. All three models however share a physiological association of central dopaminergic circuits as part of their phenotypic array.

<i>Bmal1<sup>−/−</sup></i>

BMAL1 is a fourth major transcription factor within the TTFL whose functional loss results in a mania-like phenotype (Kondratova et al., 2010). Loss of Bmal1 results in arrhythmicity in wheel-running experiments and unstable generation of PER2::LUC rhythms within the SCN and complete absence in peripheral tissues (Bunger et al., 2000; Ko et al., 2010a). Bmal1 animals exhibit a hyperactive phenotype in an open-field, reduced immobility although without changes to anxiety behaviours (Kondratova et al., 2010; Leliavski et al., 2014). As anxiety was measured only through duration within the centre of an open-field arena, more rigorous tests would be required to dismiss anxiety phenotypes in this model. Part of the behavioural phenotype of Bmal1<sup>−/−</sup> animals appears due to hypocortisolism that underlies changes in immobility behaviour (Leliavski et al., 2014). Bmal1<sup>−/−</sup> animals therefore share low-amplitude behavioural and central circadian output with ClockΔ19 and Per2<sup>Brdm−/−</sup> models as well as reduced immobility and hyperlocomotor behavioural phenotypes with the former example.
Outside of disruption within transcription factors of the TTFL, mutations or loss of key elements regulating clock protein localisation and degradation also result in altered mood phenotypes. The Fbxl3 mutant also known as after-hours (Afh) exhibit a long free-running period, altered phase re-setting to light and reductions in behavioural amplitude when assessed by drinking activity (Godinho et al., 2007; Guilding et al., 2013). The pacemaking of these animals is affected by delayed degradation of CRY1 and CRY2 proteins and results in dampened molecular output in situ and reduced network factors defining pacemaking within the SCN ex vivo. Interestingly the Afh mutation also results in dampened clock gene expression in the habenula, a key area in the expression of depression-like behaviour (Guilding et al., 2013). Afh animals exhibit a consistent reduction in anxiety-like behaviours across several behavioural paradigms including elevated plus maze, light-dark box and open-field as well as reduced immobility in a force-swim test (Keers et al., 2012). The phenotypic repertoire in the Afh model appears most similar to that of ClockΔ19 with long tau, low amplitude and shared type 0 phase shifting responses to light. Afh also exhibit dampened SCN molecular output and changes to anxiety-like behaviours that are similar to ClockΔ19 animals.

CK1ε/δ

Other elements of the clock protein degradation pathway CK1ε/δ that also regulate cellular localisation can also influence affective behaviours (Etchegaray et al., 2009). Loss of CK1ε−/− results in the lengthening of period and in gain-of-function mutations, there is a marked shortening of tau, altered phase re-setting and accelerated re-entrainment to altered LD cycles (Meng et al., 2008; Pilorz et al., 2014). Changes to the amplitude of SCN output is not reported in CK1ε−/− animals, although firing rate rhythms from in vitro preparations are lengthened (Meng et al., 2008). The CK1ε−/− knockout line exhibit altered reward-related behaviours with hyperlocomotion and heightened sensitivity to psychostimulants such as methamphetamine (Bryant et al., 2012). Unfortunately CK1δ−/− animals die shortly after birth although targeted overexpression of CK1δ in the forebrain of mice results in a mania-like phenotype (Zhou et al., 2010). Interestingly changes to dopaminergic systems are implicated in mechanism of CK1δ with altered dopaminergic receptor expression and methamphetamine response. Pharmacological inhibition of both
CK1ε and CK1δ concomitantly in rats also alters reward-seeking measured as saccharine intake and alcohol consumption (Perreau-Lenz et al., 2012).

**GSK3β**

The loss or gain of function of another clock-associated kinase, GSK3β also can manifest in changes to affective behaviour. GSK3β interacts with the TTFL through the phosphorylation of PER2, providing a signal for re-entry into the cell nucleus (Iitaka et al., 2005). This broadly-functioning enzyme is heavily implicated in the pathophysiology of BPD through its hypothesised role in the mood-stabilising effects of lithium (O’Brien and Klein, 2009). Partial loss of function of GSK3β results in decreased immobility in a forced-swim paradigm and increased exploratory behaviours (O’Brien et al., 2004). Overexpression of GSK3β results in mania-like phenotype including hyperactivity, decreased habituation and increased startle response (Prickaerts et al., 2006). Disinhibition of GSK3β through changes to phosphorylation sites results in heightened sensitivity to methamphetamine and increased depression-like behaviour in response to stress. Altered GSK3β activity therefore can have a bi-directional effect on mood-associated behaviours (Polter et al., 2010). There is yet to be a circadian investigation into the behavioural or physiological pacemaking in any of the GSKβ transgenic lines and so phenotypic correlates of circadian properties cannot be made. Given the widespread and diverse role of GSK3β within the CNS would perhaps be difficult to derive any behavioural effect specifically to the molecular clock yet these findings still maintain relevance given the role of GSK3β in the TTFL (Rowe et al., 2007).

**Dbp**

The final example that will be made reference towards is the D-box binding protein, Dbp, double knockout mouse. DBP is an output factor of the molecular clock and Dbp/−/− animals exhibit shortened free-running rhythms, reduced wheel-running activity and increased light-time activity under LD conditions (Lopez-molina et al., 1997). These animals also exhibit a depression-like phenotype with reduced activity and decreased responses to psychostimulants (Le-Niculescu et al., 2008). When exposed to sleep deprivation however, these mice exhibit hyperactive behaviour and an increase in alcohol
consumption. *Dbp*<sup>−/−</sup> therefore appears to represent an environmentally sensitive model that expresses both depression and mania-like states and provides strong face validity for the *Dbp*<sup>−/−</sup> mouse as a model of BPD. Physiological studies into the central pacemaking in *Dbp*<sup>−/−</sup> mutants are lacking, but at a behavioural level the *Dbp*<sup>−/−</sup> bares closest similarity to *Rev-erba*<sup>−/−</sup>. Under basal conditions however these animals exhibit a depression-like phenotype unlike *Rev-erba*<sup>−/−</sup> highlighting a further discordance between circadian phenotype and mood.

The diverse array of clock gene mutants described express varied circadian phenotypes and different levels of disruption to central pacemaking. There is also a divergence in the specific type of mood behaviours exhibited across different models. There appears a clear trend towards mania-like endophenotypes with associated behaviours appearing in baseline conditions in 7 of the 8 clock targeted models and mania-like behaviours inducible in the final example (*Dbp*<sup>−/−</sup>). What remains a challenge however is to draw comparisons between circadian phenotype and specific mood characteristics. There is a broad diversity in the range of circadian phenotypes both *in vivo* and *in vitro* within the cohort available alongside a lack of continuity in the experimental paradigms to which each model is exposed. Generally there does appear a trend for low amplitude free-running rhythms (*ClockΔ19, Per2<sup>Brdm−/−</sup>, *Bmal1*<sup>−/−</sup>, *Afh*) with many also showing blunted molecular oscillations within the SCN. Dampened central pacemaking represents a primary candidate factor in aberrant affective behaviours exhibited in these models. Despite their roles within the TTFL, clock genes can also interact with a broad range of transcriptional and post-translational targets in a tissue-specific manner (Miller et al., 2007; Panda et al., 2002b). This means that changes in behaviour of clock gene models cannot be prescribed exclusively to changes in the regulation of biological timing. Greater insight into the role of these lesions particularly within extra-SCN circuits will be required to build a greater understanding into specific changes to rhythms and effects upon distinct behaviours. Dissecting potential brain circuits affected by deficits in circadian time-keeping however, represents a daunting task given the vast number of central regions exhibiting circadian influence (Abe et al., 2002; Guilding and Piggins, 2007).
The *Myshkin* model of mania

Circadian models of affective behaviours have augmented the study into the pathophysiology of mood, yet outside of these examples there are various other genetic, pharmacological and environmental models utilised to study these phenomena (Kato et al., 2007). Specific examples include forebrain glucocorticoid receptor overexpression and neuron-specific mutPOLG knockout mice that affects mitochondrial DNA expression (Kasahara et al., 2006; Wei et al., 2004). It is interesting to note that this latter example exhibits altered monoamine levels and turnover across the brain as well as altered diurnal wheel-running activity. Pharmacological models include administration of methamphetamine, ouabain or 6-OH-dopamine with environmental stressors such as sleep deprivation, social isolation and social defeat also capable of inducing BPD-associated phenotypes (Gessa et al., 1995; Gould et al., 2001; Kato et al., 2007; Petty and Sherman, 1981; Willner, 1991).

In a recent study, a mouse possessing a mutation in the alpha 3 subunit of the Na\(^+\)/K\(^+\)-ATPase (NKA) was characterised as a model of the manic phase of BPD, satisfying face, construct and predictive validity (Kirshenbaum et al., 2011). The NKA is responsible for the active transport of Na\(^+\) and K\(^+\) across the plasma membrane and this mutation affects the movement of K\(^+\) along the pump’s transport pathway, rendering the subunit inactive and a 42% reduction in whole-brain NKA activity (Clapcote et al., 2009; Heinzen et al., 2014). Due to hippocampal epileptiform activity and susceptibility to seizures, the initial colony of animals was back-crossed on to a seizure resistant C57BL/6N line resulting in a 6% increase in whole-brain NKA activity.

This model known as *Myshkin* (*Myk/+*) exhibited a range of mania-like phenotypes including hyperactivity in an open-field, increased exploratory activity and reduced anxiety in both a light-dark box and elevated-plus maze paradigm. *Myk/+* animals also displayed heightened reward-seeking behaviour in sucrose preference tests, reduced immobility and impaired pre-pulse inhibition and startle response. In relation to sleep and circadian rhythms *Myk/+* animals displayed altered free-running rhythms and reduced total sleep, with attenuation in both REM and non-REM bouts and decreased REM latency. The face validity of *Myk/+* animals is very strong and manifests across a broad
range of behavioural tests. Treatment with the mood stabilisers lithium or valproic acid reduced hyperlocomotor and anxiety-like behaviour and support predictive validity in the \textit{Myk/+} model of mania.

The construct of the \textit{Myk/+} mutation is also represented across the clinical literature of BPD. Altered expression of NKA subunits are reported in post-mortem studies of BPD patients, with decreased expression of $\alpha_2$ and $\alpha_3$ subunits in the temporal and pre-frontal cortex (Rose et al., 1998; Tochigi et al., 2008). BPD patients also exhibit altered levels and binding of ouabain, a cardiotonic steroid and endogenous ligand for NKA alpha subunits that act to inhibit pump activity (El-Mallakh et al., 2010; Goldstein et al., 2006). In genetic studies allelic associations of alpha3 subunits are reported in BPD patients as well as other NKA alpha subunits (Goldstein et al., 2009; Mynett-Johnson et al., 1998). Further evidence for the role of NKA activity in BPD include state-dependent changes in pump activity of erythrocytes sampled from BPD patients (el-Mallakh and Wyatt, 1995). There are also interesting clinical observations that digitalis toxicity from the use of cardiotonic steroids such as ouabain and its homologs can induce depression and mania-like behaviour in human subjects (Keller and Frishman, 2003). Animal models also provide strong evidence for a role of NKA pumps in affective behaviours, as direct pharmacological targeting of NKA activity through ICV infusion of ouabain is recognised as a pharmacological model of mania (Herman et al., 2007).

\textbf{Role of the $\text{Na}^+$/K$^+$-ATPase in the CNS and disease}

Clinical and laboratory evidence supports a role for NKA expression and activity in the pathology of BPD, yet a functional role of NKA activity in mood regulation has yet to be accurately described. Across the body, the ATP-dependent activity of NKA results in the transport of three sodium ions out of the cytoplasm in exchange for two potassium ions across each catalytic cycle (Kaplan, 2002). Alpha subunits, of which there are four major isoforms, are the catalytically active regions of the NKA complex with each possessing unique expression and biochemical properties. The alpha3 subunit has a particularly distinct expression profile as it is found exclusively within neurons of the CNS and PNS (Blanco, 2005; Bøttger et al., 2011; Juhaszova and Laustein, 1997; Shyjan and Levenson, 1989). Although neuron-specific the alpha3 isoform is not ubiquitous across all of the
CNS, instead exhibiting different patterns of expression across neuronal populations and complete absentia from others (Bøttger et al., 2011).

The understanding of the roles individual NKA alpha subunits play within the brain is in its infancy, although certain theories have been postulated for the role of alpha3 in neuronal function based upon its expression and biochemical profile (Dobretsov and Stimers, 2005). The alpha3 subunit has a reduced affinity for Na⁺ yet a shallow voltage dependency, that latter which results in faster transport kinetics, than other NKA alpha subunits (Munzer et al., 1994; Zahler et al., 1997). This specialisation would theoretically optimise the efficacy of the pump during times of high Na⁺ load such as during action potential firing in neurons. These properties suggest that alpha3 subunits are specialised as reserve pumps to restore ionic gradients during action potential firing and explain their unique expression within neurons (Dobretsov and Stimers, 2005). Interestingly this functional role during high Na⁺ loads has recently been empirically demonstrated in rat hippocampal and striatal neurons (Azarias et al., 2013).

Part of the reason that the functional role of alpha3 subunits has not been intensively studied across different neurons and circuits of the CNS is attributable to difficulties in pharmacologically isolating alpha2 and alpha3 activity due to similar binding affinities for ouabain and cardiotonic steroids (Juhaszova and Laustein, 1997; Sweadner, 1985; Urayama and Sweadner, 1988). A role of non-isoform specific NKA pumps however is evident in electrical activity and the accurate encoding of neuronal information in diverse neuronal populations.

NKA subunits are critical in generating large electrochemical ionic gradients and in doing so generate a negative outward current from the asymmetrical exchange of Na⁺ and K⁺ cations. This action has a profound effect on the RMP and therefore excitability of neurons. NKA turnover can variably contribute to RMP of neurons that range between 9 and 45% depending on the type and locality of the neuron studied (Azarias et al., 2013; Dobretsov and Stimers, 2005; Molnar et al., 1999). NKA activity is also critical as it represents the only mechanism of extracellular Na⁺ clearance in neurons as well as contributing to the clearance of extracellular K⁺ (D’Ambrosio et al., 2002).
Pharmacological inhibition of NKA pumps can result in large rises of intracellular Na\textsuperscript{+} and can alter the firing properties of neurons from bursting to singles-spikes or the induction of repetitive firing (Azarias et al., 2013; Johnson et al., 1992; Senatorov et al., 1997; Shen and Johnson, 1998). In direct relation to mood circuitry, NKA activity regulates the burst firing behaviour of midbrain dopamine neurons in the rat, a characteristic electrical property of these neurons that is attenuated in the presence of the cardiotonic steroid strophanthidin (Johnson et al., 1992). NKA inhibition can have potentially pathological consequences when applied within the hippocampus. In the CA1 layer of hippocampal slices, inhibition of NKA activity results in neuronal hyperexcitability and expression of epileptiform activity (Vaillend et al., 2002). Interestingly this epileptiform activity was observed in hippocampal slices from Myk/+ animals prior to long-term back-crossing on to the C57BL/6N background (Clapcote et al., 2009). In terms of behavioural effects, haploinsufficiency of either α2 or α3 subunits results in altered behaviour in mice with deficits in learning and also in mood behaviours including anxiety and responses to methamphetamine (Lingrel et al., 2007; Moseley et al., 2007). NKA activity is therefore critically involved in the excitability and output of central neurons and either pharmacological or genetic lesions have significant effects on learning and affective behaviours.

The function of the NKA complex is not limited to effects on neuronal excitability, with a recently established role as a signal receptor that can induce various downstream signalling cascades through interactions with ouabain and other cardiotonic steroids as well as the proteoglycan agrin (Nesher et al., 2007; Tidow et al., 2011). Membrane-bound NKA pumps can form large signalling complexes involving ankyrin, the tyrosine kinase SRC, inositol trisphosphate receptor (IP3R), phosphotidylinositol-3 kinase (PI3K) and cavoloine-1 (Silva and Soares-da-Silva, 2012). Extracellular activation of NKA by ouabain can result in induction of AKT, ERK and intracellular Ca\textsuperscript{2+} signalling pathways across various cell types (Kim et al., 2008; Miyakawa-Naito et al., 2003; Yu et al., 2010). In Myk/+ animals hyperlocomotor and anxiety phenotypes can be partially attenuated using rostafuroxin, a compound that displaces ouabain at the site of NKA binding and the inhibitor of ERK activity SL327 (Kirshenbaum et al., 2011). This highlights that effects of
NKA activity on behaviour can manifest through intracellular signalling pathways of the NKA complex.

Although there is much evidence of the central physiological function played by NKA pump activity, evidence for the specific role played by the alpha3 subunit, in which the Myk/+ mutation is found, remains relatively sparse. In hippocampal neurons the alpha3 subunit regulates Na\(^+\) clearance during high action potential firing rates (Azarias et al., 2013). Within cerebellar neurons the loss of alpha3 activity results in elevated inhibitory synaptic transmission on to molecular layer interneurons. This altered inhibitory signalling arises from enhanced pre-synaptic signal propagation from upstream Purkinje neurons (Ikeda et al., 2013). A role for alpha3 activity has also become apparent in network function through the regulation of dendritic signalling (Blom et al., 2011). Loss of alpha3 subunits exclusively at dendrites that modulate post-synaptic fast depolarisations can result in altered post-synaptic signal propagation and the breakdown of network dynamics in cortical neurons (Shiina et al., 2010).

In light of this emerging physiological role for alpha3 subunits in the CNS, genetic studies have implicated alpha3 dysfunction into the pathophysiology of other non-affective disorders. Very strong genetic associations are apparent between alpha3 subunits and rapid dystonia parkinsonism (RDP) and alternating childhood hemiplegia (ACH), with the Myk/+ mutation itself residing in an identical amino acid residue observed in an AHC patient (Heinzen et al., 2014). Alpha3 NKA expression and activity therefore play a critical role within basic cellular functions of the CNS and changes in activity result in deficits in learning, affective behaviour and within diverse neural pathologies.

In relation to the circadian system, no studies have focused on the role of the NKA alpha3 subunit on circadian physiology or behaviour. In rats there is a diurnal rhythm in non-isoform specific NKA pump current that peaks during the day and reaches a nadir at night (Wang and Huang, 2004). The activity of the NKA pump is also dependent on intracellular glycolysis and oxidative phosphorylation (Wang et al., 2012b). Blockade of NKA activity with the cardiotonic steroid strophanthidin or zero K\(^+\) extracellular fluid affects spontaneous firing in the rat SCN, resulting in increased depolarisation rates, changes to afterhyperpolarisation amplitude and a decrease in action potential firing (Wang and
Huang, 2006). These studies currently represent the sole insights into NKA pump activity in mammalian circadian physiology.

The Myk/+ mouse model therefore presents a novel opportunity to study circadian abnormalities in a model of mania with powerful behavioural, construct and pharmacological validity. Although the NKA plays a functional role within the SCN, it has yet to be described as a critical element of the TTFL that underlies circadian output. Previously described clock gene models of affective behaviours possess lesions within critical TTFL elements that underpin fundamental circadian timekeeping within cells. The Myk/+ mouse model exhibits a broad array of mania-like phenotypes including a long free-running period, yet it is not known how this mutation affects cellular or network-level pacemaking that may contribute to these behaviours. This study shall address how the Myk/+ mutation alters circadian timekeeping mechanisms and to observe in detail the overt effects these changes have on circadian behaviour.

**Aims**

This thesis represents an investigation into the behavioural and neurobiological interactions of the circadian system with affective disorders. The first two chapters are focused upon the Myk/+ mouse model of mania. Chapter one provides an in-depth characterisation of circadian, metabolic and light-dependent responses of Myk/+ animals and identifies novel behavioural phenotypes important in the profile of affective behaviours. The second chapter utilises an *in vitro* and *ex vivo* approach to investigate the underlying molecular and physiological function of the Myk/+ SCN. By harnessing bioluminescent, patch-clamp and calcium-imaging techniques the molecular, electrical and synaptic properties of the Myk/+ SCN are characterised. These studies reveal that despite an intact molecular pacemaker *ex vivo*, there remains a dampened electrical output and altered time-dependent synaptic signalling in the Myk/+ SCN that underlie abnormal circadian behaviour. The third chapter focuses on a separate aspect of circadian associations with BPD by investigating the biochemical interactions of lithium with molecular rhythms of the SCN. This chapter uses *ex vivo* SCN bioluminescence recordings and pharmacological tools to antagonise specific intracellular pathways that are known targets of lithium.
References


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General Methods
General Methods

Experimental Approach

The approach of the first two chapters of this thesis is multi-disciplinary, utilising a broad range of tools to identify changes to the circadian system and underlying SCN physiology in *Myshkin* mutants. The methods used can be broadly split into *in vivo* and *in vitro* elements.

The final chapter of this thesis uses a single *ex vivo* model, PER2::LUC organotypic bioluminescence recording, to study the response of the molecular time-keeping mechanisms of the SCN to the mood stabiliser lithium.

**In vivo approach**

A well-established method to study circadian rhythms *in vivo* is through the observations of wheel-running behaviour under different lighting conditions. When presented with a running-wheel, individually-housed mice and other rodents exhibit rhythmic bouts of activity that provide a representation of the output of the intrinsic circadian system (Pittendrigh and Daan, 1976).

Manipulations of the light-dark cycle provide further indications as to the function of the intact circadian system. Under constant dark conditions, light pulses can be given to animals across different points of the circadian cycle to determine the phase-shifting properties of the SCN to their major zeitgeber; light (Golombek and Rosenstein, 2010; Johnson, 1999; Roenneberg et al., 2003).

Considerations are required when utilising this method to study rhythmic behaviour. Running wheel behaviour has been suggested to be a form of stereotypy that occurs only in captivity (Mason, et al 2007) and that such records may not represent the animal’s natural behaviour, or only a very specific sub-type of behaviour. Exercise is also known to feedback directly to the SCN from known afferent inputs, which affect both *in vivo* electrical activity and behavioural rhythms (Hughes and Piggins, 2012; van Oosterhout et
al., 2012; Yamazaki et al., 1998). This poses questions into the effects that such high-intensity activity may have on the SCN’s timekeeping properties.

Despite these considerations, wheel-running is a clearly established model to study in vivo circadian rhythms (Jud et al., 2005). This method is consistently able to highlight intrinsic circadian behavioural deficits associated with disruption to the circadian system. These include animal models with molecular lesions of core clock genes, essential neuropeptides and output factors, and is capable of screening animals for specific circadian deficits and is widely utilised in the literature (Aton et al., 2005; Ko and Takahashi, 2006; Prosser et al., 2007; Vitaterna et al., 1994).

Recently, the coupling of circadian rhythms with metabolic processes, from cellular to organismal level, has also become firmly established in the literature (Asher and Schibler, 2011). Assessment of metabolic activity can therefore provide further information on the regulation of circadian output processes, such as behaviour (indirectly via O₂ consumption) as well as feeding and drinking activity (Bechtold, et al., 2008; Guilding et al., 2013). Indirect calorimetry and the monitoring of feeding and drinking patterns were therefore used to compliment wheel-running studies to aid the characterisation of the Myshkin circadian system.

**Animals Used**

The majority of the experiments in this study were performed on Myk/+ mutant mice or their congenic +/+ littermates, generously provided by Dr Steve Clapcote (University of Leeds, UK). Myk/+ mutants were backcrossed on to a seizure resistant C57BL/6 (6NCR) background for over 20 generations prior to arrival in Manchester and maintained as such in the University’s animal care facility (Kirshenbaum et al., 2011). Myk/+ mutants survive only as heterozygotes, and within their mania-like endophenotypes, exhibit abnormal behavioural responses to different environmental conditions. Heightened responses to novel and stressful situations were observed during initial behavioural experiments and as a result certain handling and housing conditions had to be considered in behavioural experiments, which are described where appropriate.
A further consideration when breeding Myk/+ animals was that litters from female Myk/+ mothers do not survive. As a result all breeding colonies were established with a single Myk/+ male mutant housed with one or two +/+ females.

PER2::LUC knock-in animals, used principally in the final chapter and as the background for establishing the Myshkin x PER2::LUC line (MykP2), were originally generated as described in Yoo et al. (Yoo et al., 2004). PER2::LUC animals are bred on to a C57BL6/J background and do not show any alterations in wheel-running behaviour to that of WT C57BL6/J littermates.

MykP2 animals were generated by crossing heterozygous Myk/+ male animals with age-matched female PER2::LUC animals. F1 generation animals were used for all MykP2 due to time restrictions on experiments. All Myk/+ x PER2::LUC and +/+ x PER2::LUC animals were behaviourally screened in wheel-running cages prior to use in any experiment to validate the expression of Myk/+ circadian phenotypes.

Male and female mice were group-housed on a 12:12 light-dark cycle at constant temperature (~18°C), humidity (~40%) and fed with a standard lab chow diet ad libitum (5/20/75% calories from fat, protein and carbohydrate). All animals used in the study were adult mice, aged between 8 weeks and 6 months old.

**Wheel-Running Behaviour**

The circadian profile of +/+ , Myk/+ and MykP2 animals was assessed through monitoring of wheel-running behaviour from single-housed cages maintained under controlled lighting conditions. Animal’s phase-shifting responses to light were also assessed across different portions of the circadian cycle under Aschoff type I and type II protocols (Jud et al., 2005).

Prior to single housing, animals were placed inside the light-tight cabinet, in which they were to be maintained, in group-housed home cages for 2 days to acclimatise to the surroundings. Cages were environmentally enriched with a single cardboard tube, bedding and a small blue plastic shelter and wet mash provided for the first week following isolation.
During each experiment, cages were arranged in rows of 6 in light-tight cabinets fitted with 16W low-heat emitting light bulbs of >250 lux (Kosnic Ltd, Newbury, UK). Animals were disturbed as infrequently as possible during experiments and when necessary cage changes were performed during the animal’s active phase to minimise arousal-induced phase shifting effects.

Male mice (aged 2-6 months) were individually-housed in solid-based plastic bases (410 x 247 x 124mm) and the surrounding cage was equipped with a 16cm-diameter lined steel running wheel and standard lab chow and water provided *ad libitum*. Wheels were fitted with magnets and a magnetic micro-switch connected to an acquisition PC running Chronobiology Kit Software (Stanford Software Systems, Santa Cruz, CA) was attached above the wheel. Each complete wheel revolution was recorded as a binary signal, by the creation of a circuit on the micro-switch, and the number of revolutions stored in 5-minute interval bins.

To assess the phase shifting effects of light, both Aschoff type I and type II protocols were applied, due to the difficulty of defining CT12 under DD conditions in some *Myshkin* animals. For type II phase responses, animals were maintained under a 12:12 LD cycle for 2-weeks and released into DD. During the second cycle of subjective night under constant darkness, the lights fitted within the cabinets were turned on for one hour and the animals then left to free-run in DD for 14 days.

To evaluate the effects of the type I protocol, each animal’s tau was calculated as described below and a one-hour light pulse given at the appropriate circadian phase measured from the predicted onset of activity (CT12). For the pulse, animals were carefully transported to a room adjacent to the recording room, where lights (>250 lux) were turned on for one hour and the mice returned to the same position within the light-tight cabinet with minimal disturbance.

**Data Analysis**

To assess circadian behavioural phenotypes of animals in wheel-running experiments, a range of parameters were used to determine individual responses to LD and DD conditions. All parameters described were measured in 10 day epochs and analysis
performed in Kit Analyze version 1.06 (Stanford Software Systems, Santa Cruz, CA) and Microsoft Excel 2010 (Microsoft, USA):

- Period (tau): Tau was calculated by chi-squared periodogram to give a quantitative measure of the dominant periodic component (Sokolove and Bushell, 1978). Free-running period was validated through the fitting of a regression line to the phase angle of daily onsets of behaviour. The period was taken as the mean value between the manually and quantitatively defined tau.

- Phase shift: Regression lines were eye-fitted to the phase angle of each animal’s free-running rhythm for the 10 days before and after exposure to light. The size of each phase shift was calculated as the distance between the two fitted regression lines on the first behavioural cycle following the pulse. When fitting regression lines to the free-running rhythm following a light pulse, transient behavioural shifts prior to establishment of a stable free-running rhythm are discounted. Phase shifts were independently assessed by experienced circadian researchers.

- Rhythm amplitude: The inter-daily variability of each rhythm was calculated as percentage variance from the power of the chi-squared periodogram:

  \[ \%V = \left( \frac{Q_p}{n} \right) \times 100 \]

  Where \( Q_p \) is the power of the chi-squared rhythm and \( n \) total number of bins.

- Wheel revolutions: Wheel revolutions were calculated by exporting wheel-running values from Kit Analyze and dividing total revolutions by the number of hours of a given epoch, thus presenting data as wheel revolution hr\(^{-1}\).

- Percentage of activity in light-phase: The relative amount of an animal’s wheel-running activity during lights-on was calculated by manually dividing data into light and dark phases and the proportion of activity within the light-phase calculated.

**Metabolic Activity Rhythms**

The metabolic profile of male *Myshkin* and +/+ littermates (aged 2-6 months) was assessed using a Complete Laboratory Animals Monitoring System (CLAMS, Columbus Instruments, Ohio, USA). Animals were individually-housed in clear, sealed perspex metabolic cages connected with insulated tubing at input and output terminals, creating
an active, open-circuit of gas flow. Gas concentrations flowing from the output port can be compared against that of a known concentrations pumped into the cage’s input valve as an indirect measure of metabolic activity (Columbus Instruments, Columbus, Ohio).

Feeding was measured by an infra-red beam across the food hopper, where visits are recorded as number of beam breaks. Drinking was measured by an electrical contact between the drinking spout, mouse and a conductive steel base. Each sip induced a small current that was recorded as a single visit to the sipper. All parameters were recorded in 10-minute bins over the course of the experiment.

Animals were monitored for 7 days with food and water provided ad libitum. Each animal was weighed and cages calibrated accordingly prior to the start of each experiment. Calorimetric cages were maintained in a dedicated room under a 12:12 light-dark cycle (>250 lux), at constant temperature (~18°C) and humidity (~40%) with a small amount of bedding provided. Metabolic characterisation under constant conditions was not possible due to limitations on animal husbandry conditions in the storage facility for the CLAMS system. Food and water were manually replaced when necessary during the course of the experiment, although this was done as infrequently as possible to minimise disturbance.

**Data Analysis**

All data was recorded directly by a personal computer running CLAMS custom software and recorded as a CSV time series allowing for easy manipulation. The following parameters were used to assess the metabolic profile of the tested animals:

**Basal Metabolic Rate (VO\(_2\))**: The overall mean of oxygen consumption (VO\(_2\)) per hour was calculated for each animal over the entire duration of the experiment to assess basal metabolic rate.

**VO\(_2\) Amplitude**: The amplitude of metabolic rhythm was defined as the difference between the daily peak and trough of VO\(_2\) following a 3-hour smooth to account for inherent noise in the data.

**Feeding and Drinking**: Data was recorded as beam breaks/electrical contacts and the total sum over 7 days was recorded as a measure of feeding and drinking activity.
Percentage light activity (feeding and drinking): All parameters of interest were manually divided into periods of lights-on and lights-off. The relative percentage of activity during lights was used to assess the entrainment of ingestion behaviours to the light-dark cycle.

Metabolic alpha: VO$_2$ metabolic profiles in mice are clearly defined by a daily peak in phase with lights-off and lights-on respectively. To assess the length of this daily peak and trough in VO$_2$, individual records were smoothed over 3-hours and the duration of daily peak and trough VO$_2$ measured manually in GraphPad Prism Version 6 (GraphPad Software, La Jolla, USA). Peak-trough duration was calculated for every cycle over the 7-day period and expressed as an average duration.

**In vitro approach**

The field of chronobiology benefits from the acute and long-term preservation of circadian properties of central and peripheral tissues *in vitro*. For example, hypothalamic SCN slices maintain rhythmic electrophysiological properties in culture that are observed *in vivo* (Belle et al., 2009; Groos and Hendriks, 1982; Kuhlman and McMahon, 2004; Wang et al., 2012). Such slices also maintain distinct spatiotemporal architecture of clock gene rhythms across the SCN network, with precise environmentally inducible changes visible when using single-cell imaging protocols (Evans et al., 2013; Guilding et al., 2013; Yamaguchi et al., 2003). Intracellular calcium rhythms across the SCN network are also maintained in acute slice preparations (Brancaccio et al., 2013; Enoki et al., 2012; Ikeda et al., 2003). This property allows the use of tissue culture as a viable method to study molecular and cellular mechanism of circadian timekeeping *in vitro*.

**Current Clamp Electrophysiology**

Current clamp recordings were made from intact hypothalamic slices containing the midrostro-caudal portion of the SCN. Recordings were made across the diurnal cycle (ZT0-24) from individual neurons across the dorso-ventral SCN axis for a maximum of 8 hours prior to the initiation of recordings.

Male and female animals (aged 2-6 months) were group housed in home cages and maintained under a 12:12 light-dark cycle above. All slices were prepared during the light
phase to avoid possible phase-shifting effects that can occur during the subjective night (Gillette et al., 1995).

Each mouse was placed in an anaesthetic chamber filled with isofluorane and killed by cervical dislocation. Following decapitation, the brain was removed within 60 seconds with due care taken to gently cut the optic nerves. Whole-brains were immediately covered in oxygenated (95% O₂; 5% CO₂), ice-cold incubation artificial cerebrospinal fluid (aCSF) with low sodium, low calcium and high magnesium (NaCl 95mM; KCl 1.8mM; KH₂PO₄ 1.2mM; CaCl₂ 0.5mM; MgSO₄ 7mM; Glucose 15mM; Sucrose 50mM; Phenol Red 0.05mg ml⁻¹; pH 7.4; 300-310 mosmol kg⁻¹) to minimise the anoxic and neurotoxic effects of slice preparation (Moyer and Brown, 1998). Intact brains were blocked coronally using a fresh razor blade, cutting approximately at the anterior extremity of the cerebellum and through the basal forebrain. The blocked brain was then attached to a vibratome stage using cyanoacrylate glue and allowed to dry, placed in a vibratome bath and filled with ice cold, oxygenated incubation aCSF.

250µm coronal slices were cut using a Campden Instruments automated vibratome (Campden Instruments 7000smz, Loughborough, UK) as a trade-off between optical transparency and the need to maintain the SCN as intact as possible (approximate rostro-caudal length; 600µm; Abrahamson and Moore, 2001). Once hypothalamic slices containing the SCN were apparent, each was transferred into a chamber containing room temperature recording aCSF (NaCl 127mM; KCl 1.8mM; KH₂PO₄ 1.2mM; CaCl₂ 4.8mM; MgSO₄ 1.3mM; NaHCO₃ 26mM; Glucose 15mM; Phenol Red 0.05mg ml⁻¹; pH 7.4; 300-310 mosmol kg⁻¹) continuously bubbled with 95% O₂; 5% CO₂. This solution has higher sodium and calcium content and a lower magnesium concentration than incubation solution as a closer mimic of the true ionic composition of the extracellular space of the CNS. Slices were maintained at room temperature during all subsequent experiments (20-23°C).

Whole-cell recordings were performed on a current clamp electrophysiology rig consisting of an upright microscope equipped with filters for differential interface contrast imaging (Olympus BX51WI, Essex, UK) mounted on to a vibration-free air table (TMC 63-500 series, MA, USA). Slices were placed in a specialised bath allowing continual flow of
oxygenated recording aCSF and secured with a platinum harp fitted with fine nylon threads. Slice were visualised through a 40x water-immersion lens, using a high-resolution camera (Orca R², Hamamatsu, Japan), mounted to the microscope’s camera port. The recording headstage was mounted to a micromanipulator, allowing extremely fine control up to 20nm resolution (PatchStar, Scientifica, East Sussex, UK).

The electrical recording apparatus was a dedicated current-clamp configuration, consisting of an npi BA-01X bridge amplifier (npi electronics, Tamm, Germany). Output signals from the bridge amplifier were routed through an acquisition interface with a sample rate of 30kHz (Micro 1401, CED System, Cambridge, UK) and stored on a PC running to Spike2 Version 6.02 (CED System, UK) for analysis.

Individual neurons were recorded through tight-seal patch-clamp recordings using high impedance (7-10MΩ) borosilicate thick-walled glass capillaries (Harvard Apparatus Ltd, Kent, UK) from a two-stage vertical micropipette puller (PC-10, Narishige, Tokyo, Japan). Pipettes were half-filled with 0.22µm filtered (Millex-GV, Millipore, UK) intracellular solution (K-gluconate 120mM; KCl 10mM; MgCl₂ 2mM; K₂-ATP 2mM; Na-GTP 0.5mM; HEPES 20mM; EGTA 0.5mM; pH 7.28 with KOH; Osmolarity 295-300mosmol kg⁻¹) stored on ice to prevent ATP and GTP degradation. The presence of ATP and GTP in this solution was to prevent rundown of the cell, by providing energy for phosphorylation reactions and G-protein-mediated cellular functions. EGTA (ethylene glycol tetraacetic acid) was used in the pipette solution to buffer and prevent the build-up of potentially toxic levels of calcium during action potentials.

Due to the transfer of electrons between salt solutions and metal surfaces, solid-liquid junction potentials occur during patch-clamp recordings and are exacerbated by the difference in ionic concentrations between recording aCSF and intracellular solution. This phenomenon was attenuated through the use of silver-chloride pelleted reference electrodes and the coating of the pipette electrode with chloride by immersion in bleach. Using a bridge amplifier, any visible junction potential, typically ~13mV, could be offset to 0mV at the start of each recording to account for these differences.

To establish a tight-seal configuration, neurons were approached at 40x resolution, using DIC to improve visualisation of cell membranes. Positive pressure was applied to the
pipette prior to entry into the bath to avoid blockages. Cells were chosen according to visual inspection and approached with the application of 110pA positive step currents at 1s intervals until a visible indent could be seen in the membrane. A gigaohm seal was created through the release of positive pressure and could be visualised in Spike2 by the appearance of a large voltage deflection in phase with the timed step currents in accordance with Ohm’s laws (V=IR). Step currents were reduced to 10pA and whole-cell configuration established at a negative holding current potential of ~50mV by the application of manual suction via a mouthpiece attached to the pipette holder. Whole-cell configuration was confirmed by the appearance of action potentials and/or a sudden reduction in voltage deflections induced by the applied step-current.

Once break-in was confirmed, the holding current was slowly removed and neurons were left with zero applied current to observe their intrinsic behaviour. Input resistance was measured by the use of negative step currents of -10 and -20pA applied to the cell.

Data Analysis

Cell selection: SCN neurons are small, high input resistance neurons that show a broad range of electrophysiological behaviours (Belle et al., 2009; Kononenko and Dudek, 2004; Pennartz et al., 1998). Due to this nature and other considerations of whole-cell recordings, such as cellular rundown, SCN neurons can alter their behaviour during recording and the point at which to measure neuronal behaviour has been open to debate (Belle et al., 2009; Kostyuk, 1984; Schaap et al., 1999). To maintain objective analysis a defined set of criteria was used, in agreement with published methods from our group, to determine the intrinsic behaviour of the cell (Belle et al., 2009):

1. Following break-in, the cell must achieve a stable RMP, or in the case of regular firing neurons a stable centre of oscillation.
2. Cells must remain stable (ΔV<5mV) in the recorded state for 30s and achieve this state within 3 minutes of break-in.
3. The first stable behavioural state that the cell achieves for >30s is defined as the intrinsic behaviour of the neuron in question.
All single-cell recordings were analysed using Spike 2 software, version 6.2 (CED Systems, Cambridge, UK) and the following parameters measured:

Resting membrane potential: Calculated by using an in-built Spike2 script that quantifies the mean potential difference across all points at which the derivative of the phase-plane plot (ΔdV/dT) is <4% maximum; to exclude action potentials from the calculation.

Firing frequency: Action potentials from representative traces of 60 seconds were extracted as time-stamped waveforms with a low cut-off threshold (~10mV) and long duration (30ms) defined to include all waveforms for analysis using Spike2’s waveform average script. Waveforms were then exported as an inter-spike interval histogram from which firing frequency was calculated as 1/mean interval.

Coefficient of variation (CoV): Calculated as standard deviation of inter-spike interval/mean inter-spike interval.

Input resistance: Measured manually from the voltage deflections from applied -20pA step currents on the recording trace using Ohm’s law (ΔV/I) where ΔV = RMP/Peak of -20pA deflection.

Waveform average: Spike2’s waveform average function was used to generate a typical action potential (AP) waveform from all spikes included in a representative time window using the same parameters for inclusion (30ms, -10mV). From the mean waveform the afterhyperpolarisation size could be measured as the voltage difference between action potential threshold and peak of post-spike hyperpolarisation.

Currently, there is a lack of consensus in the literature on the definition of SCN neurons exclusively by their electrophysiological properties. In an attempt to roughly categorise sub-types of neurons, cells were classified based on their action potential firing properties:

1. Regular firing: Coefficient of variation (CoV) <0.25 and spontaneous firing rate (SFR) > 0.3Hz.
2. Irregular Firing: CoV > 0.25; SFR > 0.3Hz.
3. Hyperpolarised: CoV <0.25; SFR < 0.3Hz; Resting membrane potential (RMP) < -45mV
4. Depolarised: Resting membrane potential (RMP) >-35mV; No action potentials.

**Calcium Imaging**

Calcium imaging was performed using dual wavelength excitation of intact hypothalamic slices containing the mid-rostro-caudal SCN loaded with the membrane-permeable (acetoxymethyl ester) form of the calcium indicator dye Fura-2 (Bootman et al., 2013; Irwin and Allen, 2009).

Coronal hypothalamic slices for calcium imaging were prepared under the same conditions as described for electrophysiological recordings, except slices were cut at 200µm to increase the tissue’s transparency for fluorescent imaging. Following dissection, slices were loaded with Fura-2(AM) ratiometric calcium imaging dye (TEFLabs, Austin, USA) by incubation at 37°C for 15 minutes in recording aCSF at a concentration of 10µM; Fura-2(AM) pre-diluted in 50µl Pluronic F-127; 20% solution in DMSO (Molecular Probes, Life Technologies, USA) to aid solubility. Slices were further incubated for an hour at room temperature and left for 2 hours in continuously gassed recording aCSF (95% O₂; 5% CO₂) for de-esterification prior to imaging.

Calcium imaging was performed on an upright Olympus BX51 WI microscope mounted to a vibration-free air table (TMC 63-500 series, MA, USA). Slices were placed in a bath, suspended between lens and condenser, with unidirectional solution flow to allow constant perfusion of the tissue with oxygenated aCSF and secured with a platinum harp. Slices were left for an hour before the start of experiments to provide maximum stability during recording.

Image capture was performed using a water-immersion UV objective (UMplanFL N 20x/0.5 Olympus, Japan) and dual-excitation from two opto-LEDs (Cairn Research, Kent, UK) at 365nm – the isosbestic point of Fura-2’s dual emission spectra - and 385nm – a stable absorption wavelength for unbound fura-2 with pulses were emitted every 2s. Excitation emissions were filtered at 510nm and captured by a high-sensitivity QImaging Rolera EM C2 CCD camera (QImaging, Surrey, Canada) attached to the microscope’s video port. The high sensitivity of this camera reduced the intensity of light required for signal detection, optimised for long-term recording to reduce photo-bleaching of fluorophores.
The recording camera was connected to a PC running Optofluor Version 7.7.5.0 (Cairn Research, Kent, USA) imaging software, capturing separate 365 and 385nm images for offline analysis. Prior to each experiment, images were calibrated to optimise signal to noise ratio according to the strength of Fura-2 intensity, this was done by altering the exposure time of the camera shutter.

Drug applications were performed using a solenoid-valve controlled perfusion system. All drugs were bath applied and flow rate was controlled at the start of every experiment to 2.5mlmin\(^{-1}\). A minimum of five minutes was allowed for washouts between drug treatments although the length of washout varied according to observations of cellular responses.

**Data Analysis**

Data analysis was performed offline using Optofluor Version 7.7.5.0 software. From captured images (14-bit, 1.24x1.24 Megapixels), somatic regions of interest were manually drawn on to cells, identified by high concentrations of Fura-2 emission. The intensities of both 365nm and 385nm wavelengths for individual ROIs were then recorded as an average over the number of pixels within the ROI acquired at each image over time course of the experiment.

Background subtraction of all ROIs was performed by deduction of raw 365 and 385nm intensities from a defined background ROI, taken adjacent to the SCN. Under 20x magnification, subtraction of scattered background light, such as at the edge of the image is appropriate rather than proximal to the cell of interest – as used under higher resolution protocols (Bootman et al., 2013; O’Connor and Silver, 2007). Final Fura-2 ratios were produced by manually subtracting time-matched 365nm/385nm background intensities from the raw 365/385nm intensity values for each ROI across the duration of the experiment.

Subtracted ratios were exported in a time series to Origin Pro 9.0 (OriginLab, Northampton, USA) and plotted for visual inspection. Each neuron’s response to drugs was assessed by manually sampling 10 consecutive points of the ROI prior to drug application and 10 points during the observed peak response. Following drug application,
intracellular calcium responses were defined if the mean value of the peak response >2 standard deviations from the mean prior to drug application (Irwin and Allen, 2009, 2010). Peak response and washout times of each drug application were measured manually as the latency from application of the drug to the peak response and return to baseline.

**Bioluminescence Tissue Culture**

Bioluminescence recording of core clock gene oscillations was performed with male and female *Myk/+ x PER2::LUC, +/- x PER2::LUC* or WT C57BL6/J PER2::LUC SCN tissue explants. Explants of the mid-rostro-caudal SCN were prepared under the same conditions described for electrophysiology with certain alterations.

Ice-cold tissue culture media (Hanks Balanced Salt Solution; HBSS; NaHCO₃ supplemented, Sigma, UK) supplemented with 10mgml⁻¹ penicillin-streptomycin (Gibco Invitrogen, Paisley, UK) and 0.01M HEPES (Sigma, UK) was used to store and dissect tissues in place of aCSF. A manually-controlled vibratome (HA752; Campden Instruments, UK) was used to cut the slices to increase the speed of preparation as multiple brains were prepared at any one time.

SCN tissue explants were excised manually from hypothalamic slices using surgical scalpels under a dissecting microscope. Slices were dissected approximately half way between the dorsal tip of the third ventricle and SCN, and at the distal ends of the lateral anterior hypothalamic nuclei.

The proceeding steps were all performed under sterile conditions in a cell culture hood (Astec Microflow, Hampshire, UK). Explants were transferred on to permeable 30mm, 0.4µM PTFE inserts (Millipore, Watford, UK) inside 35mm culture dishes (Corning, Tewksbury, USA) that contained luciferin-supplemented, sterile neuronal culture media; Dulbecco’s Modified Eagle Medium (D-2909 Sigma, Gillingham, UK) 3.5g/L D-glucose (Sigma) 0.035% NaHCO₃ (Sigma) 0.1M HEPES (Sigma) B27 serum-free media (Gibco Invitrogen, UK), 0.1mM beetle luciferin potassium salt (Promega, Southampton, UK). The edge of each dish was lined with autoclaved high vacuum grease (Dow Corning Ltd,
Coventry, UK) and dishes immediately sealed with a UV-treated borosilicate glass, 0.13mm coverslip (VWR, Lutterworth, UK).

Sealed culture dishes were placed inside light-tight incubators maintained at 37°C and 5% CO₂. Bioluminescence was recorded by photon-multiplier tube assemblies (H8259/R7518P, Hamamatsu, Welwyn Garden City, UK) encased within steel chambers to reduce background noise. Total photon counts were recorded in 5-minute intervals and stored as a time-series .txt file on PMTMonTL software (Hamamatsu, UK).

For the application of drugs, cultures were left incubating for 96 hours prior to treatment. During application, tissues were transferred to a cell culture hood where fresh 35mm dishes containing the drug or vehicle were pre-prepared. Each SCN explant resting on PTFE inserts was transferred into the fresh 35mm dishes containing drug or vehicle and sealed with vacuum grease and a fresh UV-treated cover slip. Cultures were immediately placed back within the incubator and left undisturbed for the remainder of the experiment.

**Data Analysis**

Raw bioluminescence values were automatically recorded as a time-series in 5-minute intervals. Data was transformed in Microsoft Excel by subtracting a 24-hour moving average from individual time-points to de-trend the data. This produces a data set oscillating around y=0 to allow comparison of waveform parameters. Data sets were then subjected to smoothing over a 3-hour interval when curve-fitting was performed.

Smoothed time-series were exported to GraphPad prism and the following parameters derived manually and from a non-linear regression with the function:

\[Y = \text{Amplitude} \times \exp(-K \times X) \times \sin((2\pi \times X)/\text{Wavelength}) + \text{PhaseShift}\]

This equation is simply a sine function with K acting as a decay constant describing the rate at which the oscillation damps over time. Estimated parameters from this regression were:

- **Period:** The duration of a single circadian PER2::LUC oscillation (wavelength constrained at 18 hours)
- Amplitude: The distance from y=0 of the peak of the first cycle.
- Half-life: The time taken for the amplitude of the first cycle to reduce by 50%.

Parameters were also estimated by eye using y intercepts as phase markers. Final values presented are the mean value of both fitted and manually scored values. From the manual method, parameters estimated were:

- Period: The mean duration of all y intercepts greater than 18 hours apart.
- Amplitude: The height of the estimated peak of the first oscillation from y=0.
- Phase: The time at which the oscillation first intercepts the y axis. To assess phase accurately, the same portion of the waveform was always used for direct comparison.

To compare the effect of drug treatments, parameters were calculated both before and after application of the drug. The first 12-hours following drug application were left out of the analysis due to the transient peaks of bioluminescence due to the process of treating cultures.
References


Chapter 1

*Myshkin* mice exhibit disruption to circadian, metabolic and light-dependent behaviours.
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Introduction

Rhythmicity and the temporal segregation of behaviour and physiology within distinct daily epochs is a feature of the biological composition of nearly all species on earth. In mammals, intrinsic daily rhythms are driven by a hierarchical system of single-celled oscillators that manifest across a broad range of tissues including the liver, retina, skeletal muscle and the central nervous system (Mohawk et al., 2012). These oscillations, known as circadian rhythms, are co-ordinated by a master pacemaker that reside within the hypothalamus of the CNS, the suprachiasmatic nuclei (SCN; Welsh et al., 2010). These densely packed structures synchronise cellular and tissue-level rhythms across the brain and body into a coherent temporal relationship. The SCN also act as an interface between external and internal time, receiving and responding to environmental stimuli, predominantly light and arousal signals (Golombek and Rosenstein, 2010).

The near 24-hour biological oscillations that arise within single cells of the circadian system are driven by a well-characterised genetic transcription-translation feedback loop (TTFL) (Partch et al., 2014). The major components of the TTFL are the heterodimeric partners, CLOCK and BMAL1, that together promote the expression of PERIOD1,2,3 (PER) and CRYPTOCHROME1,2 (CRY), which function as the transcriptional repressors of the TTFL (Bae et al., 2001; Bunger et al., 2000; Gekakis et al., 1998; Vitaterna et al., 1999). Following dimerisation the PER-CRY complex returns to the nucleus to inhibit Clock and Bmal1 and complete the transcriptional cycle. Various subsidiary components contribute to the timing of this genetic clock including through modulation of transcription; Rev-erba/β and Rora/β (Cho et al., 2012; Guillaumond et al., 2005); phosphorylation of core clock proteins; Casein Kinase 1ε/δ, Glycogen synthase kinase 3 (Iitaka et al., 2005; Pilorz et al., 2014); chromatin re-modelling; Sirtuin1 (Chang and Guarente, 2013; Nakahata et al., 2009; Ramsey et al., 2009) and protein degradation; Fbxl3 (Godinho et al., 2007).
timed interaction of these different clock factors result in a near 24-hour oscillation and provides a temporal control of cellular transcriptomes across the brain and body (Miller et al., 2007; Panda et al., 2002a).

Within the SCN, isolated neurons can maintain intrinsic molecular rhythms and electrical firing patterns, thus acting as self-sustaining oscillators (Webb et al., 2009; Welsh et al., 1995). To generate coherent tissue-level oscillations however, single neurons rely on locally-derived cues to re-enforce their rhythm stability and maintain a coherent phase relationship with other neurons across the SCN (Brown and Piggins, 2007; Liu et al., 2007; Nakamura et al., 2002). These network properties arise through a variety of synaptic and paracrine network interactions, including rhythmic and inducible neuropeptide release (VIP, AVP, GRP; Brown et al., 2005; Maywood et al., 2011; Yamaguchi et al., 2013), endogenously synthesised GABA (Evans et al., 2013; Freeman et al., 2013) and afferent neuropeptide/neurotransmitter release (Glutamate, NPY, GABA, orexin) (Belle et al., 2014; Besing et al., 2012).

Direct disruption of the core molecular clockwork or coupling factors that maintain accurate timekeeping can have profound effects on behaviour and physiology. Mutations in key clock genes such as ClockΔ19 and Per2Brdm mice or targeted knockout of neuropeptides signalling including Vip−/− and Vip2r−/− mice induce major disruptions to circadian wheel-running behaviour (Aton et al., 2005; Hughes and Piggins, 2008; Vitaterna et al., 1994; Zheng et al., 1999). Many of these clock-disrupted strains also exhibit severe pathophysiological abnormalities with particularly strong associations between molecular clock disruption, metabolic syndrome and tumorogenesis (Fu et al., 2002; Kondratov et al., 2006; Takahashi et al., 2008; Turek et al., 2005). Observations across many of these rodent lines, most notably ClockΔ19 and Rev-erba−/− mice, have revealed a pervasive association between disruption to circadian timing and neuropsychiatric disorders (Chung et al., 2014; Foster et al., 2013; Landgraf et al., 2014; Roybal et al., 2007). These demonstrations in laboratory animal models come alongside decades of research that highlight changes to sleep-wake and circadian cycles in patients with major affective disorders including schizophrenia, major depressive disorder (MDD) and what will remain the focus of this study, bipolar disorder (BPD; Gonzalez, 2014; McCarthy and Welsh, 2012; McClung, 2007).
Bipolar disorder (BPD) is a neuropsychiatric condition that has a profound pathophysiological association with sleep and circadian rhythm disruption (Gonzalez, 2014; Harvey, 2008). BPD is affects an estimated 0.5-1.5% of the global population and is placed within the top ten of the world’s most debilitating non-communicable diseases (Craddock and Sklar, 2013; Lozano et al., 2012; Wittchen et al., 2011). From the first appearance of the disease, BPD patients cycle between long-term episodes of mania and depression interspersed with states of euthymia, defined as clinically normal mood-state (DSM-V, 2013; ICD-10, 2014; Phillips and Kupfer, 2013). Episodes of mania specifically are defined by heightened mood-related behaviour including; hyperactivity, a reduced need for sleep, increased self-esteem and elevated reward-seeking behaviour.

Changes to underlying sleep and circadian rhythms have been long-recognised as a clinical feature of BPD, with sleep disruption a key marker in the aetiology and pathology of both manic and depressive episodes (Harvey, 2008). Various changes to the phase and stability of sleep and activity patterns are often observed in BPD patients including during euthymia and sleep-wake disturbances often precede the onset of episodes (Brietzke et al., 2012; Jones et al., 2005; Rock et al., 2014; Wood et al., 2009; Zeschel et al., 2013). In humans further evidence has emerged from genetic studies highlighting various clock gene SNPs such as Clock, NPAS2, Arntl1 and Per2 as risk-factors within BPD patient populations (Etain et al., 2011). Therapeutic intervention within of mood disorders also targets the circadian system via chronotherapeutics, the manipulation of sleep and zeitgeber cues, to stabilise mood symptoms (Benedetti, 2012). Pharmacologically the circadian system is also profoundly affected by mood stabilisers used in the treatment of BPD, such as lithium and valproic acid (Johansson et al., 2011; Li et al., 2012). Despite consistent genetic and clinical associations, it remains unclear how alterations to the circadian system may be involved in the development or pathophysiology of bipolar disorder and their upstream regulation of mood-related behaviours.

The appearance of mania-like phenotypes in numerous animal models with targeted mutations, knockdown or knockout of core clock genes has allowed some of these mechanisms to be dissected (Landgraf et al., 2014). The well-studied ClockΔ19 mouse displays a variety of mania-like phenotypes, including hyperlocomotion, increased reward-seeking behaviour and reductions in sleep (Roybal et al., 2007). In the ClockΔ19
model these behavioural changes are heavily regulated through mesolimbic dopaminergic structures, namely the VTA. ClockΔ19 animals exhibit altered dopamine release, increased burst-firing of dopamine neurons and altered coupling between the VTA and nucleus accumbens (NAcc) (Coque et al., 2011; Dzierza et al., 2011).

Recently Rev-erbα−/− knockout mice were described to possess mania-like behavioural traits and attenuation in the rhythmic expression of mood behaviours. These behavioural alterations were similarly ascribed to changes in the rhythmic regulation of dopamine synthesis within VTA also, via interactions of Rev-erbα with tyrosine hydroxylase (Chung et al., 2014). These intriguing studies have begun to reveal a circadian regulation of mesolimbic reward systems that impact directly on to mania-like behaviour. Various other clock gene models are also reported to display mania-like phenotypes include Afterhours (Fbxl3) (Keers et al., 2012), Per2−/− mutant (Hampp et al., 2008) and Bmal 1−/− knockout mice (Kondratova et al., 2010; Leliavski et al., 2014).

In another recent study mice with a mutation in the neuron-specific α3 subunit of the Na+/K+-ATPase (NKA), known as Myshkin (Myk/+), were shown to have a variety of mania-like behavioural phenotypes (Clapcote et al., 2009; Kirshenbaum et al., 2011). These traits included hyperlocomotion, reduced anxiety, reduced sleep and elevated reward-seeking behaviour that were responsive to the mood stabilisers lithium carbonate and valproic acid. Interestingly, these mice also showed alterations to free-running rhythms that suggested changes to the intrinsic circadian system. Unlike other clock gene models of mania-like behaviour such as ClockΔ19, Rev-erbα and Afterhours, the NKA α3 subunit is not recognised as an integral part of the core molecular circadian oscillator. The Myk/+ mutant thus represents a model of BPD in which to study change to the circadian system may arise without direct molecular lesions within core clock genes.

Using an in vivo approach, we have characterised the circadian and metabolic profile behaviour of Myk/+ mutants, including entrainment, free-running, phase res-setting, negative masking and ingestion behaviour. Myk/+ animals displayed a broad range of abnormal circadian behaviours characterised by a continuum of labile phenotypes. Abnormal circadian traits included long tau, long alpha duration, unstable free-running rhythms, reduced behavioural masking by light and altered phase re-setting to photic
stimuli. Myk/+ mutants also displayed a range of metabolic abnormalities including an elevated basal metabolic rate, increased light-phase ingestion behaviour and reduced overall feeding. Myk/+ mice therefore exhibit an array of behaviours that suggest severe disruption to central circadian time-keeping and metabolic regulation. These behaviours also present interesting phenotypic similarities with other reported clock gene models of mania, namely ClockΔ19.
Methods

Animal breeding and housing: Myk/+ animals were originally generated through an ENU mutagenesis screen and backcrossed on to a seizure-resistant C57BL6/NCr for 20 generations. From this background, a Myk/+ breeding colony was generously provided by Dr Steve Clapcote (University of Leeds) and maintained on the C57BL6/NCr background. Male and female +/+ and Myk/+ mice (aged 2-6 months) were group-housed in a 12:12 LD cycle at constant temperature (~18°C) and humidity (~40%) with food (standard lab chow) and water provided ad libitum. All animal protocols were in accordance with guidelines of the UK Animal (Scientific Procedure) Act 1986.

Wheel-Running Behavioural Characterisation: Male and female +/+ (n=37) and Myk/+ (n=38) mice were individually housed in plastic-based cage-bases (410 x 247 x 124mm) equipped with 160mm lined steel running wheels with food and water provided ad libitum. Animals were initially exposed to a 12:12 LD cycle (>250 lux) for a minimum of 14 days before release into DD >14 days to assess free-running rhythms. For characterisation of wheel-running behaviour under different photoperiod +/+ (n=5) and Myk/+ (n=4) animals were also maintained for 14 days in a 16:8 LD cycle.

Phase shifting protocol: Animals were exposed to either an Aschoff type I (+/+ =18; Myk/+ =15) or a type II light phase shifting protocol (+/+ =11; Myk/+ =11). Under type I conditions animals were allowed to free-run in DD for 14 days prior to receiving a light pulse. On the day of the pulse, CT12 for each animal was predicted by fitting a regression line to a wheel-running actogram. At the predicted circadian time (CT; CT6, CT14, CT20, CT23) animals were carefully transferred to an adjacent room with the lights on (>250 lux) for one hour before return to home cages. For type II pulses, animals were released into DD and the lights within cabinets turned on for one hour at the appropriate CT, calculated relative ZT0 under 12:12 conditions, after one full circadian cycle.

Re-entrainment protocol: To assess the speed of re-entrainment to a new light-dark cycle, male and female (+/+ =5; Myk/+ =5) were individually housed in a 12:12 LD cycle for 14 days. After 14 days the light dark was advance 8 hours, with lights coming on at ZT16 relative to the previous LD cycle. Animals were maintained in a 12:12 LD cycle at the new
phase for a further 14 days before the LD cycle was delayed for 8 hours. To delay the light cycle lights remained on for 8 hours into the dark phase of the previous regime. The new 12:12 LD cycle was again maintained for 14 days.

**Masking protocol:** To assess the negative effects of masking on animal behaviour, after 14 days under a 12:12 LD cycle were given a 1-hour light-pulse without physical disturbance at either ZT14 or ZT20 (+/+: n=18; Myk/+: n=23). In addition a cohort of +/+ and Myk/+ animals were exposed to an 8 hour light pulse in which lights were turned on between ZT16 – ZT24.

**Data Analysis:** Data was collected in 5-minute time bins on a personal computer running Chronobiology Kit software (Stanford Software Systems, Santa Cruz, CA). Data and statistical analysis was performed in Kit Analyze version 1.06 (Stanford Software Systems, Santa Cruz, CA), Prism6 (GraphPad Software, La Jolla, USA) or Microsoft Excel 2010 (Microsoft, USA). Period was calculated as a mean of chi-squared periodogram calculated in Kit Analyze and eye-fit regression lines. Chi-squared periodogram was also used to provide a measure of rhythm amplitude and was applied to 240-hour (10-day) epochs and converted to percentage variance. Alpha duration was calculated manually using major circadian onset and offset bouts of activity as markers to fit parallel regression lines over stable 10-day epochs. Phase-shifts were calculated by manually fitting regression lines to a minimum of 7-day epochs pre and post light-pulse. Magnitude of phase shift was then recorded as the difference between regression lines on the day following the pulse. Phase shifts were independently verified by other experienced circadian researchers. Re-entrainment was assessed manually as the number of days to fully entrain both onset and offset of activity to the new light phase. Genotype comparisons were made using t-test, one-way anova or non-parametric equivalents where necessary.

**Feeding, drinking and metabolic activity assessment:** Male +/- and Myk/+ animals (+/+=10; Myk/+ =12) were individually housed in sealed Complete Laboratory Animals Monitoring System (CLAMS, Columbus Instruments, Ohio, USA) perspex metabolic cages for 7 days and maintained under a 12:12 LD cycle at constant temperature (~18°C) and humidity (~40%). Due to restrictions to animal husbandry practised, metabolic activity could not be assessed under DD conditions. Metabolic cages were connected through an
open-circuit gas flow system provided with a known concentration of oxygen and carbon dioxide to allow constant, indirect calorimetric assessment. Standard lab chow was provided at an open access food hopper with an infra-red beam across the opening. Feeding behaviour was recorded as number of beam breaks. Water was provided *ad libitum* through a plastic water bottle in the roof of the cage with a metallic sipper. When drinking, a small current passed through the mouse and the conductive steel-based floor and recorded on a personal computer. All activity was recorded in 10-minute bins for analysis.

*Data Analysis:* All data was recorded directly by a PC running CLAMS custom software and exported to Microsoft Excel (USA) and Prism6 (USA) for analysis. VO₂ rhythms were smoothed over 3-hour epochs to allow analysis of trends in the data where necessary due to the inherent noise in calorimetric data, otherwise raw values were used. T-tests and non-parametric equivalents were used to compare genotype groups.
Results

*Myk/+ animals show normal entrainment under a 12:12 LD cycle but increased light-phase activity:* To characterise the *Myk/+* behavioural phenotype, mice were initially housed for a minimum of 14 days under a 12:12 LD cycle in cages equipped with stainless steel running-wheels. Under LD conditions, the majority of *Myk/+* (35 of 38; 92%) mice entrained to the lighting schedule, with activity bouts initiating at the start of the dark phase as observed in *+/+* animals (Fig 1A,B). Occasionally *Myk/+* animals exhibited poor entrainment with unstable activity bouts that were phase advanced (between 1-4h) of lights-off (Fig 1C). The majority of other *Myk/+* behavioural phenotypes under 12:12 conditions were comparable to *+/+* including the amplitude of rhythmic behaviour (Fig 1D).

The LD actograms of *Myk/+* animals consistently appeared different to *+/+* in the amount of activity spent in the light phase. This was visible by a continuation of wheel-running activity at ZT0, the beginning of the light phase (Fig 1B,C). At ZT0 *+/+* locomotor behaviour was typically suppressed with little visible wheel-running activity. *Myk/+* animals consistently displayed a characteristic tail in their activity bout that continued past ZT0 and into lights-on. When quantified, the relative amount of daily wheel-running activity in the light phase for *Myk/+* animals was more than double that observed in *+/+* mice (Fig 1G). This propensity was also clear in the duration of daily activity bouts, known as alpha, that was over 2 hours longer in *Myk/+* animals due to the continuation of wheel-running activity past ZT0 (Fig 1F).

*Absence of locomotor suppression and poor entrainment in Myk/+ animals under 16:8 conditions:* Due to heightened light-time activity observed under a 12:12 LD cycle, a sub-group of *Myk/+* mice were exposed to long day conditions (16h light:8h dark). Under this lighting schedule *Myk/+* animals exhibited highly abnormal behaviour. All *+/+* animals showed clear entrainment and compression of nearly all (>99%) wheel-running activity to the dark phase (Fig 2A,B). *Myk/+* animals were instead characterised by poor entrainment to the 16:8 schedule and high variability in their daily onset and offset of activity. 50% of animals demonstrated particularly unstable behavioural rhythms in this lighting paradigm (Fig 2C-E). In addition all *Myk/+* animals demonstrated high levels of activity during the
light phase with greater than 40% of wheel revolutions occurring during lights-on relative to less than 1% observed in +/+ (Fig 2F). Although not approaching statistical significance there was a clear trend in Myk/+ animals to express lower amplitude behavioural rhythms under 16:8 (Fig 2G). These data suggest that Myk/+ animals capacity to entrain and consolidate activity to the dark phases of an LD cycle is decreased in-line with lengthening photoperiod.

Figure 1: Myk/+ animals show increased activity during lights on in a 12:12 LD cycle; A; Example of +/+ animal entrained to a 12:12 LD cycle, the majority of wheel-running activity ends prior to lights-on. B: Example of Myk/+ animal under 12:12 conditions. Myk/+ animals show clear entrainment to the LD cycle, but with a tendency for wheel-running activity to continue into the light phase, highlighted by the red boxes in A-C. C: Occasionally Myk/+ animals do not entrain to a 12:12 LD cycle and show unstable onset of activity in advance of ZT12. D: +/+ and Myk/+ animals show similar rhythm amplitude (+/+: 38.38 ±2.4%; Myk/+: 42.32 ± 2.9%; p=0.30), as measured by chi-squared periodogram, E: Myk/+ animals show longer activity bouts than +/+ under 12:12 conditions (+/+: 11.95 ± 0.12h; Myk/+: 14.07 ± 0.32h; p<0.0001) F: Myk/+ animals spend a higher proportion of their total wheel-running activity during the light phase relative to +/+ animals (+/+: 5.8 ± 1.2%; Myk/+: 12.7 ± 2.7%; p<0.05).
Figure 2: *Myk/+* animals show poor entrainment and high light-phase activity under a 16:8 LD cycle: A: Example of +/+ actogram under 16:8 photoperiod. Entrainment to the beginning of the dark phase is clear and the vast majority of locomotor behaviour is constrained within the dark phase. B: Rhythm profile plot of +/+ animal in panel A, nearly all activity is within the dark phase. C: Example of *Myk/+* animal in 16:8 LD cycle characterised by poor entrainment, unstable locomotor rhythms and high light-time activity, particularly in the early subjective day (ZT0-6). D: Rhythm profile plot of animal from actogram C. Activity is poorly consolidated within the dark phase. E: Example of *Myk/+* animal under 16:8 LD cycle. This animal shows more stable wheel-running rhythm than panel C but again much less stable than +/+. Rhythm onsets are phase-advanced of lights-off with high subjective day activity. Most light-time activity is again towards the end of alpha bouts (ZT0-6). F: Percentage of wheel-running activity during lights-on. *Myk/+* animals spend a much greater proportion of their daily wheel-running activity during the light phase (+/+; 0.80 ± 0.42%; *Myk/+*; 41.76 ± 10.94%; *p*=0.016). G: Rhythm amplitude in 16:8 photoperiod. *Myk/+* animals show a non-significant trend towards lower amplitude rhythms (+/+; 53.06 ± 10.59%; *Myk/+*; 34.07 ± 9.14%; *p*=0.23).
**Impaired negative masking in Myk/+ mutants:** Due to increased light-phase activity in both 12:12 and 16:8 LD cycles, the role of behavioural masking by light was assessed by quantifying wheel-running activity during epochs of light exposure. One-hour light pulses were applied to both cohorts maintained in a 12:12 LD cycle either at ZT14 or ZT20, the early or late subjective night (Fig 3A-B). To assess the suppressive effects of light over a longer time course, animals were also exposed to an 8-hour pulse of light between ZT16-24 (Fig 3C). Under both short and long-term exposure Myk/+ animals demonstrated a visible absence of light-induced inhibition of locomotor activity. Wheel revolutions during lights on were increased in Myk/+ animals both in total amount of locomotor output (+/+; 45 ± 13 revs hr⁻¹; Myk/+; 238 ± 62 rev hr⁻¹; p=0.005) and normalised wheel running activity (Fig 3D-E). It should be noted that there was a continuum of responses in Myk/+ animals in the relative amount of negative masking observed. Some animals did exhibit strong reductions in behaviour akin to +/+ responses, while other Myk/+ examples displayed increased activity in response to light. These data demonstrate that increased light phase activity in Myk/+ animals is directly influenced by changes to the mechanisms of negative masking.

**Myk/+ mice display abnormal and unstable free-running rhythms:** Following entrainment to 12:12 LD cycle, animals were allowed to free-run in constant dark conditions for a minimum of 14 days. A previous investigation (Kirshenbaum et al., 2011) reported that Myk/+ animals exhibit a lengthened period and long alpha duration under circadian conditions. These phenotypes were again apparent, with elongation of tau observed in Myk/+ mice (+/+; 23.72 ± 0.03h; Myk/+; 24.22 ± 0.06; p<0.0001) (Fig 4A-D). Alpha duration in Myk/+ animals was also greatly increased, with actograms characterised by long, consolidated bouts of activity over the circadian cycle (+/+; 13.14 ± 0.26h; Myk/+; 18.99 ± 0.37; p<0.0001; Fig 4E).
Figure 3: Myk/+ animals show deficits in the negative masking effects of light: A: Example of +/+ and Myk/+ animal receiving a one hour light pulse – indicate by red box – at ZT14 (+/+: n=11; Myk/+: n=13) and (B) ZT20 (+/+: n=7; Myk/+: n=10). C: Example of +/+ (n=5) and Myk/+ (n=4) animal receiving 8h of light from ZT16-24. Note in all example the increased activity of Myk/+ animals in each lighting condition in which the locomotor activity of +/+ mice is rapidly suppressed. D: Wheel revolutions per hour of +/+ and Myk/+ animals during lights-on across all paradigms A-C (+/+: 45 ± 13 revs hr⁻¹; Myk/+: 238 ± 62; Mann-Whitney; p=0.005). E: Relative wheel revolutions during masking pulses, normalised in each animal by mean wheel revs hr⁻¹ (+/+: 0.13 ± 0.05 relative wheel revs; Myk/+: 0.52 ± 0.12 relative wheel revs; Mann-Whitney; p=0.002).
Previous report suggested Myk/+ animals possessed an intrinsic free-running tau of 25 hours and alpha duration of less than 15 hours. Our data suggests quite different behavioural parameters with a marked reduction in period yet much longer alpha duration in our Myk/+ cohort.

Under free-running conditions Myk/+ animals exhibited further free-running behavioural deficits not previously reported. Myk/+ animals exhibited a consistent reduction in free-running amplitude; a direct measure of the strength of the circadian system’s output (Fig 4F). Decreases in rhythm amplitude were evident across a continuum of phenotypes with different degrees of stability in behavioural onset and offsets (Fig 4B,C). Some animals showed very poor rhythm consolidation that broke down into very weak or arrhythmic behaviour (Fig 4C). Interestingly a sub-group of Myk/+ animals demonstrated stronger free-running rhythms (7 of 38; 18%) than the majority of the littermates. Observations of the data revealed a relationship of the rhythm strength with the length of alpha. This association was apparent in both +/+ and Myk/+ cohorts in which alpha inversely correlated with rhythm amplitude (Fig 4G,H). In Myk/+ animal this inverse relationship was stronger and a clear bi-modal distribution of animals was apparent; the sub-group of Myk/+ animals with high amplitude behavioural rhythms also expressed short alpha bouts. Conversely animals with longer activity durations formed a separate population with lower rhythm strength. It appears therefore that a relationship exists in Myk/+ animals between alpha length and rhythm amplitude, with shorter bouts of activity beneficial in maintaining stable free-running rhythms.

Another trait of Myk/+ animals was reductions in the intensity of wheel-running activity over time (Fig 4I). When wheel-running activity across day 1-5 in DD was compared to day 10-14, Myk/+ animals demonstrated a typical 33% decrease in total wheel revolutions, whereas +/+ animals show little change (5% increase). The intrinsic drive on psychomotor output in Myk/+ animals therefore appears to dissipate over time in wheel-running cages.
Figure 4: Myk/+ animals show abnormal and low-amplitude free-running behaviour in DD; A: Example of +/+ animal released from a 12:12 LD cycle into DD for 20 days with a typical period <24h and typical alpha duration. B: Example Myk/+ actogram. Upon release into DD the animal runs with a long tau, very long alpha and relatively stable onsets of activity. C: The Myk/+ phenotype is variable with some animals becoming arrhythmic and showing very long alpha duration once released into DD. D: Myk/+ animals show long free-running period in DD (+/++; 23.72 ± 0.03h; Myk/++; 24.22 ±0.06h; p<0.0001); E: Alpha duration is greatly lengthened in Myk/+ animals upon release into DD (+/++; 13.14 ± 0.26h; Myk/++; 18.99 ± 0.37h; p<0.0001). F: Myk/+ animals show reduced amplitude free-running rhythms, suggesting that Myk/+ behavioural rhythms are less stable than +/+. Note that a sub-group of Myk/+ animals show higher amplitude rhythms than the majority of animals; (+/++; 43.8 ± 3.1%; Myk/++; 27.73 ± 2.4%; p=0.001) G-H: Alpha duration negatively correlates with free-running amplitude in +/+ (G; R²: 0.28; p=0.002) and with a stronger relationship Myk/+ animals (H; R²: 0.66; p<0.0001). Myk/+ animals with long alpha duration consistently show lower amplitude rhythms than those with short alpha duration. I: Change in wheel-running intensity between day 1-5 and day 6-10 in DD. Myk/+ animals show a large decrease in wheel-running activity that is absent in +/+ animals (+/++; 1.04 ± 0.07 fold change; Myk/++; 0.67 ± 0.06 fold change; p=0.0002).
An unusual trait evident in Myk/+ animals was a propensity to exhibit rapid alterations in tau under free-running conditions (Fig 5). When presented with an arousal promoting stimulus such as changing the base of a cage, or a light pulse, some individuals displayed immediate or delayed changes to their intrinsic free-running period (Fig 5A,B). This behaviour was even observed seemingly spontaneously in some animals (Fig 5C,D). These observations suggest that the Myk/+ SCN is less stable in maintaining its pacemaking properties and possesses a heightened sensitivity to external perturbation.

Figure 5: Myk/+ animals display alterations in tau under DD: A-C: Actograms of Myk/+ animals displaying changes in tau under DD conditions A: 2 days after a one hour light pulse (white box), the Myk/+ animal displays a change in period from 24.11h to 22.99h B: Example of Myk/+ animal showing a change in tau following a non-photic stimulus. After the animal’s cage base was replaced (white box), a large increase in activity is seen followed by a change in tau from 24.16h to 23.60h. C: Example of “spontaneous” tau change. Left undisturbed the Myk/+ animal shows an alteration in tau from 23.59h to 24.61h. This seemingly spontaneous change is also accompanied by a phase shift so an unspecified stimulus could have driven this example. D: Time-matched actogram of +/- animal during the same experiment as panel C that displays no change in free-running behaviour. The red box indicates the day at which tau appears to spontaneously change in the Myk/+ animal.
Fast re-entrainment to phase shifts of the LD cycle: One of the major features of the circadian system in relation to health and disease is the capacity to re-align to changes in the LD cycle that simulate jet-lag and social work. To examine this property in Myk/+ animals were exposed to sudden 8 hour shifts in the phase of LD cycle. Following advances of the LD cycle Myk/+ animals exhibited faster re-entrainment than +/- cohorts (Fig 6A-C). Some animals displayed immediate re-entrainment to the new phase of lights-off and all Myk/+ animals demonstrated large initial shifts in the onset of activity on day one of the new LD regime (Fig 6D). Due to a low sample size this data requires further validation, although the average number of days to re-entrain was significantly reduced in Myk/+ animals (+/+; 5.25 ± 0.75; Myk/+; 2.60 ± 0.51; p=0.02).

Following delays to the LD cycle major differences were not evident in re-setting responses of +/- and Myk/+ genotypes. It should be noted that assessment of Myk/+ actograms under the delayed paradigm proved difficult. Due to lengthened alpha durations the end of the active phase could often not be used to determine the speed of re-entrainment as it can in +/- animals (Fig 6A-C). These data on the re-entrainment properties of Myk/+ animals suggest a change in the physiological mechanisms governing normal speed of adjustment to advances in the LD cycle. Interestingly this property is apparent exclusively during advances of the LD cycle and highlights potentially distinct mechanisms in the direction of behavioural responses to jet lag protocols.

Altered phase re-setting responses to light: In addition to the generation and maintenance of stable rhythms, the SCN must integrate and respond to environmental cues to maintain synchrony between internal and external time. To assess the response of the Myk/+ SCN to the major circadian zeitgeber light, animals were exposed to a one hour light pulse at different times across the circadian cycle. +/- and Myk/+ animals received light pulses at CT14, CT20 and CT23 using an Aschoff type I protocol.
Figure 6: Myk/+ animals re-entrainment to advances of the light-dark cycle at a faster rate than +/-: 

A: Example actogram of +/- animal following an 8h advance followed by 8h delay in the LD cycle. Red boxes indicate calculated day of full re-entrainment. 

B-C: Example actograms of Myk/+ animals that exhibit very fast re-entrainment of activity to LD cycle advances and gradual re-alignment to delays. 

D: Mean phase shift of all +/- (n=5) and Myk/+ (n=5) animals to an 8h phase advance of the LD cycle. Myk/+ animals show characteristically faster re-alignment of their activity rhythms with the new lighting conditions. Behavioural onsets were used as phase markers in these calculations. 

E: Mean number of days to re-entrain following 8h shifts in LD cycles. Myk/+ animals exhibit significantly faster re-entrainment to 8h phase advances (+/+: 5.25 ± 0.75 days; Myk/+: 2.6 ± 0.51 days; p=0.02). +/- and Myk/+ animals exhibit similar phase re-entrainment to 8h delays of the LD cycle.
and Myk/+ animals exhibited phase delays of 1-3h in wheel running activity during the early subjective night (CT14). The average magnitude of phase shift was greater in Myk/+ animals relative to congenic littermates (+/++; -1.63 ± 0.14h; Myk/+; -2.28 ± 0.09; Fig 7A-E). At CT20, towards the end of the subjective night, +/+ animals demonstrated slight phase advances in response to light pulses, appearing to transition into the advance portion of a putative PRC (Fig 7F). In the Myk/+ cohort however, 5 of 6 animals persisted in responding with phase delays in response to the light pulse (Fig 7G), ranging from 0.8 to 2.7h.

In attempts to identify if Myk/+ mice exhibit phase advances to light later into the subjective night, animals received one-hour pulses at the end of the subjective night (CT23). +/- animals responded with small phase advances of 0.56h and were still within the advance portion of their putative PRC. At CT23 one third (2 of 6) Myk/+ animals also showed phase advances up to 1.1h in magnitude. Half of the tested Myk/+ cohort displayed minimal phase re-setting response and one animal maintaining a small phase delay of 0.22h. Due to the different response directions in the Myk/+ at this circadian time, these data suggest that the Myk/+ phase re-setting response is a transition point moving towards phase advance responses. Overall the type I phase re-setting responses observed in Myk/+ animals revealed a putative PRC with distinct characteristics; exhibiting a lengthened delay portion with phase advances occurring later into the circadian night than observed in +/- animals.

This atypical pattern of phase re-setting to light was tested in a separate cohort of animals using an Aschoff type II protocol. Both genotype groups were given one-hour pulses at both CT14 and CT20 on the second behavioural cycle after release into DD. The phase re-setting responses observed using a type II protocol resembled the shape previously demonstrated utilising a type I method (Fig 8A,B).
Figure 7: Abnormal phase re-setting response to light in Myk/+ animals in Aschoff type I protocol: A: Phase shift response of individual +/+ and Myk/+ animals in response to 1h light pulse following 14 days in DD. Note the clear difference in responses at CT20 B: Mean phase shifts of +/+ animals (CT 14; -1.63 ± 0.14h; CT20; 0.49 ± 0.11h; CT23; +/++; 0.56 ± 0.07h; ANOVA p<0.0001; Tukey post-hoc; ****p<0.0001) C: Myk/+ mean phase shifts; Myk/+; CT14; -2.28 ± 0.09h; CT20; -1.4 ± 0.42h; CT23; 0.21 ± 0.23; ANOVA; p=0.0009; Tukey post-hoc; *p<0.05; ***p<0.001). D: Example phase shift actograms of +/+ (left, blue line) and E: Myk/+ (right, red line) animals to type I light pulse at CT14. Myk/+ animals show 0.65h larger phase delays relative to +/+. F: Example phase shift actograms of +/+ (left, blue line) and G: Myk/+ (right, red line) to light pulse at CT20. At this time +/+ animals respond to light with small phase advances whereas Myk/+ animals show phase delays of variable magnitude.
At CT14 both +/+ and Myk/+ animals demonstrated phase delays in response to light, with slight increased magnitude in the response of the Myk/+ cohort; 0.70h greater than +/+ (Fig 8C,D). At CT20 +/+ animals exhibited small phase advances of 0.76 hours (± 0.08) (Fig 8B,E), similar to observations in type I conditions. At the same phase of the later subjective night, 80% (4 of 5) Myk/+ mice maintained phase delay responses at this portion of the subjective night, although of smaller magnitude (-0.48h ± 0.18). This confirmed persistence of abnormal direction of phase re-setting at CT20 in Myk/+ mice under type II conditions although of reduced magnitude (Fig 8D,F).

Hyperlocomotor phenotype accompanied by elevated metabolic rate: One of the major behavioural phenotypes that define Myk/+ animals as a model of mania is a high locomotor drive that results in hyperambulation in an open field. In wheel-equipped cages however, no genotype differences were observed in total wheel-running activity (Fig 9B). To confirm this phenotype and identify potential circadian variation in this behaviour, animals were placed in a pre-habituated open-field and activity was monitored for 15 minutes. This was done during the dark-phase of a 12:12 LD cycle. In this paradigm Myk/+ animals travelled almost 4 times the distance of +/+ mice (Fig 9A-E). Previous reports demonstrated this behaviour only during the light phase of the circadian cycle therefore in addition to validating this phenotype, we confirmed that genotype differences in open-field locomotor output are maintained across the diurnal cycle (Kirshenbaum et al., 2011).

Due to increased open-field activity and the visually small size of Myk/+ animals, metabolic activity of the mice was assessed using indirect calorimetry (Fig 9F). In all example Myk/+ animals demonstrated a large increase in basal metabolic rate, measured as relative oxygen consumption (Fig 9G-H). Interestingly, even during nadirs that occur during the subjective day oxygen consumption in Myk/+ animals was generally maintained above peak levels of +/+ animals (Fig 9G).
Figure 8: Abnormal phase re-setting response to light in Myk/+ animals in Aschoff type II protocol; A: Individual phase shift response of +/- and Myk/+ animals to one-hour Aschoff type II light pulses at CT14 and CT20. Myk/+ animals show a similar pattern of responses as observed with type I pulses. B: Myk/+ animals display larger magnitude phase shifts at CT14; (+/+: -1.15 ± 0.14; Myk/+ -1.81 ± 0.21h; ANOVA; p=0.03). At CT20 +/- and Myk/+ animals show phase shifts of opposing direction (+/+: 0.76 ± 0.08; Myk/+; -0.48 ± 0.18; p=0.003). C-D: Example actograms of +/- (C) and Myk/+ (D) exposed to one-hour light pulse at CT14 (white box) after one full circadian cycle in DD. Both animals respond with phase delays. E-F: Example actogram of +/- (E) and Myk/+ (F) animals given light pulses at CT20. +/- animal exhibit a phase advance relative whereas Myk/+ animal responds with a phase delay. Bars under actograms represent zeitgeber time relative to initial LD cycle.
As circadian and metabolic pathways are heavily inter-dependent, the rhythmic properties of VO$_2$ activity were also quantified. In Myk/+ mice the daily amplitude of peak-trough VO$_2$ rhythms was increased (Fig 9H). The duration of the daily peak in VO$_2$ consumption was also lengthened while the nadir in activity was reduced in Myk/+ mice (Fig 9I). The duration of peak VO$_2$ activity was designated metabolic alpha and Myk/+ animals thus exhibited lengthened duration of metabolic alpha, mimicking increased wheel-running alpha durations observed under both LD and DD conditions. These data reveal alterations in both basal and rhythmic properties of metabolic profiles in Myk/+ animals.

Throughout calorimetric assessment both feeding and drinking activity were continuously monitored in the same cohort of animals. Feeding activity in Myk/+ mice proved to be abnormal with hypophagia indicated by a marked reduction in overall feeding activity (Fig 10D). Myk/+ animals also exhibited increased feeding during the light phase (Fig 10B-F). In relation to drinking activity the data presented revealed an opposite tendency in Myk/+ animals with increased total drinking activity relative to +/- mice. In agreement with the patterns of feeding behaviour however, the Myk/+ cohort also displayed higher drinking activity during the light phase (Fig 10G). The decreased feeding and changes to the phase of ingestion behaviours alongside an elevated basal metabolic rate may explain the reduced size and body weight of Myk/+ animals (Fig 10A).

*Environmentally-induced depression-like behaviour:* As described in figure 8 Myk/+ behaviour is marked by a large and persistent increase in locomotor activity that represents a behavioural endophenotype of mania. In figure 4I monitoring of wheel-running behaviour revealed a general tendency for Myk/+ animals to decrease wheel-running activity over time. In some examples, individual Myk/+ animals exhibited severe reductions in wheel-running activity that manifested in almost total cessation of activity (Fig 11). Hypolocomotion is indicative of a reduced motivational state and is associated with MDD and depressive episodes of BPD. Reduced locomotor activity is also a characteristic phenotype used in the definition of animal models of depression. Myk/+ animals may therefore represent a model in which environmental stressors such as social isolation can induce behavioural switching towards depression-like behaviours.
Figure 9: Dark phase hyperlocomotion and elevated metabolic rate in Myk/+ mice:
A: Experimental paradigm of open-field test (15 minutes filming). B: In wheel equipped cages Myk/+ animals do no show differences in total activity over the day in a 12:12 LD cycle (+/+; 364 ± 58 wheel revs hr⁻¹; Myk/+; 332 ± 33 wheel revs hr⁻¹; p=0.58) C: In a habituated open-field environment, Myk/+ animals exhibit a large increase in distance travelled (+/+; 3.20 ± 0.16m; Myk/+; 12.79 ± 0.36m; p<0.0001). D: Path of exploration in locomotor behaviour in +/+ and E Myk/+ animal. F: Experimental paradigm for metabolic assessment. Mice were maintained in a 12:12 LD cycle for 7 days. G: Smoothed mean VO₂ trace of +/+ and Myk/+ animals over 7 days in metabolic cages. H: Mean VO₂ consumption is elevated in Myk/+ animals (+/+; 2908 ± 138ml kg⁻¹ hr⁻¹; Myk/+; 3856 ± 129 ml kg⁻¹ hr⁻¹; p<0.0001). I: Peak-trough amplitude of smoothed VO₂ traces. Myk/+ animals show increased peak-trough amplitude in VO₂ consumption (790 ± 35ml kg⁻¹; Myk/+; 1107 ± 132ml kg⁻¹; p=0.02). J: Duration of daily peak and trough in VO₂ activity, measured from individual traces. Myk/+ animals display lengthened peak and shorted trough in VO₂ activity (Peak; +/+; 10.37 ± 0.23h; Myk/+; 13.00; ± 0.75h; p=0.003 Trough; +/+; 8.59 ± 0.30h; Myk/+; 5.05 ± 0.75h; p=0.0004).
Figure 10: *Myk/+* mice with lower body weight display decreases in feeding activity yet increased drinking and light phase ingestion: A: *Myk/+* have a lower body weight than +/+ animals (+/+; 25.8 ± 1.5g; *Myk/+*; 21.1 ± 2.0; p=0.03). B: Daily patterns of feeding behaviour averaged over 7 days in +/+ and *Myk/+* C: animals. Note the even spread over the LD cycle and less distinct night-time peak of feeding bouts over in *Myk/+* example. D: *Myk/+* animals exhibit reduced overall feeding relative to +/+ animals (+/+: 93 ± 4 visits hr⁻¹; *Myk/+*: 64.61 visits hr⁻¹; p=0.0005). E: *Myk/+* animals exhibit increased drinking activity relative to +/+ animals (+/+: 90 ± 13 visits hr⁻¹; *Myk/+*: 173 ± 32 visits hr⁻¹; p=0.03). F-G: *Myk/+* animals designate a greater amount of both overall feeding (F) and drinking (G) activity to the light phase (Feeding; +/+: 25.0 ± 1.9%; *Myk/+*: 36.32 ± 1.3%; p<0.0001; Drinking; +/+: 20.6 ± 2.4%; *Myk/+*: 33.8 ± 2.9%; p=0.003).
Figure 11: Examples of Myk/+ animals exhibiting hypolocomotion: A: Actogram of +/- animal from same experiment as example (E). B: Wheel revolutions per day normalised to maximum revolutions in a single day from animal displayed in (A). +/- animal exhibits daily variability in wheel-running. C: Example actogram of Myk/+ animal exhibiting a gradual decrease in wheel-running activity over time in isolation. D: Total wheel revolutions per day demonstrate a gradual decrease and almost total cessation by day 27. E-F: Myk/+ actogram and wheel revolution plot exhibiting similar reduction wheel-running behaviour as example (C). Note that wheels were checked for functionality throughout the duration of experiment.
Discussion

Increased light-phase activity in Myk/+ mice: In experiments assessing both wheel-running and metabolic activity Myk/+ animals consistently demonstrated increased activity during the light phase. Myk/+ mice displayed a lengthened metabolic alpha and increased ingestion behaviour during lights-on. In wheel-equipped cages mutant cohorts exhibited increased wheel-running activity during the light phase that was generally confined within a distinct tail at the end of daily alpha bouts.

Under long photoperiods (16:8) Myk/+ animals exhibited a greater relative proportion of activity during the light phase and rhythm instability. One explanation for this behaviour is a deficit in the negative masking effects of light. This was tested through exposure of +/+ and Myk/+ animals to light pulses during the night. Myk/+ animals displayed a clear deficit in the suppressive effects of light on locomotor output thus revealing an important attenuation of the physiological pathways governing negative masking.

As the effects of light are increasingly recognised as major contributory factor to mood and mood-related behaviours (LeGates et al, 2014) this abnormal interaction with photic stimuli is of particular interest in a behavioural model of mania. Negative masking is a feature of the nocturnal mammalian response to light, separate and complementary to the circadian system, and results in rapid suppression of activity and avoidance of photic stimuli (Redlin, 2001).

In rodents the neural mechanisms of light-induced behavioural suppression are not fully delineated, but a major influence of this pathway is imparted through both classical visual as well as non-imaging forming (NIF) photoreceptors and their downstream projections (Mrosovsky and Hattar, 2003; Panda et al., 2003; Thompson et al., 2008). The major sensory input of the latter of these pathways arises from melanopsin-expressing intrinsically photosensitive retinal ganglion cells (ipRGCs; Berson et al., 2002; Güler et al., 2008; Lucas et al., 2014). There exist various ipRGC sub-classes projecting to diverse brain regions that influence a range of behaviours in addition to light-aversion and circadian entrainment (Johnson et al., 2010; Thompson et al., 2010; Schmidt et al., 2011).
The NKA α3 subunit within which Myk/+ mice possess an inactivating mutation is known to be expressed throughout the retina in both the mouse and rat, including along axonal projections of ipRGCs (Blanco, 2005; Specht and Sweadner, 1984). Due to this expression profile in the retina, the Myk/+ mutation may alter ipRGC function, affecting transduction of non-visual light information. This may in turn contribute to alterations observed in the activity-suppressing effects of light. As the NKA α3 also has a broad expression profile in the CNS (Bøttger et al., 2011), the possibility also exists that changes to NIF-dependent masking arise within downstream target regions.

Potential changes to NIF-function in the Myk/+ represents an extremely interesting line of enquiry into the possible role of NIF actions on mood-related behaviours. Changes to light exposure are known to influence mood states in human and animal models (LeGates et al., 2014). Light-dependent control of mood is believed to be exerted by ipRGCs via innervations across CNS regions that are associated with affective disorders; the lateral habenula (LHb) and the amygdala (Hattar et al., 2006; Lecca et al., 2014; Li et al., 2013; Roozendaal et al., 2009; Schmidt et al., 2011). Both of these regions play important roles in emotional and aversive behaviour and memory and have multiple interactions with key regions the brain’s reward pathways such as the ventral tegmental area (VTA) and nucleus accumbens (Russo and Nestler, 2013; Stamatakis and Stuber, 2012). If NIF pathways are indeed hyper or hypo-functional as postulated by the masking deficits in Myk/+ animals then signalling to the LHb and amygdala may contribute to mood regulation and mania-like behaviours in Myk/+ animals.

Outside of the visual system increased light-phase activity may be explained by a heightened arousal system that may supersede the suppressive effects of light. Myk/+ animals exhibit reduced sleep and a hyperlocomotoric phenotype that we have confirmed experimentally across both the day and night (Kirshenbaum et al., 2011). The major arousal-promoting circuits in the CNS are; hypocretin, histaminergic and most interestingly in the setting of this study, dopaminergic (DA) systems (Panula and Nuutinen, 2013; Sakurai, 2007). Due to the association of aberrant dopamine regulation in animal models of mania and heightened responses to d-amphetamine in Myk/+ animals this circuit represents a candidate mechanism underlying masking deficits (Chung et al., 2014; Coque et al., 2011; Kirshenbaum et al., 2011; Roybal et al., 2007). Negative
masking in mice is dependent on dopamine D2 receptors and methamphetamine can attenuate behavioural suppression by light (Doi et al., 2006; Vivanco et al., 2013). Pharmacological manipulation of DA systems through drugs of abuse such as cocaine and methamphetamine induces heightened locomotor and motivational states that mimic locomotor and arousal phenotypes of Myk/+ animals (Di Chiara, 1995; Cousins et al., 2009). Interestingly NKA α3 subunits co-localise with tyrosine hydroxylase neurons of the mouse VTA highlighting a potential direct involvement of the Myk/+ mutation in DA physiology (Bøttger et al., 2011). Increased DA signalling agrees with a behavioural model underlying changes to negative masking in Myk/+ animals and would also explain lengthened alpha durations and open-field hyperlocomotion.

Abnormal phase re-setting to light reveals altered PRC shape in Myk/+ mice: Both entrainment and rhythm stability in Myk/+ animals appeared disrupted under long 16:8 photoperiods with some animals even unable to entrain to 12:12 LD cycles. These data suggest that light exhibits an abnormal interaction with circadian systems in addition to masking behaviour. To test the impact of light input to the circadian system animals were subjected to one hour light pulses using Aschoff type I and II protocols. Under both experimental paradigms Myk/+ animals exhibited larger magnitude phase shifting responses during the early subjective night (CT14). Myk/+ mice also displayed an extension in the delay portion of their putative PRC to light, with phase delays persisting into the late subjective night (CT20 ) and small advances appearing at the very end of the night phase (CT23).

Changes to phase re-setting can be attributed to various factors that affect SCN function including altered clock gene function, peptide signalling (VIP), electrical and intercellular communication and light input via the retinohypothalamic tract (RHT; Albrecht et al., 2001; Han et al., 2012; Hughes and Piggins, 2008; Panda et al., 2002b). Of these pathways the obvious candidate to explain alterations to PRC would be changes to light input or integration within the SCN. Firstly, our data indicates that increasing photoperiod disrupts Myk/+ entrainment. In addition, we have demonstrated that light pulses can induce sudden changes in tau under free-running conditions, further highlighting an unusual sensitivity of the circadian system to photic input. Coupled with altered negative masking behaviour described above, these data suggest that changes to the processing or strength
of afferent photic input arriving to the Myk/+ SCN may explain heightened phase-shifting at CT14. Behavioural phase shifts in rodents are mediated by glutamatergic release and the induction of intracellular signalling cascades that alter the clock gene expression and neuropeptide-mediated re-organisation of the SCN network (Ding et al., 1994; Shearman et al., 1997; Shirakawa and Moore, 1994; Vindlacheruvu et al., 1992). Extending the deductions made in relation to negative masking, alterations to NIF photic signalling to the SCN from iPRGCs could explain a heightened glutamatergic input on to the SCN and a subsequent heightened phase shift.

Changes in light input however fail to explain the full spectrum of phase-shifting abnormalities observed in Myk/+ behaviour. Phase-specific differences to the direction of behavioural re-setting revealed alterations in the shape of a putative PRC in Myk/+ mice (Fig 12A). These changes would rather suggest that the organisation and pacemaking of the SCN in Myk/+ animals is abnormal and result in different phase shifting responses.

![Figure 12: Putative PRC of Myk/+ mice](image)

**Figure 12: Putative PRC of Myk/+ mice:** A: Based upon initial phase re-setting responses to light, predicted model of +/- and Myk/+ PRC. Myk/+ mice (red) appear to have a larger magnitude delays response that is elongated in its duration over the early to late subjective night. This results in a later transition to phase advances relative to response observed in +/- animals (blue). B: Complete PRC of rats exhibiting long alpha (open circles) vs. short alpha (closed circles) (from Honma et al., 1984). Extended delay portion mimics Myk/+ phase re-setting at approximately CT20.

From the traditional view on photic re-setting in mice, the Myk/+ PRC shape may be surprising given that rodent species tend to show an inverse relationship between tau, which is elongated in Myk/+ mice, and the relative size of the delay portion of PRC (Pittendrigh & Daan, 1976b). This unusual re-setting may instead be explained by the exceptionally long alpha observed in these animals.
Honma and colleagues demonstrated in rats, a species with a period typically greater than 24 hours, that individuals with longer alpha durations exhibited similar increases in the duration of the delay portion of their PRC to those observed in the Myk/+ cohort (Fig 12B; Honma et al., 1984). As Myk/+ animals show a distinctive and marked increase in alpha duration relative to +/- (~5.85h) this relationship may similarly result in such changes to PRC shape. The theoretical basis of this model lies within the conventional idea of dual morning and evening oscillators within the SCN, each differentially contributing to phase shift responses. The relationship of these oscillators can be altered by interactions with light and are affected by the intrinsic tau and alpha of the circadian system (Honma et al., 1984; Pittendrigh and Daan, 1976a, 1976b). Locomotor activity is known to feedback to the SCN and dampens electrical activity via NPY and GABAergic projections (Besing et al., 2012; Houben et al., 2014; Morin and Allen, 2006; van Oosterhout et al., 2012; Yamazaki et al., 1998). We postulate that increased locomotor activity across the circadian cycle due to lengthened alpha bouts may interact with the physiological mechanisms underlying coupling of morning and evening oscillators in the SCN and result alterations to the Myk/+ PRC.

Recent work has begun to elucidate what the neurophysiological substrate of morning and evening oscillator models in rodents. The general consensus of this work suggests that alterations to the phase relationship between spatially distinct populations of SCN neurons (Golombek and Rosenstein, 2010). Within the SCN these clusters can be separated physically or through changing photoperiod (Evans et al., 2013; Jagota et al., 2000; de la Iglesia et al., 2004). The result of these manipulations are distinct dorso-ventral or antero-posterior neuronal populations that exhibit disparate peaks in molecular and electrical rhythms that appear to act as the physiological manifestation of morning and evening oscillators (Brown and Piggins, 2009; Inagaki et al., 2007; de la Iglesia et al., 2004). Recent studies have also determined that this spatiotemporal relationship is heavily dependent on two major SCN neurochemicals; GABA and VIP (Evans et al., 2013; Farajnia et al., 2014). Investigations of the neurophysiological properties of the Myk/+ SCN will be required to determine if altered spatiotemporal and synaptic mechanisms empirically support these predictions underlying altered phase resetting responses.
Abnormal free-running rhythms in Myk/+ animals; In addition to responses to external stimuli, the other key property of the circadian system is long-term stability and pacemaking of biological rhythms. To fully characterise behavioural circadian output, Myk/+ animals were allowed to free-run in constant dark for a minimum of 14 days. Under DD Myk/+ animals exhibited a range of deficits, including long tau and impressively lengthened alpha duration. Behavioural rhythms were also characterised by reduced amplitude and when presented with external stimulation, the propensity to show rapid alterations in tau. To identify further changes in pacemaker properties, Myk/+ animals were also subjected to jet lag paradigms and displayed an increase in the speed of re-entrainment following advances of the LD cycle.

During the initial characterisation of Myk/+ mutant animals as a model of mania, the description of circadian behaviour suggested a longer period (~25h) and shorter alpha duration (<15h) than observed in this study (Kirshenbaum et al., 2011). The report from the 2011 paper however, did not characterise the low amplitude and unstable free-running rhythms in the Myk/+ animals. The current study presented here utilised a higher sample size of animals with longer durations under free-running conditions to establish a more accurate description of circadian behaviour. We propose that instead of the stable behaviour described previously, the Myk/+ phenotype manifests across a continuum of labile phenotypes that can be generalised to a moderate increase in period, long alpha, low amplitude rhythms with an unusual degree of malleability in pacemaking properties.

The output of the Myk/+ circadian system therefore appears to have an altered oscillatory waveform of reduced amplitude that in turn fails to generate a signal sufficient to terminate daily wheel-running activity. Behavioural changes to the circadian system can be due to loss or mutation of core clock genes, ion channels or coupling factors that directly affect pacemaking of the SCN (Aton et al., 2005; Bunger et al., 2000; Granados-Fuentes et al., 2012; Vitaterna et al., 1994). Such genetic deficits can manifest within the SCN as changes to the period of molecular or electrical rhythms or altered cellular synchrony that reduces output of the tissue (Cutler et al., 2003; Guilding et al., 2013; Ko et al., 2010; Vitaterna et al., 2006). Within the current literature however, the physiological role of the NKA α3 subunit within cellular or tissue-level pacemaking is unclear. It is therefore difficult to make assumptions upon the physiological role of the...
Myk/+ mutation within the SCN. In light of the unusual behavioural trait of tau switching in Myk/+ animals, predicting effects within pacemaking structures is further complicated as typically the SCN is resistant to perturbation and changes in network properties (Liu et al., 2007).

The only studies into the role of NKA pumps in the SCN are not isoform-specific and instead utilise general NKA inhibition to determine potential roles in neuronal physiology. These investigations revealed a diurnal peak in NKA activity in rats, which arrives during the day (Wang and Huang, 2004). Pharmacological blockade of NKA activity also affects depolarisation rates and reduces spontaneous firing activity in SCN neurons (Wang and Huang, 2006). Outside of the SCN, changes to NKA and α3 isoform activity can alter electrical activity, neural coding and network dynamics in different structures across the CNS (Dobretsov and Stimers, 2005; Ikeda et al., 2013; Johnson et al., 1992; Shiina et al., 2010; Vaillend et al., 2002). Concomitantly the NKA can function as a signal receptor for endogenous ligands such as ouabain and agrin, activating a range of signalling cascades relevant to SCN neuronal function including ERK, AKT and intracellular Ca\(^{2+}\) release (Khodus et al., 2011; Kirshenbaum et al., 2012; Miyakawa-Naito et al., 2003; Obrietan et al., 1998; Silva and Soares-da-Silva, 2012; Yu et al., 2010; Zheng and Sehgal, 2010). The Myk/+ mutation has the potential to impact on either electrical or intracellular signalling cascades within the SCN. Characterisation of the SCN network is therefore to elucidate how the Myk/+ mutation may impact upon central pacemaking and ultimately behaviour.

Circadian behaviours of Myk/+ animals in relation to circadian models of affective behaviours: To place these observations of altered circadian behaviour in a wider context, the circadian phenotype of Myk/+ exhibits similarities to other clock gene models of affective behaviours. Tau in Myk/+ animals was generally lengthened and this phenotype is shared with other models of mania-like behaviours including ClockΔ19 and Afh models although shortening of tau also appear in similar behavioural models (Rev-erba\(^{-/-}\), Per2\(^{Brdm}\), Chung et al., 2014; Godinho et al., 2007; Hampp et al., 2008; Keers et al., 2012; Roybal et al., 2007; Vitaterna et al., 1994; Zheng et al., 1999). ClockΔ19, Bmal1\(^{-/-}\), Per2\(^{Brdm}\) and Afh are all examples of mania-like behavioural models that concomitantly exhibit similar reductions in behavioural amplitude as Myk/+ (Bunger et al., 2000; Guilding et al., 2013; Kondratova et al., 2010). Interestingly ClockΔ19, Rev-erba\(^{-/-}\), Bmal1\(^{-/-}\), Afh and
Per2Brdm also display alterations in phase shifting responses or re-entrainment to light (Albrecht et al., 2001; Pfeffer et al., 2014; Preitner et al., 2002; Vitaterna et al., 2006). Identifying correlations in behaviour are important in the continued attempts to define the circadian system in models of BPD and affective behaviours. The Myk/+ behavioural phenotype adds to a growing data set supporting the idea that low-amplitude circadian rhythms and altered phase re-setting to light are common across a range of models with mania-like behaviours. These overt circadian phenotypes are also described in humans exhibiting affective disorders, including unstable daily behavioural rhythms and altered phasing of daily activity and physiological output factors such as body temperature and melatonin (Jones et al., 2005; Rock et al., 2014; Emens et al., 2009; Mansour et al., 2005; Souqtre et al., 1989; Wong et al., 2000). Within this literature therefore, dampened circadian amplitude, altered photic re-setting and re-entrainment, resulting in possible phase mis-alignment, appear as common phenotypes in Myk/+ and other models that are shared with human affective disorders. It remains unclear however if these specific changes to circadian system act as key effectors of changes in mood behaviours.

**Elevated metabolic rate and ingestion behaviour in Myk/+ animals:** Some research implies that wheel-running activity provides reinforcing feedback and is a form of stereotypy in laboratory conditions (Mason et al., 2007). To dispel this idea and compliment behavioural data obtained from wheel-running paradigms, animal activity was further assessed in open-field and metabolic cages. Due to the close association of metabolic and circadian systems, metabolic activity can provide further insights into circadian regulation of behaviour and metabolism (Asher and Schibler, 2011). For example animals with genetic lesions that result in altered circadian behaviour, VIP-VPAC2 knockout and Afh animals, express a metabolic phenotypes that accompany circadian wheel-running changes (Bechtold et al., 2008; Guilding et al., 2013). Under these conditions Myk/+ animals demonstrated activity that mimicked observations made in wheel equipped cages, including lengthened metabolic alpha and increased light-time feeding and drinking. Indirect calorimetric assessment revealed a further range of metabolic abnormalities in Myk/+ animals. These phenotypes included change to basal and rhythmic metabolic output; elevated BMR, hypophagia and increases in amplitude of daily
fluctuations in VO₂. The combination of increased metabolic rate and reduced feeding reveals a likely major factor in the low body weight and small size of Myk/+ animals.

As the NKA α3 is a neuron-specific isoform of the NKA complex, metabolic phenotypes must be centrally-derived (Bøttger et al., 2011; Shyjan and Levenson, 1989). Due to both basal and rhythmic changes in metabolic activity observed in Myk/+ animals, the candidate pathways that underlie these alterations may include neural centres of metabolic and ingestion behaviours under circadian control. The SCN projects directly to regions of the hypothalamus critical in the regulation of metabolic and ingestion behaviours including the paraventricular nucleus (PVN), arcuate nucleus (ARC) and dorsomedial hypothalamus (DMH) (Buijs et al., 1994; Sohn et al., 2013; Yi et al., 2006). These regions also express circadian rhythms in PER2 expression and neuronal activity while direct lesions can lead to alterations in both metabolic and feeding activity (Chou et al., 2003; Gooley et al., 2006; Guilding et al., 2009; Tousson and Meissl, 2004). Potential changes to local (ARC, PVN, DMH) or SCN-derived circadian rhythm generation may contribute to the altered metabolic and ingestion phenotypes.

Parts of the metabolic spectrum exhibited by Myk/+ animals however do not appear to correlate with circadian phenotypes. Increased day-night amplitude and an overall elevation of BMR appear contradictory to low amplitude circadian behaviour. Instead this suggests fundamental changes to basic metabolic physiology within the CNS. The potential mechanisms of these changes are potentially vast. Basic changes to the functional output of hypothalamic structures such as the ARC, PVN and DMH or autonomic regulation of peripheral metabolism may underlie disrupted metabolism in Myk/+ animals (Sohn et al., 2013).

A further explanation may also be an interaction of arousal systems impacting upon metabolic regulation. Myk/+ arousal phenotypes have been discussed and part of this behavioural spectrum includes hyperlocomotion and reduced REM and non-REM sleep (Kirshenbaum et al., 2011). Increased overall activity may simply provide an increased metabolic demand contributing to elevations in BMR. In addition, across both humans and rats, decreases in REM-sleep from chronic sleep deprivation result in a marked rise in metabolic rate, with an increase in VO₂ consumption of even greater magnitude than
observed in Myk/+ animals (Bonnet and Arand, 2003; Koban and Swinson, 2005). This sleep-dependent element of the Myk/+ arousal phenotype may feasibly interact with similar metabolic circuits and contribute to elevated BMR.

Interesting the metabolic phenotype in Myk/+ animals draws further comparisons to other clock gene models described above that also exhibit various forms of metabolic disruption. ClockΔ19, Rev-erba<sup>−/−</sup>, Afh, Bmal1<sup>−/−</sup> models all demonstrate metabolic abnormalities alongside changes to affective behaviours, with altered food anticipatory behaviour also exhibited in Per2<sup>Brdm</sup> mice (Duez et al., 2008; Feillet et al., 2006; Guilding et al., 2013; Kondratov et al., 2006; Turek et al., 2005). There is strong evidence in epidemiological studies of increased susceptibility to metabolic syndrome and metabolic abnormalities in human BPD patients (Taylor and MacQueen, 2006; Turek et al., 2005; Vancampfort et al., 2013). The consistent appearance of metabolic abnormalities across clock gene laboratory models opens up the question as to whether metabolic phenotypes may act – in association with circadian pathways - within the symptomatology or pathology of BPD.

**Myk/+ animals may represent a switching model of BPD episodes:** In the search for pre-clinical models of BPD the gold standard of face validity would be an animal that changes between mood states representative of mania and depression. In long-term wheel-running experiments some Myk/+ animals exhibited profound reductions in wheel-running activity. These data suggest that Myk/+ animals possess an increased sensitivity to environmental stressors that may arise during wheel-running experiments. Hypokinetic behaviour is a key clinical symptom of depression recognised as a major factor in the diagnosis of depression or depressive episode (DSM-V, 2013; ICD-10, 2014). Reduced activity also represents a distinctive endophenotype in the identification of depression-like behaviour in laboratory models (Willner, 1991). The environmental factor that may explain these behaviours include changes to lighting conditions – the transition from LD to DD - or social isolation. The latter explanation appears likely in the Myk/+ model as severe reductions in locomotor behaviour were observed in the absence of transitions to DD. Social isolation is a known stressor with the capacity to induce depression-like behaviours in rats and so appears a likely factor mediating these behaviours in Myk/+ animals (Willner, 1991).
There currently exist animal models that can display environmentally-induced opposing BPD-like behavioural states including clock associated models; $Dbp^{-/}$ animals and mice with a mutation in the inhibitory serine-phosphorylation site of GSK3α/β (Le-Niculescu et al., 2008; Polter et al., 2010). This potentially exciting new insight into $Myk/+$ behaviour will require further investigation as we have observed differences in kinetic behaviours depending on method of assessment - open-field vs wheel-running activity. The severity of the hypokinesia observed however appears very promising towards further validation of the $Myk/+$ mouse as a model of both manic and depressive mood aspects of BPD.

**Conclusions:** The association between BPD and sleep and circadian rhythm disruption in both laboratory and clinic appears inseparable. Understanding the interactions between the central circadian system and mood-related behaviour is of great importance and preclinical models represent an important tool, permitting the dissection of genetic and physiological mechanisms that underpin this association.

The $Myk/+$ is a model of BPD that recapitulates an impressive array of endophenotypes associated with episodes of mania. In this study the $Myk/+$ circadian phenotype has been well characterised to reveal novel deficits in pacemaking and phase re-setting to light. In addition profound alterations in metabolic phenotypes have been demonstrated alongside potential environmentally induced changes in mood-associated behaviours. This behavioural characterisation has re-enforced associations between dampened circadian output, light interactions and metabolic pathways observed in other models of altered affective behaviours.

The $Myk/+$ model however is distinct from these circadian-mood models. Despite various sleep and circadian deficits $Myk/+$ animals possess a mutation in the NKA α3 subunit. Neither NKA pumps nor α3 subunits are currently regarded as critical elements that define or interact with the molecular pacemaker, the fundamental machinery of circadian rhythm generation in mammals. As questions persist into the role of circadian rhythm alterations in BPD and affective behaviour, the $Myk/+$ model represent a potentially novel insight into mechanisms of circadian disruption outside of genetic lesions within the TTFL.

Moving forward the next key question to address will be trying to understand the neurophysiological mechanisms that underpin the behavioural deficits observed in $Myk/+$
mice. How does the NKA α3 affect fundamental pacemaking at molecular and cellular levels and how does this translate to network-level rhythm generation within the SCN and downstream oscillators? Elucidating changes to the circadian system and circadian regulated pathways will aid our understanding into how central timing mechanisms contribute to mood regulation and affective disorders.
References


Chapter 2

Altered central pacemaking in the *Myshkin* model of mania.
Altered central pacemaking in the Myshkin model of mania.

Introduction

Daily rhythms in physiology and behaviour are highly conserved features of species on earth, manifesting in various forms across distinct phylogenetic kingdoms. These biological oscillations, known as circadian rhythms, evolved as an adaptive response to daily environmental cycles on earth. The planet’s geometric rotation results in approximately 24-hour cycles of light, temperature and many other factors critical to an individual’s survival. In mammals the circadian system that has evolved to anticipate these fluctuations is hierarchical in nature; initiating from molecular and physiological oscillations within single cells that are re-enforced at the tissue level by local synchronising cues and ultimately from systemic signals from a master pacemaker (Mohawk et al., 2012). The dominant rhythm-generating structures that govern the temporal organisation of brain and body lie within the hypothalamus of the CNS and are known as the suprachiasmatic nuclei (SCN; Ralph et al., 1990; Stephan and Zucker, 1972).

The molecular clock that drives oscillations and rhythmic physiology at the level of single cells is regulated through by an inter-locked transcriptional translational feedback loop (TTFL) (Partch et al., 2014). Various molecular and enzymatic elements modify the post-transcriptional activity and cellular localisation of key transcription factors of the TTFL via phosphorylation, ubiquitin-dependent proteasome degradation, sumoylation and chromatin re-modelling. The net result of these interactions is a near 24-hour oscillation in clock genes and their downstream targets (Koike et al., 2012; Panda et al., 2002a). The basic helix-loop-helix transcription factors CLOCK and BMAL1 function as the positive elements of the TTFL, acting as a PAS-dependent heterodimer to drive the transcription of clock-controlled genes (Bunger et al., 2000; Gekakis et al., 1998). The targets of the CLOCK-BMAL1 protein complex include the negative regulators of molecular rhythms, Period1,2 and 3 (Per) (Bae et al., 2001; Zheng et al., 1999)and their heterodimeric partners Cryptochrome1 and 2 (Cry) (Vitaterna et al., 1999). PER:CRY complexes translocate back to the nucleus following heterodimerisation to inhibit the activity of
CLOCK-BMAL1, in turn reducing their own expression and liberating CLOCK-BMAL1 once more to re-initiate the TTFL cycle.

Subsidiary transcriptional loops interact with these key elements including the nuclear receptors REV-ERBα/β and RORα/β that play an antagonistic role in the expression of Bmal1 (Cho et al., 2012; Guillaumond et al., 2005; Preitner et al., 2002). Localisation and degradation of clock proteins is regulated by phosphokinases including CK1ε/δ (Etchegaray et al., 2009; Pilorz et al., 2014) and glycogen synthase kinase 3β (GSK3β) (Iitaka et al., 2005). Ubiquitin-dependent proteasome degradation is known to act directly upon CRY1 and CRY2 via Fbxl3. Chromatin re-modelling and epigenetic factors act to generate a transcriptionally active window regulated in part by histone-acetyl transferase activity of CLOCK and its interaction with the histone-deacetylase Sirtuin1 (SIRT1; Chang and Guarente, 2013; Nakahata et al., 2009; Ramsey et al., 2009).

Although the basic elements of circadian pacemaking exist within single cells, in isolation these oscillations lack robustness and rapidly fall out of phase with their neighbours (Freeman et al., 2013; Webb et al., 2009; Welsh et al., 1995). Within the SCN, tissue-level synchrony depends on network interactions that couple weaker individual oscillations into a robust, high amplitude population-level output.

Cellular interactions within the SCN depend the activity of synaptic, paracrine and gap junction-dependent signals (Baba et al., 2008; Long et al., 2005; Maywood et al., 2011). The blockade of action potentials alters both in vitro pacemaking of the SCN and behavioural output with the loss of gap junction structural proteins affecting rhythmic behaviour (Baba et al., 2008; Enoki et al., 2012; Long et al., 2005; Schwartz et al., 1987; Yamaguchi et al., 2003). Population-level pacemaking therefore begins with the electrical activity of single cells. Neurons of the SCN express circadian rhythms in spontaneous firing rates (SFR), resting membrane potential (RMP) and electrical behaviours that translate into a day-night rhythm in firing activity of the SCN in vivo or in vitro (Belle et al., 2009; Groos and Hendriks, 1982; Inouye and Kawamura, 1979; Kuhlman and McMahon, 2004; VanderLeest et al., 2007). The cells of the SCN achieve this feat through the activity of a range of ion channels and currents that are either rhythmically or non-rhythmically expressed with each imparting a distinct contribution to cellular excitability.
The major conductances underlying these behaviours include L-type Ca\(^{2+}\) (Pennartz et al., 2002), persistent Na\(^{+}\) (Jackson et al., 2004), small and large conductance Ca\(^{2+}\)-dependent K\(^{+}\) (Belle et al., 2009; Pitts et al., 2006), T-type Ca\(^{2+}\) (Huang, 1993), A-type K\(^{+}\) (Itri et al., 2010) fast-delayed rectifier K\(^{+}\) currents (Kudo et al., 2011) and an un-specified TEA-sensitive K\(^{+}\) current. Genetic loss of underlying ion channels alters the both pacemaking properties of the SCN behaviourally and physiologically (Granados-Fuentes et al., 2012; Han et al., 2012; Meredith et al., 2006).

Molecular and electrical rhythms within neurons of the SCN are believed to interact through fluctuations of intracellular Ca\(^{2+}\) (Colwell, 2011). The role of Ca\(^{2+}\) as an integrator of both processes has recently emerged as an equally vital component of SCN pacemaking. Ca\(^{2+}\) levels can be altered transiently or over longer time courses through post-synaptic interactions of SCN neurons with various neurotransmitters and neuropeptides and through a peak in daily Ca\(^{2+}\) currents (Colwell, 2000; Irwin and Allen, 2009, 2010; Pennartz et al., 2002). Ca\(^{2+}\) interactions at the membrane are augmented by a circadian rhythm from intracellular stores and result in a day-night oscillation in Ca\(^{2+}\) (Ikeda et al., 2003). Molecular oscillations within single-cells depend upon this rhythm in Ca\(^{2+}\) and peak Ca\(^{2+}\) activity appears as a dorso-ventral wave across the spatiotemporal architecture of the SCN network in vitro. This wave is much-like the spatiotemporal progression of molecular oscillations across the SCN although Ca\(^{2+}\) rhythms phase-lead CRE-dependent and clock gene expression across the SCN (Brancaccio et al., 2013; Enoki et al., 2012; Evans et al., 2013; Lundkvist et al., 2005; Travnickova-bendova et al., 2002).

Ca\(^{2+}\) and electrical rhythms act to maintain intercellular synchrony across the SCN through pre-synaptic vesicle release. The SCN represent highly heterogeneous populations with a diverse profile of neurochemical expression including arginine vasopressin (AVP), gastrin-releasing peptide (GRP), prokineticin2 (PK2), vasoactive intestinal polypeptide (VIP) and GABA (Abrahamson and Moore, 2001). In recent years VIP and GABA have emerged as particularly critical elements of the SCN network. Loss of VIP and its receptors VPAC2 profoundly alters behavioural rhythms and responses to environmental stimuli, which also manifest as attenuated pacemaking and network synchrony in vitro (Aton et al., 2005; Cutler et al., 2003; Evans et al., 2013; Hughes and Piggins, 2008; Maywood et al., 2011). GABA signalling, specifically through actions on GABA\(_A\)-receptors, functions to
synchronise or de-synchronise spatially distinct populations of SCN neurons depending on the current state of the network (Evans et al., 2013; Freeman et al., 2013). The mechanism of this state-dependent change in the role of GABA appears due to alterations of post-synaptic response of GABA_A receptors via NKCC1 modulation of intracellular Cl⁻ concentrations (Choi et al., 2008; Farajnia et al., 2014).

Every level of the SCN pacemaking apparatus is critical to circadian rhythm generation from molecular to circuit-level. Due to the fundamental role of biological timekeeping across organisms, disruption to individual elements of the clock can result in diverse pathophysiological consequences. Genetic lesions of core clock genes or circadian disruption in humans result in a variety of disease-associated phenotypes including metabolic syndrome, cardiovascular disease and increased risk of cancer (Fu et al., 2002; Maury et al., 2010; Takeda and Maemura, 2011; Turek et al., 2005). Various clock gene knockout and mutant strains also result in behavioural phenotypes that mimic aspects of affective disorders, most notably mania-like symptoms (Landgraf et al., 2014). These pre-clinical models include ClockΔ19, Rev-erbα⁻/⁻, Per2Brdm⁻/⁻, Bmal1⁻/⁻ and Afterhours (Afh) (Chung et al., 2014; Hampp et al., 2008; Keers et al., 2012; Kondratova et al., 2010; Roybal et al., 2007).

Circadian rhythm disruption has a long-held clinical association with neuropsychiatric disorders and particularly bipolar disorder (BPD; Gonzalez, 2014). BPD is a condition that affects between 0.5-1.5% of the global population with an increased risk of development across families that highlight the heritable nature of the condition (Craddock and Sklar, 2013; Lozano et al., 2012; Wittchen et al., 2011). BPD patients suffer from exaggerated mood and motivational states that cycle between episodes of mania and depression (DSM-V, 2013; ICD-10, 2014; Phillips and Kupfer, 2013). Sleep and circadian rhythm disruption is a facet of BPD pathology wherein patients report a reduced need for sleep in 69-99% of cases of mania and insomnia or hypersomnia in 28-100% during episodes of depression (Harvey, 2008; Hudson et al., 1993). Circadian rhythm abnormalities are also apparent across activity cycles and output parameters of the circadian system such as melatonin and corticosterone (Jones et al., 2005; Rock et al., 2014; Schnell et al., 2014; Wong et al., 2000). Further clinical evidence support this profound association including the use of chronotherapeutics in mood management, genetic associations of clock genes.
and the response of the circadian system to mood stabilisers such as lithium and valproic acid (Benedetti, 2012; Etain et al., 2011; Johansson et al., 2011; Li et al., 2012). Although this association is now well-established in patients the specific role of clocks in the aetiology and pathology of mood disorders is still unclear.

Recent years have seen some of the mechanisms through which changes to the circadian system becoming elucidated through the use of pre-clinical models. As alluded to above an interesting trend has emerged in which a number of animal models with lesions in core clock genes display an array of changes to affective behaviours (Chung et al., 2014; Landgraf et al., 2014; Roybal et al., 2007). Work specifically on ClockΔ19 and Rev-erba models has revealed a clock-dependent regulation of dopamine synthesis and release within the VTA as part of the mechanism through which the circadian system can directly impart influence mood (Chung et al., 2014; Coque et al., 2011; Dzirasa et al., 2011; Mukherjee et al., 2010). Despite a clear role of the circadian system in the dopaminergic activity within the VTA the rhythmic activity of the VTA appears dependent on input from the SCN as autonomous rhythms have yet to be revealed ex vivo (Abe et al., 2002; Baltazar et al., 2013; Luo et al., 2008). Questions still therefore remain over the role of central vs local clock dysregulation within the circadian influence on mood.

Recently a mouse model possessing a mutation within the Na⁺/K⁺-ATPase (NKA) α3 subunit known as Myshkin (Myk/+) was described as a model of bipolar disorder with face, construct and predictive validity (Kirshenbaum et al., 2011). The NKA α3 subunit is one of four NKA α subunits and is expressed almost exclusively within neurons of the CNS (Blanco, 2005; Bøttnæs et al., 2011; Shyjan and Levenson, 1989). The α subunit is the enzymatically active element of the NKA pump, the functions in concert with β and FXYD subsidiary cofactors to actively transport Na⁺ and K⁺ ions across the plasma membrane via ATP hydrolysis (Kaplan, 2002). Part of the behavioural phenotype observed in these animals was changes to the free-running behavioural rhythms suggesting that the Myk/+ mutation can affect the circadian system. No specific role for α3 subunits of the NKA complex has ever been demonstrated within cellular or behavioural pacemaking and so this represented an interesting model to investigate potential changes to circadian system.
Building on these data we fully characterised the diurnal, circadian and metabolic behaviour of these animals revealing further profound deficits in rhythm stability, phase re-setting to light, negative masking and metabolic activity. Although isoform specific roles of NKA activity in the circadian system are lacking a rhythm in non-subunit specific NKA activity occurs within the rat SCN, blockade of which can alter firing and electrical properties of SCN neurons (Wang and Huang, 2004, 2006). Again however no role of the NKA in pacemaking was revealed from these investigations.

In this study the electrical, synaptic and molecular properties of the central circadian system of +/+ and Myk/+ animals were investigated using in vitro slice and explant preparations of SCN tissue. Electrical and synaptic properties of individual and population-level SCN neurons were determined using whole-cell patch clamp electrophysiology and fura-2(AM) based calcium imaging. Molecular rhythms were assessed through long-term tissue culture of SCN explants from Myk/+ mice crossed on to the bioluminescent clock gene reporter line PER2::LUC.

Our results revealed that long-term molecular rhythms in Myk x PER2::LUC animals were comparable to +/+ animals and did not recapitulate the behavioural phenotypes of Myk/+ and Myk x PER2::LUC observed in vivo. Whole-cell current clamp recordings from Myk/+ SCN neurons revealed single cell electrical output comparable to +/+ neurons. When analysed at population level however, a dampening of diurnal electrical rhythms was apparent suggesting at changes to network properties and pacemaker output in the Myk/+ SCN. Fura-2 imaging further revealed phase-specific changes to post-synaptic Ca$^{2+}$-dependent response to the major SCN neurotransmitter GABA and the synthetic glutamate receptor agonist AMPA.

Together these data suggest that despite an ex vivo molecular oscillator that is robust and comparable to +/+ and normal single-cell electrical output, the network properties of the SCN are altered with a reduction in output that may underlie abnormal free-running and phase re-setting behaviour in the Myk/+ model. We hypothesise that rather than a direct effect within the SCN clockwork these effects may be due to altered afferent signals to the SCN and opens up the question as to whether the circadian disruption can be induced through external system perturbation in models of neuropsychiatric disorders.
Methods

Animal Housing: Myk/+ animals were originally generated through an ENU mutagenesis screen and backcrossed on to a seizure-resistant C57BL6/NCr for 20 generations. From this same background line, a Myk/+ breeding colony was generously provided by Dr Steve Clapcote (University of Leeds, UK) and maintained on a C57BL6/NCr background.

Myk x PER2::LUC (MykP2) animals were generated through crosses of heterozygous male Myk/+(C57BL6/NCr) and female PER2::LUC animals. All behavioural and in vitro studies were performed on F1 generation animals. PER2:LUC mice are a C57BL6/J mice with a fusion luciferase protein attached to the 3’ end of the mammalian PER2 protein allowing real-time bioluminescent monitoring of clock gene expression (Yoo et al., 2004)

Male and female animals of all backgrounds (aged 2-6 months) were group-housed in a 12:12 LD cycle at constant temperature (~18°C) and humidity (~40%) with food (standard lab chow) and water provided ad libitum. All animal protocols were in accordance with guidelines of the UK Animal (Scientific Procedure) Act 1986.

Wheel-Running Behavioural Characterisation: Male and female +/+ (n=37) and Myk/+ (n=38) mice were individually housed in plastic-based cage-bases (410 x 247 x 124mm) equipped with 160mm lined steel running wheels with food and water provided ad libitum. Animals were maintained in a light-tight wooden coffin and exposed to a 12:12 LD cycle (>250 lux) for a minimum of 14 days before release into DD for a minimum of 14 days to assess free-running rhythms.

Phase Shifting Protocol: Male and female +/+ and Myk/+ animals were exposed to either an Aschoff type I (+/+ =18; Myk/+ =15) or a type II (+/+ =11; Myk/+ =11) phase shifting protocol. Under type I conditions animals were allowed to free-run in DD for 14 days prior to receiving a light pulse. On the day of the pulse, CT12 for each animal was predicted by fitting a regression line to a wheel-running actogram. At the predicted CT (CT14, CT20, CT23) animals were transferred to an adjacent room with the lights on (>250 lux) for one hour before being returned to the coffin. For type II pulses animals were released into DD and the lights in cabinets turned on for one hour at the appropriate CT, calculated relative to ZT0 under 12:12 conditions after one full circadian cycle in DD.
Whole-cell electrophysiology: Hypothalamic coronal brain slices (250µm thick) containing the mid-SCN were prepared from male and female +/+ (n=17) and Myk/+ (n=18) animals (aged 2-6 months) housed under 12:12 LD conditions. To minimise phase-shifting associated with slice preparation, animals were culled between ZT 1-4 and ZT 10-11 (Gillette 1995). Animals were deeply anaesthetised with isofluorane and killed by cervical dislocation and brains removed. Whole-brains were blocked and placed in ice-cold, low Na+, low Ca²⁺, high Mg²⁺ artificial cerebrospinal fluid (aCSF); NaCl 95mM; KCl 1.8mM; KH₂PO₄ 1.2mM; CaCl₂ 0.5mM; MgSO₄ 7mM; NaHCO₃ 26mM; Glucose 15mM; Sucrose 50mM; Phenol Red 0.005mg L⁻¹; pH 7.4; 300-310 mosmol kg⁻¹ pre-bubbled with 95% O₂; 5% CO₂. 250µm slices were cut on an automated vibroslicer (Campden Instruments 7000smz, Loughborough, UK) and continuously perfused with room temperature (20-23°C) recording aCSF; NaCl 127mM; KCl 1.8mM; KH₂PO₄ 1.2mM; NaHCO₃; 26mM; CaCl₂ 4.8mM; MgSO₄ 1.3mM; Glucose 15mM; Phenol Red 0.005mg ml⁻¹; pH 7.4; 300-310 mosmol kg⁻¹) bubbled with 95% O₂; 5% CO₂.

Whole-cell current-clamp recordings were made on an upright light microscope (Olympus BX51WI, Essex, UK) using an npi BA-01X bridge amplifier (npi electronics, Tann, Germany). Gigaohm seal configuration and membrane rupture were performed using high impedance (7-10MΩ) borosilicate glass capillaries from a two-stage pipette puller (PC-10, Narishige, Tokyo, Japan) under differential interface contrast using a 40x water-immersion lens. Pipettes were half-filled with 0.22µm filtered intracellular solution; K-gluconate 120mM; KCl 10mM; MgCl₂ 2mM; K₂-ATP 2mM; Na-GTP 0.5mM; HEPES 20mM; EGTA 0.5mM; pH 7.28 with KOH; Osmolarity 295-300mosmol kg⁻¹; stored on ice to prevent ATP and GTP degradation. Signals were sampled at 30KHz and stored on a personal computer running Spike2 software for analysis (Cambridge Electronic Design, Cambridge, UK).

Calcium Imaging: Hypothalamic coronal brain slices were prepared as described above although cut at 200µm to enhance transparency for fluorescent imaging. Slices were immediately transferred into recording aCSF bubbled with 95% O₂; 5% CO₂ and incubated in leak-resistant Fura-2(AM) (TefLabs, Austin, USA) dissolved in 50µl Pluronic F-127; 20% solution in DMSO (Molecular Probes, Life Technologies, USA) at 37°C for 15 minutes, then at room temperature (20-23°C) for one hour. Once loaded slices were perfused
continuously (2.5ml min\(^{-1}\)) with recording aCSF for 1 hour for de-esterification prior to recording. Imaging of intracellular calcium levels in single neurons and their responses to pharmacological stimulation was performed on an upright Olympus BX51 WI microscope mounted to a vibration-free air table (TMC 63-500 series, MA, USA). Image capture was performed using a water-immersion UV objective (UMplanFL N 20x/0.5 Olympus, Japan) and dual-excitation from two opto-LEDs (Cairn Research, Kent, UK) at 365nm and 385nm every 2 seconds. Excitation emissions were filtered at 510nm and captured by a cooled, high-sensitivity QImaging Rolera EM C2 CCD camera (QImaging, Surrey, Canada) connected to a PC running Optofluor Version 7.7.5.0 (Cairn Research) for offline analysis.

AMPA (Tocris) (5µM, 10µM and 20µM) and GABA (Tocris) (200µM) were bath applied via a gravity-driven perfusion system for 60s and 90s respectively. Responses to drug application was defined as a ratio change of >2 S.D. of 10 consecutive background subtracted points from manually-determined somatic ROIs of individual neurons (Irwin and Allen, 2009).

**Bioluminescence Imaging:** Male and female Myk x PER2::LUC and +/- x PER2::LUC mice were individually-housed in plastic-based cages equipped with 16cm steel, lined running wheels and maintained in a 12:12 LD (>250 lux) cycle for 2 weeks before being released into DD for 14 days to monitor free-running rhythms. On day 15 under DD animals were culled in darkness at CT8 and brains removed and immediately placed in ice-cold Hank’s Buffered Saline Solution (NaHCO\(_3\)-supplemented HBSS, Sigma, UK) supplemented with 10mg\(\text{ml}^{-1}\) penicillin-streptomycin (Gibco Invitrogen, Paisley, UK) and 0.01M HEPES (Sigma, UK). Brains were blocked and mid-coronal hypothalamic slices (250µm) cut using a manual vibroslicer and placed in a sterile 6-well holding chamber (Campden Instruments, Loughborough, UK) and bilateral SCN explants, dissected approximately half way between the dorsal tip of the third ventricle and SCN, and at the distal ends of the lateral anterior hypothalamic nuclei, were made using a dissecting microscope. Explants were transferred on to permeable 30mm, 0.4µM PTFE inserts (Millipore, Watford, UK) inside 35mm culture dishes (Corning, Tewksbury, USA) that contained 1ml luciferin-supplemented, sterile neuronal culture media; Dulbecco’s Modified Eagle Medium (D-2909 Sigma, Gillingham, UK) 3.5g/l D-glucose (Sigma) 0.035% NaHCO\(_3\) (Sigma) 0.1M HEPES (Sigma) B27 serum-free media (Gibco Invitrogen, UK) 0.1mM beetle luciferin potassium salt (Promega,
Southampton, UK). Dishes were lined with autoclaved vacuum grease (Dow Corning Ltd, Coventry, UK) and sealed with glass UV-treated borosilicate glass, 0.13mm coverslips (VWR, Lutterworth, UK). Dishes were then transferred into light-tight incubator units (Galaxy-R+RS Biotech) maintained at 37°C and 5% CO₂ and left undisturbed for a minimum of 7 days. Bioluminescence was recorded as total photon counts using Photon-Multiplier Tubes (H8259/R7518P, Hamamatsu, UK) and integrated every 299s for 3 minutes.

Data Analysis: Behavioural data was analysed using Kit Analyze version 1.06 (Stanford Software Systems, Santa Cruz, CA) and statistically analysed by un-paired t-test or non-parametric equivalents (SPSS Version 16, GraphPad Prism Version 6.0). Analysis of bioluminescence data was performed in GraphPad Prism V6.0 and genotype differences assessed by un-paired t-test. Electrophysiological data was analysed in Spike 2 Version 6.08 and compared statistically by un-paired t-test or one-way ANOVA in Prism or SPSS. Calcium imaging data was extracted in Optofluor software Version 7.7.5.0 and statistically analysed by un-paired t-test and one-way ANOVA in Origin Pro Version 9 (OriginLab, Northampton, USA). All tests performed were parametric unless specific non-parametric equivalents are stated. Significance thresholds were set at p=0.05 and corrected by Bonferroni or Tukey post-hoc for pair-wise comparisons when necessary.
Results

Myk/+ animals show disrupted and unstable behavioural rhythms, altered phase resetting and masking response to light: By using a greater number of animals under longer durations in DD several novel phenotypes were apparent in Myk/+ animals not previously reported (Kirshenbaum et al., 2011). Under a 12:12 LD cycle Myk/+ animals generally entrained their wheel-running activity to the onset of the dark phase, with a minority (3 of 38) unable to align their rhythms with the LD cycle. Under this schedule however, Myk/+ animals displayed higher light-time activity with a characteristic lengthened alpha duration that continued into the light-phase (Fig 1A-C). Upon release into DD Myk/+ animals demonstrated lengthened tau (+/+; 23.72 ± 0.03h; Myk/+: 24.22 ± 0.06; p<0.0001) and a marked increase in alpha duration, with some animals running almost continuously across the circadian day (Fig 1D-E; +/+; 13.14 ± 0.26h; Myk/+; 18.99 ± 0.37h; p<0.0001). In free-running conditions Myk/+ animals also consistently displayed low amplitude free-running rhythms, which in some animals degenerated into arrhythmia (Fig 1F; +/+; 43.8 ± 3.1%Var; Myk/+; 27.73 ± 2.4%Var; p=0.0001). Behavioural rhythms also demonstrated rapid alterations in tau that could appear following environmental stimulation by light, arousal or seemingly spontaneously.

The responses of the circadian system to light Myk/+ animals was assessed through both Aschoff type I and type II photic phase re-setting protocols. Across both paradigms Myk/+ mice exhibited small increases in the magnitude of phase delays to light pulses applied during the early subjective night (CT14; Fig 1I-J). Myk/+ cohorts also exhibited increases in the length of the delay portion of a putative phase response curve (PRC; Fig 1G-J). This trait is evident as a persistence of phase delays in Myk/+ mice into the late subjective night (CT20). At this same circadian time point, +/+ animals exhibited a clear transition into the advance portion of their putative PRC.

At CT23 Myk/+ animals exhibited either small phase advances up to 1.1h or no discernible shift. These data suggest that CT23 as a transition point for Myk/+ animals towards the advance portion of their PRC that arises much later into the subjective night (CT23) than +/+ (Fig 1G). These data together demonstrate a putative PRC in Myk/+ animals with an
Figure 1: Circadian behavioural summary of Myk/+ animals: A: Example +/+ actogram in LD and DD. B-C: Example Myk/+ actograms. Note the higher light-time activity often observed at the end of the active phase (red boxes). D: Free-running tau is longer in Myk/+ animals (+/+, 23.72 ± 0.03h; Myk/+, 24.22 ±0.06h; p<0.0001). E: Lengthened alpha duration in Myk/+ mice (+/+, 13.14 ± 0.26h; Myk/+, 18.99 ± 0.37h; p<0.0001). F: Reduced amplitude free-running rhythms in Myk/+ cohort under DD (+/+, 43.8 ± 3.1%; Myk/+, 27.73 ± 2.4%; p=0.0001). G-H: Examples of altered phase re-setting in Myk/+ animals at CT20. Myk/+ mice exhibit phase delays at this time of the subjective night as +/+ animals display phase advances. I: Individual response to type I light pulses. J: Individual response of animals to type II light pulses. Myk/+ mice maintain phase delay responses at CT20. K: Example of impaired negative masking in Myk/+ animals exposed to 8h light during the dark phase. L-M: Myk/+ mice show increased wheel-running activity when light is applied at night in both total (+/+, 45 ± 13 revs hr⁻¹; Myk/+, 238 ± 62; Mann-Whitney; p=0.05) and relative wheel revolutions (+/+, 0.13 ± 0.05 relative wheel revs; Myk/+, 0.52 ± 0.12 relative wheel revs; Mann-Whitney; p=0.02).
altered shape owing to an extended delay portion and heightened early amplitude of phase shifts.

Given increased light-time activity and abnormal phase re-setting in $\text{Myk}^+/+$ mice, acute behavioural responses to light were determined through photic exposure during the dark phase of a 12:12 LD cycle for short (1h) or long (8h) durations. $\text{Myk}^+/+$ animals displayed a variable although marked reduction in the suppressive effects of light on locomotor output, with very little inhibition observed in some examples (Fig 1K-M). These data suggest that alongside altered circadian responses to photic input, $\text{Myk}^+/+$ animals exhibit a general deficit in the negative masking effect light typically exerts on locomotor output.

*The molecular clock of $\text{MykP2}$ animals does not mimic circadian behavioural phenotype and maintains stable and robust oscillations ex vivo:* Circadian behaviour is regulated at the level of single cells by an intricate molecular clock. Aberrant function of elements within this oscillator directly results in changes to SCN function and behavioural output. Due to the range of abnormal behavioural phenotypes observed in vivo, we hypothesised that molecular rhythms and ex vivo pacemaking of the $\text{MykP2}$ SCN may be altered. To determine the oscillatory properties of the $\text{MykP2}$ master pacemaker, the behaviour of individual animals was characterised followed by long-term bioluminescence recordings of both $\text{MykP2}$ and $+/+P2$ SCN explants.

To confirm similarities to the $\text{Myk}^+/+$ behavioural phenotype both $+/+$ and $\text{MykP2}$ animals were individually-housed in wheel-equipped cages and allowed to free-run for 14 days prior to removal of SCN tissue. $\text{MykP2}$ animals exhibited long tau, long alpha and reduced amplitude rhythms in free-running conditions (Fig 2A-D). Induced or spontaneous alterations were not observed in $\text{MykP2}$ animals, but due to the lack of external stimulation administered to this cohort the presence of this trait cannot be discounted.

Following 14 days of free-running SCN explants were removed and bioluminescence recorded for a minimum of 7 days. Interestingly there were no major deficits in the expression of clock gene expression in $\text{MykP2}$ SCN in relation to the rhythms expressed by $+/+$ tissue (Fig 2E-I). Since $\text{MykP2}$ mice exhibit lengthened free-running tau, it is possible that the $\text{MykP2}$ SCN cycles with a longer period than the $+/+$ SCN. The period of PER2::LUC oscillations however was 0.65h shorter in $\text{MykP2}$ animals relative to $+/+$.
Figure 2: Wheel-running behaviour but not SCN molecular pacemaking properties of MykP2 animals reflects Myk/+ circadian behavioural phenotype: A: Example actogram of male MykP2 animal maintained in a 12:12 LD cycle then released into DD for 14 days before the SCN was removed for bioluminescence recording. MykP2 behavioural phenotype reflect those of Myk/+ animals; B: MykP2 animals exhibit longer free-running period in DD (+/+; n=11; 23.88 ± 0.03h; MykP2; n=5; 24.38 ± 0.05h; p<0.0001) C: Alpha duration is consistently lengthened in MykP2 animals (+/+; 14.16 ± 0.40h; MykP2; 19.09 ±0.83h; p<0.0001). D: Free-running amplitude, measured by chi-squared periodogram, is lower in MykP2 animals in DD conditions (+/+; 57.2 ± 4.3%V; MykP2; 32.8 ± 5.6%V; p=0.005). E: Raw bioluminescence traces from +/+ (blue) and Myk/+ (red) SCN explants. F: Example de-trended trace of +/+ and Myk/+ SCN bioluminescent recordings. G: Mean period of PER2::LUC oscillations (+/+; n=11; 24.69 ± 0.10h; MykP2; n=5; 24.39 ± 0.12; p=0.09) H: Mean amplitude of SCN explant molecular oscillations (+/+; 6123 ±512 A.U.; MykP2; 4878 ± 472 A.U.; p=0.15). I: Half-life of PER2::LUC oscillations exhibits no genotype differences (+/+; 66.18 ± 4.6h; MykP2; 75.73 ± 7.8h; p=0.28).
although this difference was not statistically significant (Fig 2G). *In vitro* the MykP2 SCN did show slight reductions in the initial amplitude of PER2::LUC rhythms, reflective of the initial states of the SCN post-dissection, but again this deviation from the amplitude of +/+ rhythms was not statistically significant (Fig 2H).

The long-term pacemaking of the SCN can also be assessed during *ex vivo* PER2::LUC culture with compromised network function resulting in faster dampening of oscillations (Guilding et al., 2013). Once more MykP2 rhythms did not reveal any changes in function, with damping rate of tissue-level clock gene expression, measured as half-life of oscillations, comparable to +/+ (Fig 2I). These results suggest that the range of behavioural deficits seen in Myk/+ and MykP2 is not accompanied by consistent changes in the SCN molecular clock. On the contrary when the MykP2 SCN is isolated in this paradigm, the SCN of MykP2 animals appears to operate as a robust and functional oscillator, akin to those of +/+ animals.

**Myk/+ SCN neurons retain individual electrophysiological output but exhibit dampened population-level rhythms:** The principle output pathway of the SCN arises from a day-night rhythm in the electrical output of their constituent neurons. As molecular rhythms appear normal in MykP2 SCN explants and the NKA α3 subunit is known to affect membrane excitability, it was hypothesised that the electrical properties of individual SCN neurons may be altered in Myk/+ animals. To investigate single-cell and population level electrical activity, whole-cell current-clamp recordings were made over the complete 24-hour day-night cycle.

Within +/+ and Myk/+ slices, individual SCN neurons displayed electrical states during whole-cell recording that have been previously reported in the literature. These behaviours include regular and irregular firing neurons, hyperpolarised low frequency firing cells and highly depolarised neurons that arise during the day (Fig 3A-E; Belle et al., 2009; Kononenko and Dudek, 2004; Pennartz et al., 1998). When cells were analysed as an entire population between ZT0-24, no genotype differences were apparent in resting membrane potential (RMP) or spontaneous firing rate (SFR) of SCN neurons (Fig 3F,G).
Figure 3: Similar electrical behaviours in +/+ and Myk/+ SCN whole-recordings: +/+ and Myk/+ neurons exhibited similar whole-cell configuration electrical states A: Example irregular firing neuron (IF) from +/+ (left; RMP; -40mV, 0.6Hz) and Myk/+ (right; -39mV; 0.8Hz) SCN slices. B: Regular firing neuron examples (+/+; -48mV; 3.6Hz; Myk/+; -49mV, 3.4Hz). C: Depolarised silent cells (+/+; -30mV; Myk/+; -32mV). D: Depolarised cells exhibiting low amplitude membrane oscillations (+/+; -32mV; Myk/+; -32mV). E: Hyperpolarised neuron (+/+; -61mV; Myk/+; -58mV). In all examples lower dashed line represents RMP and upper dashed line 0mV. Black bars represent 1s and 10mV. F-H: Whole-cell properties of all neurons recorded across the day-night cycle in +/+ and Myk/+ SCN populations. F: Mean RMP of+/+(n=135) and Myk/+ (n=136) neurons was extremely similar (+/+; -42.97 ± 0.58mV; Myk/+; -43.02 ± 0.62; p=0.95). G: Mean SFR of all recorded SCN neurons that fired action potentials during recordings (+/+; 2.05 ± 0.14; Myk/+; 2.02 ± 0.14; p=0.85) H: Input resistance across all neurons shows an increase in Myk/+ SCN neurons (+/+; 3.37 ± 0.10GΩ; Myk/+; 3.82 ± 0.10GΩ; p=0.002). These data suggest that lnRes is increased in Myk/+ recordings, although this change is compensated to not overtly affect electrical output and behaviours.
There was however, a general increase in the input resistance (InRes) of Myk/+ neurons (Fig 3H). This suggests that there is an alteration in K⁺ conductance or driving force in Myk/+ SCN neurons. These changes appear adequately compensated so RMP and SFR, the major output of the individual neurons, are not overtly affected.

As single SCN neurons exhibit heterogeneity in their spontaneous state, neurons were categorized by behaviour to determine if the Myk/+ genotype affected a sub-type of neuronal behaviour within the SCN. Neurons were characterised based on resting membrane potential (RMP), spontaneous firing frequency (SFR) and coefficient of variation (CV) into four categories; regular firing (RF), irregular firing (IF), depolarized (DPz) and hyperpolarised cells (HPz; Fig 3; Table 1).

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>RMP (mV)</th>
<th>SFR (Hz)</th>
<th>Input Res (GΩ)</th>
<th>Afterhyp (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regular Firing</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/- (n=37)</td>
<td>-42.4 ± 0.9mV</td>
<td>3.1 ± 0.2Hz</td>
<td>3.3 ± 0.2GΩ</td>
<td>21.2 ± 0.8mV</td>
</tr>
<tr>
<td>Myk/+ (n=38)</td>
<td>-43.9 ± 0.6mV</td>
<td>3.0 ± 0.1Hz</td>
<td>3.8 ± 0.2GΩ</td>
<td>21.7 ± 0.7mV</td>
</tr>
<tr>
<td>Irregular Firing</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/- (n=42)</td>
<td>-40.7 ± 0.6mV</td>
<td>1.4 ± 0.1Hz</td>
<td>3.0 ± 0.2GΩ*</td>
<td>20.3 ± 0.6mV</td>
</tr>
<tr>
<td>Myk/+ (n=42)</td>
<td>-41.3 ± 0.5mV</td>
<td>1.3 ± 0.1Hz</td>
<td>3.7 ± 0.2GΩ*</td>
<td>20.4 ± 0.7mV</td>
</tr>
<tr>
<td>Depolarised</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/- (n=22)</td>
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<td>NA</td>
<td>3.4 ± 0.2GΩ</td>
<td>NA</td>
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<tr>
<td>Myk/+ (n=24)</td>
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<td>NA</td>
<td>3.6 ± 0.2GΩ</td>
<td>NA</td>
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<tr>
<td>Hyperpolarised</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>+/- (n=34)</td>
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<td>3.5 ± 0.2GΩ</td>
<td>NA</td>
</tr>
<tr>
<td>Myk/+ (n=32)</td>
<td>-51.9 ± 1.0mV</td>
<td>NA</td>
<td>4.0 ± 0.2GΩ</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 1: Sub-classification of SCN neurons based on electrical properties: Neurons were grouped based on basic electrical properties. Regular firing; FR>0.3Hz and CV<0.25; Irregular firing; FR>0.3Hz; CoV>0.25; Depolarised; RMP> -40mV; neuron shows low-amplitude membrane oscillatory behaviour and/or requires hyperpolarising current to fire action potentials; Hyperpolarised; SFR<0.3Hz; RMP< -45mV. Little difference was observed in the basic electrical properties of +/- and Myk/+ neurons with only input resistance in irregular firing neurons showing a significant difference between genotypes (p=0.016). RMP varied little between +/- and Myk/+ recordings, as did SFR. Afterhyperpolarisation was also unaffected in spontaneously active Myk/+ neurons, suggesting little change in calcium-dependent hyperpolarising conductance.
Although grouping SCN neurons into distinct sub-categories has proved difficult in the past (Kononenko and Dudek, 2004; Pennartz et al., 1998), these coarse sub-divisions successfully distinguished neurons into what were visibly distinct behaviours, with CoV proving a useful parameter in defining a clear boundary between IF and RF neurons.

Neither RF and IF neurons exhibited genotype differences in RMP, SFR or afterhyperpolarisation (AHP) that suggested little difference in the electrical output and calcium-dependent conductances in Myk/+ cells (Table 1). There was however, a significant increase in input resistance in Myk/+ IF neurons, and RF neurons showed higher InRes that approached but did not reach statistical significance. As HPz and DPz neurons rarely fire spontaneous action potentials, measurement of SFR and AHP was not possible, RMP and InRes however, did not significantly deviate between +/+ and Myk/+ cells. These results suggest that the population-level difference in InRes arises mainly from the spontaneously active RF and IF neurons within the SCN. No effect on RMP or SFR was apparent in any sub-population, suggesting that despite these changes the electrical output of individual Myk/+ SCN neurons is not altered.

Electrophysiological output of the SCN is not defined strictly by the activity of single neurons, rather the activity of the SCN population as a whole. The temporal organisation of the SCN determines a coherent network of electrical rhythms characterised by a peak in excitability during the day and a trough at night.

In +/+ animals, a clear day-night difference in RMP was apparent with a ~3.1mV higher RMP during the day than observed at night (Fig 4A,B,G). In Myk/+ animals this overt rhythm was dampened and statistically absent, characterised by a decrease in mean RMP of ~1.0mV in the day relative to the night (Fig 4C,D,H). This lack of diurnal rhythmicity was also apparent in the firing output of the SCN. +/+ neurons displayed a 66% overall increase in firing during the day relative to night (Fig 4E,I). Comparatively Myk/+ SCN neurons only display a 15% increase in SFR during the day which also failed to approach significance thresholds (Fig 4F,J). Given the even populations of neuronal sub-types sampled between genotypes and the similar baseline RMP and SFR of individual neurons,
Figure 4: Population-level electrical output is dampened in the Myk/+ SCN. A: RMP of all neurons recorded in +/+ animals. Open circles represent 4hr means (n=135 cells) B: RMP of +/+ neurons plotted in 4-hour bins show a clear day-night progression, increasing across the day and decreasing throughout the night C: Individual RMP of all recordings from Myk/+ SCN neurons (n=136 cells). D: RMP of Myk/+ neurons in 4 bins. There is no clear day-night difference or pattern of RMP progression as seen in +/+ SCN neurons, RMP appears less well-organised across the day-night cycle E: SFR of +/+ SCN neurons in 4-hour time bins show a clear peak that increases during the day that falls during the night (ANOVA; p=0.004; Bonferroni; ZT4-8 vs. ZT8-12 vs. ZT20-24; p<0.05; ZT8-12 vs. ZT20-24; p<0.05). F: Myk/+ SFR shows a less clear day-night difference in SFR with a pronounced nadir between ZT4-8 the time of peak SCN output (ANOVA; p=0.39) G-J: Across all recordings, +/+ neurons display a day-night difference in RMP and SFR, both of which are statistically absent in the Myk/+ SCN. G: +/+ day-night mean RMP (Day; 41.02 ± 0.80mV; Night; 44.16 ± 0.84mV; p=0.009). H: Myk/+ day-night mean RMP (Day; 43.46 ±0.83mV; Night; 42.42 ± 0.89mV; p=0.39). I: +/+ day-night mean SFR (Day; 2.01 ± 0.22Hz; Night; 1.40 ± 0.17Hz; p=0.003). J: Myk/+ day-night mean SFR (Day; 1.66 ± 0.19mV; Night; 1.44 ± 0.18mV; p=0.40).
the amplitude differences observed across the day-night cycle therefore appear due changes in the temporal expression of electrical states.

This apparent de-synchrony of electrical behaviour can be visualised by dividing electrical properties cycle into 4-hour time bins (Fig 4E-F). In +/+ mice RMP and SFR and show a clear progression of excitability, that gradually increases across the day before transitioning to more hyperpolarised, quiescent states at night (Fig 4B). In the Myk/+ SCN, a clear temporal progression of RMP is seemingly absent, with neurons lacking a coherent daily transition, yet with a tendency for more depolarised cells during the day (Fig 4D).

A similar pattern is also observed in the SFR of +/+ slices, which exhibit a gradual increase in firing rate during the day followed by a sustained decrease in night-time output, with a statistically significant time-dependent variation (Fig 4E). In Myk/+ animals a small increase in SFR is apparent during the day, although with a pronounced nadir between ZT4-8, a time at which SCN neurons typically exhibit higher firing frequencies (Fig 4F). These data support a lack of temporal organisation in the electrical states of neurons within the Myk/+ SCN over a 24-hour cycle.

*Altered early-night post-synaptic responses to GABA and AMPA in Myk/+ animals:* Inter-cellular communication and afferent synaptic inputs are critical to the network properties and phase-shifting responses of the SCN. As deficits in behavioural responses to light and altered electrical activity are observed in Myk/+ animals, changes to synaptic communication may be a crucial factor in this behaviour. To study post-synaptic responses in Myk/+ neurons, Fura-2 Ca^{2+} imaging was used to assess responses of SCN neurons to major neurochemical stimuli. As light is the major zeitgeber of the SCN, via the glutamatergic retinohypothalamic tract, the synthetic glutamate agonist AMPA was applied during the day and night. GABA is the most abundant neurotransmitter expressed within the SCN and plays a major role in determining synchrony and architecture of the SCN network and was also applied to +/+ and Myk/+ neurons.

+/+ and Myk/+ slices were treated for 60s with 5 and 10µM concentrations of AMPA between ZT14-18, the early portion of the subjective night and within the SCN’s typical window of photic sensitivity (Fig 5A). At these times Myk/+ SCN neurons displayed significantly larger rises in intracellular Ca^{2+} in response to both 5 and 10 µM
Figure 5: Heightened early-night AMPA response in Myk/+ SCN: A: Example false colour image of a single SCN nucleus loaded with the Fura-2(AM); Dashed outline represents approximate SCN outline; 3V; third ventricle; OX; optic chiasm; B: Mean Ca$^{2+}$ increase following AMPA application ZT4-10. +/- and Myk/+ SCN neurons displayed similar responses to 20µM application of AMPA (+/-; 29.8x10^{-2} ± 1.4x10^{-2}; fura-2 ratio A.U.; 32.6x10^{-2} ± 1.9x10^{-2} fura-2 A.U.; p=0.22). C: AMPA washout latency. +/- and Myk/+ SCN neurons show similar latency to recover to baseline Ca$^{2+}$ levels following 20µM treatment with AMPA (+/-; 340 ± 17s; Myk/+; 365 ±15s; p=0.27). D: Example trace of individual +/- (blue) and Myk/+(red) SCN neuronal, intracellular calcium responses to application of AMPA between ZT14-18. Myk/+ neurons show larger increases in calcium to both 5 and 10µM doses of AMPA. E: ZT14-18 mean Ca$^{2+}$ concentration change. Myk/+ SCN neurons show larger Ca$^{2+}$ increases to 5µM (ANOVA; p<0.0001; Bonferroni correction; +/-; 16.2x10^{-2} ± 0.8x10^{-2} fura-2 A.U.; Myk/+; 21.8x10^{-2} ± 1x10^{-2} A.U.; p=0.003) and 10µM (+/-; 22.1x10^{-2} ± 1.1x10^{-2} fura-2 A.U.; Myk/+; 35.2x10^{-2} ± 1.5x10^{-2}; p<0.0001) doses of AMPA. F: ZT14-18 AMPA washout latency. Myk/+ SCN neurons exhibit increased latency to return to baseline Ca$^{2+}$ following 10µM AMPA (ANOVA; p<0.0001; Bonferroni; +/-; 172 ± 4s; Myk/+; 209 ± 8s; p<0.001) although no differences were observed in washout time following treatment with 5µM AMPA (+/-; 121 ± 4s; Myk/+; 121 ± 4s; p>0.99).
concentrations of AMPA than observed in +/+ neurons. The concentration change induced by the application of AMPA was ~38 and 59% greater magnitude respectively (Fig 5B,C). The latency of cells to recover to baseline Ca\(^{2+}\) levels was also significantly longer in Myk/+ SCN neurons following 10\(\mu\)M application of AMPA (172 ± 4s vs. 209 ± 8s; p<0.001), although 5\(\mu\)M treatment exhibited no difference to +/+ neurons in washout latency (Fig 5D). During in vivo characterisation, Myk/+ animals demonstrated heightened phase shifting responses to light at CT14. A heightened post-synaptic response to glutamatergic release represents a potential mechanism of heightened phase-shifting in Myk/+ mutants.

AMPA application (20\(\mu\)M) during the subjective day (ZT4-10), a phase of the circadian cycle when photic re-setting is normally gated, elicited similar amplitude rises in intracellular Ca\(^{2+}\) from both +/+ and Myk/+ SCN neurons (Fig 5E). Latency to recover to baseline Ca\(^{2+}\) levels was also comparable between genotypes (Fig 5F). These data reveal that changes to post-synaptic AMPA responses appear restricted to the early-night of the circadian cycle and increased amplitude changes in Ca\(^{2+}\) concentration are not a constitutive property of Myk/+ SCN neurons.

To determine if post-synaptic alterations to GABA signalling arise within the Myk/+ SCN, 200\(\mu\)M doses were applied during both day and night for 90s. SCN cells can show excitatory or inhibitory responses to GABA both in terms of electrical excitability and in changes to intracellular Ca\(^{2+}\) levels (Choi et al., 2008; Irwin and Allen, 2009). Both post-synaptic tones of GABAergic responses were observed in +/+ and Myk/+ SCN neurons (Fig 6A). Between ZT4-10 +/+ and Myk/+ neurons demonstrated changes to intracellular Ca\(^{2+}\) levels in response to GABA of comparable amplitude. Similar magnitude responses were clear in the case of both rises (excitation) and falls (inhibition) in Ca\(^{2+}\) concentration (Fig 6A-B).
Figure 6: Altered early-night GABA signalling in Myk/+ SCN: (A-B; Day ZT4-10; C-F; Night ZT 14-18); A: Example traces of individual cellular responses to 200µM bath application of GABA for 90s. SCN neurons can show an excitatory increase in Ca\(^{2+}\) in response to GABA, or an inhibitory decrease of intracellular Ca\(^{2+}\), with typical examples shown. B: Amplitude of responses to 200µM GABA during the day (ZT4-10). Myk/+ SCN neurons show no difference in either increases (+/+; 14.4x10\(^{-2}\) ± 1.3x10\(^{-2}\) fura-2 A.U; Myk/+; 13.3x10\(^{-2}\) ± 1.5x10\(^{-2}\) A.U; p=0.61) or decreases (+/+; -14.2x10\(^{-1}\) ± 0.6x10\(^{-2}\) A.U; -15.5x10\(^{-2}\) ± 1.3x10\(^{-2}\) fura-2 A.U; p=0.37) in Ca\(^{2+}\) during this phase of the day. C: Representative trace of individual +/+ (blue) and Myk/+ (red) neurons responding with a Ca\(^{2+}\) increase to 200µM GABA at night (ZT14-18). Myk/+ SCN neurons show a small increase in the magnitude of intracellular Ca\(^{2+}\) rise during the early night. D: Amplitude of +/+ and Myk/+ population-level responses to GABA (ZT14-18). +/+ and Myk/+ show similar amplitude decreases in Ca\(^{2+}\) (+/+; -13.0x10\(^{-2}\) ± 0.7x10\(^{-2}\) fura-2 A.U; Myk/+; -12.0x10\(^{-2}\) ± 1.0x10\(^{-2}\); p=0.55) yet larger magnitude Ca\(^{2+}\) excitation (+/+; 9.6x10\(^{-2}\) ± 1x10\(^{-2}\); Myk/+; 12.3x10\(^{-2}\) ± 0.6x10\(^{-2}\); p=0.028). E-F: Proportion of +/+ and Myk/+ neurons displaying Ca\(^{2+}\) increases or decreases to GABA during the day (E) or night (F). During the day +/+ and Myk/+ show a greater proportion of neurons showing a decrease in Ca\(^{2+}\) (Ca\(^{2+}\) decrease; +/+; 51/66; Myk/+; 47/71; χ\(^2\) p=0.19). At night +/+ animals show a small increase in the number of neurons responding with Ca\(^{2+}\) increases yet Myk/+ animals display a complete reversal of GABAergic tone, with few cells showing decreases in Ca\(^{2+}\) and the majority exhibiting excitatory responses increases (Ca\(^{2+}\) decrease; +/+; 64/87; Myk/+; 18/78; χ\(^2\); p<0.0001).
During the early night (ZT14-18) the amplitude of responses in neurons exhibiting decreases in Ca$^{2+}$ following GABA treatment showed little difference between genotypes (Fig 6D). Within neurons that responded to GABA with a rise in intracellular Ca$^{2+}$ however, Myk/+ cells responded with larger amplitude changes in concentration than +/- (Fig 6C-D; +/-; 9.6x10$^{-2} \pm 1.0x10^{-2}$ fura-2 ratio A.U; Myk/+; 12.3x10$^{-2} \pm 0.6x10^{-2}$ fura-2 ratio A.U; p=0.03).

In addition to changes in post-synaptic amplitude of Ca$^{2+}$ in response to GABA between ZT14-18, a further change was observed in GABAergic responses within the Myk/+ SCN, with increased excitatory tone. Between ZT4-10 both genotypes demonstrated similar cellular responses, with a clear tendency for inhibitory Ca$^{2+}$ responses (Fig 6E; +/-; 77% inhibition; Myk/+; 66% inhibition; $\chi^2$ p=0.54). Between ZT14-18 +/- slices maintained this trend with a greater proportion of neurons responding with Ca$^{2+}$ decreases. During the early night however, Myk/+ neurons exhibited a reversal of GABAergic tone, with the majority of cells responding with excitatory increases in Ca$^{2+}$ (Fig 6F; +/-; 74% inhibition; Myk/+; 23% inhibition; $\chi^2$ p<0.0001).

GABAergic signalling within the Myk/+ SCN appears altered in a phase –specific manner with changes to amplitude and post-synaptic tone during the early night (ZT14-18). Interestingly changes to post-synaptic AMPA responses were restricted to this phase of the diurnal cycle in Myk/+ neurons highlighting this epoch of the early night as a potentially critical time within the physiology of the Myk/+ SCN.
Discussion

Altered behavioural pacemaking and responses to light: During initial characterisation as a model of mania Myk/+ animals were reported to exhibit long tau and alpha under free-running conditions (Kirshenbaum et al., 2011). Here we report a broader range of phase, light-dependent and free-running phenotypes that reveal greater disruption to central circadian pacemaking. Myk/+ animals express a continuum of labile phenotypes characterised by reduced rhythm stability and an increased susceptibility to external perturbation. Myk/+ animals typically express lengthened tau and a marked increases in alpha that occupies the majority of the active phase with consolidated activity. The amplitude of free-running rhythms is consistently reduced in Myk/+ animals who also exhibit induced or spontaneous changes in the length of tau. Integration of photic input was also found to be abnormal, with alterations in phase shifting responses across the subjective night. Myk/+ animals exhibited heightened early-night responses to light and changes to the shape of putative PRC with an extension of the delay portion and advances arising much later into the circadian cycle. Increased light-phase activity was also observed in Myk/+ that appears attributable at to reductions in the suppressive effects of light on locomotor activity.

Animal models that display such circadian abnormalities in behaviour often also exhibit changes to the molecular and electrical output of the SCN that can reflect both these pacemaking properties in vitro or in situ (Aton et al., 2005; Brown et al., 2007; Feillet et al., 2006; Guilding et al., 2013; Maywood et al., 2011; Meng et al., 2008; Vitaterna et al., 2006). Ex vivo monitoring of molecular rhythms from MykP2 animals revealed however that the Myk/+ behavioural phenotype is not reflected in the molecular properties of the SCN. Changes to the function of the SCN were observed in electrical output from whole-cell electrophysiology. Population-level rhythms in electrical output were dampened in the Myk/+ SCN and there was also early-night changes in the tone and amplitude of AMPA and GABAergic responses that suggest network alteration independent of the TTFL.

Pacemaking of the Myk/+ SCN is attenuated through electrical output and synaptic integration independent of the molecular clock: The circadian free-running behaviour of the MykP2 model was first validated in its phenotypic similarity to Myk/+ animals with
lengthened tau, alpha and reduced free-running amplitude. The same parameters can reflect the intrinsic pacemaking of the molecular oscillator as well as the overall molecular output of the SCN tissue. Other models such as Afh, Bmal1<sup>−/−</sup>, Vip2r<sup>−/−</sup> and CK1ε<sup>−/−</sup> exhibit changes to PER2::LUC oscillatory dynamics of the SCN that reflect the behavioural output in tau and rhythm amplitude of these animals (Freeman et al., 2013; Godinho et al., 2007; Guilding et al., 2013; Ko et al., 2010; Maywood et al., 2011; Meng et al., 2008). The lack of genotype differences in PER2:LUC oscillations or correlations with free-running behaviour strongly suggests that the Myk/+ mutations does not directly impact on central clock gene oscillations. Under this ex vivo paradigm the network properties of the SCN instead remain intact without overt deficits in the long-term persistence of oscillations.

Whole-cell recordings revealed similar output of RMP and SFR from single cells recorded from both genotypes, suggesting that dysfunction is not apparent within the membrane properties of individual neurons. Isolated SCN neurons function as independent electrical oscillators yet fall out of phase in the absence of synaptic and paracrine signals from the wider network, resulting in reduced population synchrony and amplitude (Maywood et al., 2006; Liu et al., 2007). We suggest that reduced intercellular synchrony underpins attenuated day-night output of Myk/+ electrical rhythms and is apparent in altered temporal expression of electrical properties, rather than intrinsic cellular output.

Various animal models demonstrating circadian behavioural deficits exhibit concomitant reductions in the amplitude of electrical output. Examples include those lacking key coupling factors Vip<sup>−/−</sup> and its receptor Vip2r<sup>−/−</sup>, ion channels Kcnma1<sup>−/−</sup> (large conductance Ca<sup>2+</sup> activated K<sup>+</sup> channel) or aged animals with weaker behavioural output (Aton et al., 2005; Brown et al., 2007; Cutler et al., 2003; Farajnia et al., 2012). We suggest that the mechanism that affects amplitude SCN electrical output in Myk/+ animals similarly underpins behavioural deficits in vivo.

Changes to early-night GABA signalling also supports our suggestions that the Myk/+ deficit is due to changes in the network properties of the SCN. GABA has emerged alongside VIP signalling as a major neurochemical determinant of SCN synchrony and network structure. GABA is the most abundant neurotransmitter in the SCN and acts to dampen the amplitude of SCN electrical rhythms and de-stabilises the intact SCN network
*in vitro* (Aton et al., 2006; Freeman et al., 2013). Increasing photoperiod reduces the amplitude and alters the spatiotemporal properties of electrical and molecular rhythms in the SCN by de-coupling distinct populations of neurons within the SCN (Evans et al., 2013; Inagaki et al., 2007; VanderLeest et al., 2007). The mechanism of this action has recently been demonstrated to function through GABA that acts to de-couple or synchronise SCN cellular populations depending on the current state of the network (Evans et al., 2013). These properties are accompanied by changes to both frequency and excitatory tone of post-synaptic GABA responses via GABA_\text{A} receptors (Farajnia et al., 2014). In the Myk/+ SCN altered GABAergic signalling may therefore reflect changes to the state of the network. In these photoperiod paradigms, increasing day-lengths also reduces the amplitude of electrical rhythms. The changes to the SCN observed in both GABA and electrical rhythm under long photoperiods agree with our findings to suggest that a similar model of dampened output exists within the Myk/+ SCN (vanderLeest et al., 2007).

**Altered phase re-setting to light as a consequence of network de-synchrony:** A low amplitude SCN network may explain the altered free-running rhythms in Myk/+ animals but does it also reflect changes to phase re-setting responses to light? Altered photic responses to light can be influenced by changes to the molecular, electrical or network properties within the SCN and external factors including those governing non-image forming phototransduction and arousal (Albrecht et al., 2001; Han et al., 2012; Hughes and Piggins, 2008; Panda et al., 2002b; Ramkisoensing et al., 2014). A putative PRC generated in Myk/+ animals suggested an increases in the magnitude of early night phase shifts and a lengthening of the delay portion, extending later into the subjective night. Interestingly at ZT14 Myk/+ neurons exhibited heightened post-synaptic responses to the synthetic glutamate receptor agonist AMPA. AMPA-sensitive mGLuR1, mGluR2 and mGluR3 receptors are expressed within the SCN and AMPA application *in vitro* or *in vivo* can phase shift molecular and behavioural rhythms (Michel et al., 2002; Mizoro et al., 2010). As a non-selective cation channel AMPA may induce phase shifts of molecular rhythms through Ca^{2+}-dependent signalling pathways or through synaptic signalling across the network through increased cellular excitability (Albus et al., 2005; Brown and Piggins, 2007; Hamada et al., 2004; Michel et al., 2002; Rohling et al., 2011). An increased sensitivity to glutamate here demonstrated via glutamate receptor agonists in the early-
night may represent a physiological response within the Myk/+ SCN that underpins heightened behavioural responses.

The mechanism underlying altered post-synaptic responses may also lie within changes to network properties of the SCN. In classic circadian behavioural studies the shape of the PRC in rodent species is dependent on distinct morning and evening oscillators whose interactions are defined by previous exposure to light and duration of behavioural activity (Honma et al., 1984; Pittendrigh and Daan, 1976a, 1976b). The last decade has provided evidence of a potential neurophysiological substrate for these distinct oscillators; temporally disparate populations of neurons within the SCN (Brown and Piggins, 2009; Evans et al., 2013; Inagaki et al., 2007; de la Iglesia et al., 2004). Genetic lesions or environmental perturbations that alter the amplitude and spatiotemporal dynamics of the SCN can subsequently result in changes to phase-shifting responses to light (Evans et al; 2013; Guilding et al., 2013; Ramkisoensing et al., 2014; Vitaterna et al., 2006). Such phase shifts within the SCN depend on communication across the entire network with a prominent role for GABA (Albus et al., 2005; Han et al., 2012). Different phase relationships in the Myk/+ SCN observed in electrical output and altered GABA signalling, may explain changes to a photic PRC that is reflected at a cellular level by increased sensitivity to glutamatergic inputs at phases of the circadian cycle with heightened behavioural shifts.

*Altered SCN output due to intrinsic or extrinsic dysfunction?*: Despite alterations in the output and synaptic properties of the Myk/+ SCN, the cause of underlying network disruption is not immediately apparent. From available data we hypothesise that changes to the SCN function may arise from abnormal afferent inputs relaying environmental and behavioural information to the SCN. In this model, these altered inputs act as the decoupling forces upon the SCN network. Behavioural experiments highlight an increased sensitivity of the Myk/+ SCN to external perturbation. Myk/+ animals can exhibit rapid changes in tau and therefore the pacemaking properties of the SCN following exposure to light or arousal stimulation. This property is unusual in free-running behaviour of animals and suggests either heightened external input or sensitivity of the SCN network to external cues.
In the \textit{Myk/+} model we suggest the SCN may be influenced by external factors that are absent in an \textit{ex vivo} PER2::LUC paradigm. Discrepancies between behavioural and PER2::LUC data have been reported in prokineticin2 receptor (PK2) mice. These animals exhibit altered circadian wheel-running behaviour despite intact SCN PER2::LUC oscillations (Prosser et al., 2007).

The major zeitgebers acting as inputs to the SCN are light and arousal, both of which may impact upon \textit{Myk/+} pacemaking. \textit{Myk/+} animals exhibit increased arousal and locomotor activity in open-field and wheel-running conditions as well as reductions in both REM and non-REM sleep (Kirshenbaum et al., 2011). Longer alpha durations are known to affect PRC shape, including extension to the delay portion of the PRC, and access to wheels can augment the phase shifting capacity of the SCN (Honma et al., 1984; Ramkisoensing et al., 2014). Locomotor feedback can therefore directly affect the SCN network at the level of behaviour.

At a physiological level, extended alpha bouts in \textit{Myk/+} animals may provide strong arousal feedback to the SCN across phases when +/- animals are typically inactive. The neurochemical substrates of arousal on to the SCN include serotonin, orexin, GABA and neuropeptide Y (NPY) afferents from the intergeniculate leaflet (IGL), lateral hypothalamus and raphe nuclei (Belle et al., 2014; Morin and Allen, 2006; Pontes et al., 2010). Serotonin and NPY can phase shift \textit{in vivo} behavioural rhythms and SCN output \textit{in vitro} thus impact on properties of the SCN (Horikawa and Shibata, 2004; Piggins and Antle, 1995; Prosser, 2003). In addition locomotor activity induces rapid and acute suppression of electrical activity \textit{in vivo} within the SCN that can ultimately alter the overall electrical waveform (Houben et al., 2014; van Oosterhout et al., 2012; Yamazaki et al., 1998). NPY application during the subjective day \textit{in vitro} appears as a candidate substrate that suppresses SCN neuronal activity (Besing et al., 2012). Due to lengthened alpha the \textit{Myk/+} SCN may receive abnormally phased arousal input from these centres that act to de-couple intercellular interactions within the SCN and dampen electrical output. Across \textit{Myk/+} electrophysiological recordings there is a tendency towards lower RMP and SFR between ZT0-8. This is a phase of the daily cycle during which \textit{Myk/+} animals maintain locomotor activity and +/- animals generally cease activity.
Changes to photic input may also play a role upon altered network properties in the *Myk/+* SCN. *Myk/+* animals exhibit altered light-dependent behaviours including a deficit in behavioural suppression by light. As negative masking is dependent on both visual and non-visual photic pathways, these behavioural changes may be encoded within photic afferents to the SCN in *Myk/+* animals. The NKA α3 subunit is expressed within the retina and specifically along axons of intrinsically photosensitive retinal ganglion cells that carry photic information to the SCN (ipRGC; Dobretsov and Stimers, 2005; Lucas et al., 2014; Specht and Sweadner, 1984). Increased light exposure reduces both molecular and electrical amplitude of the SCN population, and constant light lengthens tau in WT animals, a mechanism that involves the de-coupling of electrical activity of SCN neurons (Evans et al., 2013; Inagaki et al., 2007; Ohta et al., 2005; VanderLeest et al., 2007). Increased activity of RHT projections to the SCN in *Myk/+* animals therefore represents an upstream physiological factor that may impact upon the network properties of the *Myk/+* SCN.

The possibility remains that the deficit in network properties may be intrinsic to the SCN and not a function of external perturbation. Although no changes in SFR or RMP were apparent, a population-wide increase in input resistance was observed from whole-cell recordings. This suggests potential changes to neuronal SCN function in *Myk/+* animals despite an absence of overt changes to electrical output. NKA α3 subunits can alter spike output and integration of post-synaptic currents within the CNS. The NKA pump can also function within intracellular signalling cascades important within the SCN including ERK, AKT and intracellular Ca$^{2+}$ signalling (Ikeda et al., 2013; Khodus et al., 2011; Kirshenbaum et al., 2011; Shiina et al., 2010; Silva and Soares-da-Silva, 2012; Yu et al., 2010). This activity results from activity as a signal receptor for cardiotonic steroids, found within the hypothalamus, and the pre-synaptic proteoglycan agrin (Kirshenbaum et al., 2012; Murrell et al., 2005; Tymiak et al., 1993). Changes to intracellular signalling cascades or electrical and synaptic information that were not detected by our methods remain a possible factor that define alterations in *Myk/+* circadian timekeeping.

*Central circadian disruption in clock gene models of affective behaviours:* Having characterised the behavioural and neurophysiological circadian system in *Myk/+* animals it is important to present these findings within the context of human BPD and across
other animals models with altered affective behaviour. The major circadian models that exhibit mood disorders concomitantly with circadian disruption include Bmal1−/−, CK1ε−/−, ClockΔ19, Rev-erba−/−, Afh and Per2brdm−/− (Chung et al., 2014; Hampp et al., 2008; Keers et al., 2012; Kondratova et al., 2010; Pilorz et al., 2014; Roybal et al., 2007). Interestingly all of these models exhibit altered phase re-setting, or re-entrainment, to light in addition to low amplitude activity rhythms, with the exception of an amplitude effect on behaviour of Ck1ε−/− and Rev-erba−/− mice (Albrecht et al., 2001; Godinho et al., 2007; Guilding et al., 2013; Pfeffer et al., 2014; Preitner et al., 2002; Vitaterna et al., 1994; Zheng et al., 1999). These phenotypes exhibit are correlated with clinical findings made in human studies of affective disorders including unstable behavioural rhythms and altered phasing of behaviour and circadian output factors (Jones et al., 2005; Rock et al., 2014; Emens et al., 2009; Mansour et al., 2005; Souqtre et al., 1989; Wong et al., 2000). What is not apparent however, is the nature of the underlying deficit to circadian physiology. Animal models currently represent one of our best insights into these possible mechanisms. Myk/+ mutants and the majority of circadian-mood models exhibit changes to SCN function, mainly reported as deficits in molecular pacemaking in situ or ex vivo (Feillet et al., 2006; Guilding et al., 2013; Ko et al., 2010; Vitaterna et al., 2006). Some examples such as ClockΔ19 and CK1ε−/− also express altered electrical output, although with changes manifesting in tau rather than amplitude (Meng et al., 2008; Herzog et al., 1998). A deficit within the central pacemaker is therefore common and may represent the critical factor determining the involvement of circadian disruption upon mood states.

Despite these consistencies, Myk/+ animals represent a slightly different manifestation of central circadian disruption in relation to other circadian animal models. The majority of clock–affect models that are alluded to, possess mutations that result in changes to cellular rhythms or directly to the molecular pacemaking of the SCN when quantified ex vivo or in situ (Feillet et al., 2006; Guilding et al., 2013; Guilding et al., 2013; Herzog et al., 1998; Ko et al., 2010; Vitaterna et al., 2006; Preitner et al., 2012). Changes to molecular rhythms may also affect regions across the brain known to express circadian rhythms such as the hippocampus, VTA and LHB, in addition to effects within the SCN (Abe et al., 2002; Guilding and Piggins, 2007). In addition, there exist various genetic associations between specific circadian clock genes and affective disorders in humans (Etain et al.,
2011; Nievergelt et al., 2006; Shi et al., 2008). These demonstrations imply that the aetiology of circadian abnormalities in these conditions is rooted within disruption to molecular and cellular pacemaking.

*Myk/+* animals appear to possess altered electrical output from the SCN that arises despite seemingly intact molecular pacemaking. This aberrant signal directly from the SCN may represent the critical effector of circadian-mood interactions in *Myk+* mice rather than changes to localised molecular rhythms. The specific output that emanates from SCN efferents is known to be critical in higher processes such as memory formation, and may be equally important in the downstream regulation of mood centres (Fernandez et al., 2014). As the relative importance of changes to rhythms within the SCN or in extra-SCN structures play in affective behaviours remains an outstanding question, the physiology of the *Myk/+* mouse would suggest that any circadian influence on mood is most likely communicated directly via the output signal of the SCN (McCarthy and Welsh, 2012; Menet and Rosbash, 2011). As pre-clinical models express distinct mood behaviours, the nature of this output signal from the SCN may be a critical factor in determining distinct affective phenotypes such as anxiety, reward-seeking or locomotor activity.

In addition, the *Myk/+* model also poses further questions into the aetiology of circadian disruption in neuropsychiatric disorders. If changes to upstream signalling pathways rather than cellular pacemaking are determinants of SCN output as suggested, then circadian disruption may be imposed by changes across the wider CNS. As the pathology of neuropsychiatric disorders involves gross alterations to neurotransmitter signalling, such as dopamine and serotonin systems in affective conditions, then these pathways may directly or indirectly impact on central pacemaking function (Russo and Nestler, 2013; Wulff et al., 2010). Understanding how different neurotransmitter pathways may influence circadian timekeeping may prove important in determining the aetiology of sleep and rhythm disruption in affective disorders.

**Conclusions:** *Myk/+* animals are a model of the manic phase of BPD that exhibit various alterations to circadian behaviours, characterised by abnormal, low amplitude rhythms and altered interactions with light. These behavioural changes appear attributable to
altered network dynamics within the SCN that result in reduced electrical output despite and intact molecular clock.

There appears a consistent trend in many of the *Myk/+* phenotypes, including low amplitude behavioural rhythms and altered phase re-setting to light, across laboratory models shared with human BPD. At the level of the SCN, there is also a tendency towards dampened amplitude from the central pacemaker in these models, although this is reported more commonly as a deficit in molecular rhythms. These findings highlight the need to establish whether changes to SCN, and their specific output signal, or extra-SCN oscillators are more relevant in circadian regulation of mood. Establishing multi-factorial profiles of central pacemaking in these models, as has been achieved in this study, will facilitate this understanding and in establishing the role of SCN in affective behaviour. In the *Myk/+* model this can be addressed through investigations into the impact extra-SCN regions that exhibit circadian regulation and are involved in mood behaviours. These areas include the lateral habenula, HPA axis and the VTA. Determining common neurotransmitter and regional centres involved in these mood profiles will also be crucial in the long-term goal of understanding the role of circadian physiology in the control of mood and in the aetiology and pathology of affective disorders.
References


Chapter 3
The role of inositol phosphate cycling in the biochemical actions of lithium on the circadian clock.
The role of inositol phosphate cycling in the biochemical actions of lithium on the circadian clock.

Introduction

Bipolar disorder (BPD) is a neuropsychiatric condition that affects 0.5-1.5% of the global population and is one of the ten most debilitating non-communicable diseases (Lozano et al., 2012; Merikangas et al., 2011; Wittchen et al., 2011). BPD is a highly heritable condition characterised by cycling episode of mania and depression separated by periods of clinically normal behavioural states known as euthymia (Phillips and Kupfer, 2013). Episodes of depression are characterised by a low-mood state, lack of energy and poor motivation (DSM-V, 2013; ICD-10, 2014). A diagnosis of BPD requires the appearance of at least one manic episode, which is accompanied by elevated or irritable mood, excess energy and a heightened motivational state.

Treatment for BPD is complicated by the heterogeneity of symptoms shown between patients and differential responses to pharmacological intervention (Geddes and Miklowitz, 2013). The need to treat patients across opposing mood states and sustained prophylaxis between episodes adds to the difficulties that arise in the treatment of BPD. Despite its discovery as a psychopharmacological tool over 60 years ago, lithium is still widely prescribed in the treatment of BPD (Grof, 2010; Nivoli et al., 2012). One of the major reasons for its continued use is the unique efficacy of lithium in the treatment of both depressive and manic episodes (Baldessarini, 2013). Although more effective in the management of mania, lithium is able to normalise symptoms of depression and thus represents a bona fide mood stabiliser (Can et al., 2014). Lithium also represents the most effective commonly prescribed drug prescribed in preventing relapse and reducing the high suicide rates seen in BPD (Cipriani et al., 2013; Geddes, 2004). These distinctive characteristic make understanding lithium’s therapeutic mechanism of action in BPD of great interest.
The use of lithium however does come with major drawbacks as a first-line management. It possesses a very narrow therapeutic range, exceeding which can lead to a variety of problems including weight gain, renal failure and hyperthyroidism (McKnight et al., 2012). Understanding the molecular and biochemical interactions of lithium is therefore of continued importance in developing more effective drugs that can mimic and improve on lithium’s clinical application while reducing negative side effects.

One of the most common symptoms of BPD are disturbances in sleep timing, with hyposomnia or insomnia appearing in 69-99% and 23-78% of manic and depressed episodes respectively (Boivin, 2000; Harvey, 2008; Hudson et al., 1993). Direct changes to circadian rhythms arise in BPD patients including alterations to phase and rhythm stability (Jones et al., 2005; Rock et al., 2014; Wood et al., 2009) and various SNPs of core circadian clock genes show associations with BPD (Etain et al., 2011). Interestingly certain clock genes including Bmal1, Rora and Period3 exhibit specific associations with responses to lithium (McCarthy et al., 2013; Rybakowski et al., 2014). Within the clinic toolkit in the treatment of BPD and MDD, targeting of sleep and circadian systems through chronotherapy can rapidly, although transiently improve aberrant mood. (Benedetti, 2012; Wehr et al., 1998; Wu et al., 2009).

Part of lithium’s clinical action may be due to its direct interaction with the circadian system. Lithium has a powerful effect on biological timing up to the level of behaviour, consistently lengthening the period of overt rhythms across a broad range of species including humans (Klemfuss, 1992; Johnsson et al., Li et al., 2012; Welsh and Moore-Ede, 1990). As a result, the biochemical interaction of lithium with the circadian system has received much attention.

Circadian rhythms are regulated by a hierarchical system of oscillators with the master pacemaker residing within the hypothalamus, the suprachiasmatic nuclei (SCN) (Moore and Eichler, 1972; Ralph et al., 1990; Stephan and Zucker, 1972). Circadian rhythms within individual neurons of the SCN and cells throughout the brain and body are driven by well-characterised molecular oscillator, regulating cell and tissue-specific transcriptomes in a time-dependent manner (Miller et al., 2007; Panda et al., 2002; Partch et al., 2014). The major positive components of this transcription-translation feedback loop (TTFL) are the
transcription factors CLOCK and BMAL1 that function as a heterodimeric pair to drive the negative components of the TTFL; PERIOD1,2 and 3 and CRYPTOCHROME1 and 2 (Bae et al., 2001; Bunger et al., 2000; Gekakis et al., 1998; Vitaterna et al., 1999). A broad range of enzymes and transcription factors interact to regulate the accurate timing and function of the TTFL including further transcriptional loops that regulate Bmal1, driven by Rora/β and Rev-erba/β, chromatin re-modelling via histone acetylation (SIRT1), protein ubiquitination (FXBL3) and phosphorylation (CK1ε/δ) are also essential clock driven processes of the TTFL (Etchegaray et al., 2009; Godinho et al., 2007; Meng et al., 2008; Nakahata et al., 2009; Ramsey et al., 2009). A further element in regulating phosphorylation within the circadian clock is GSK3β, involved in the nuclear location of PERIOD proteins (Iitaka et al., 2005; Ko et al., 2010), is a direct molecular target of lithium (Klein and Meltont, 1996).

The circadian system therefore has a clearly defined molecular basis defining function and BPD is also a highly heritable condition, with 40-70% co-incidence in monozygotic twins (Craddock and Jones, 1999). Emerging evidence also suggests that patients responding to lithium represent a sub-group within the BPD spectrum that also appears heritable in nature (Alda et al., 2005; Grof, 2010; Rybakowski, 2013; Schulze et al., 2010). Given the association of clock gene SNPs with BPD alongside recent studies highlighting a relationship between specific clock genes – namely Bmal1- and lithium responses in BPD patients (Etain et al., 2011; McCarthy et al., 2013; Rybakowski et al., 2014), the basis of lithium’s interaction with the molecular circadian clock may be an important part of a genetic basis to patients’ responses.

Despite the emerging picture into the genetically defined efficacy of lithium, a specific mechanism behind its therapeutic properties remains unclear. Lithium’s actions are mainly manifested through inhibition of magnesium-dependent enzymes, due to a similar non-hydrated ionic radii (Mota de Freitas et al., 2006). Due to the nature of this interaction and the dependency of many enzymes on Mg2+, lithium possesses a broad range of cellular targets (quiroz et al., 2004). At therapeutic concentrations however (0.5-1.2mM), lithium’s targets can be narrowed down significantly (Table 1).
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Table 1: Therapeutically relevant molecular targets of lithium (adapted from Gould et al., 2004b)

Although multiple targets exist, the majority of research into the effects of lithium has focused on just two of these targets, GSK3β (Gould et al., 2004a; Klein and Meltont, 1996; O’Brien and Klein, 2009; Rowe et al., 2007) and inositol monophosphatase (IMPase; Agam et al., 2009; Belmaker et al., 1996; Hallcher and Sherman, 1980; Singh et al., 2013; Teo et al., 2009). Both GSK3β and IMPase, via phosphoinositide (PtdIns) signalling, play major roles within CNS function, while these other targets, mainly involving glycogen metabolism are believed to play little role in the pathophysiology of BPD (Berridge et al., 1989; Fisher et al., 2002; Quiroz et al., 2004; Rowe et al., 2007). The focus of research on GSK3β and IMPase is justified through the demonstration lithium’s direct effects on these signalling pathways in vitro and in vivo. Pharmacological and genetic manipulation of both systems has proved promising, although it remains unclear whether GSK3β or IMPase act as the major effector of lithium’ actions (Pasquali et al., 2010).

Administration of therapeutic concentrations of lithium results in alterations to GSK3β-dependent pathways in vivo (Beaulieu et al., 2004a; Gould et al., 2004a; O’Brien et al., 2004). There is strong evidence that points to a role of GSK3β inhibition in behavioural alterations mimicking those observed with lithium treatment. Whole-animal or cellular-specific GSK3β knockdown is sufficient to suppress amphetamine-induced hyperlocomotion, a widely-used model of mania-like behaviour (Beaulieu et al., 2004b;
Urs et al., 2012). Down-regulation of GSK3β can also exert an anti-depressant influence on behaviour. Either virally-targeted knockdown or haploinsufficiency can mimic the anti-depressant actions of lithium on rodent behaviour (O’Brien et al., 2004; Omata et al., 2011).

The role of IMPase and InsP signalling pathways (summarised in fig 1) as a bona fide target of lithium has also found empirical support through in vivo studies on rodents. Depletion of brain myo-inositol occurs following lithium treatment and reveals a direct effect of lithium on CNS inositol cycling (Allison and Stewart, 1971; Belmaker et al., 1998; Lubrich et al., 1997). Central (ICV) infusion of myo-inositol, depleted by IMPase inhibition, can restore behavioural alterations induced by lithium administration in vivo (Belmaker et al., 1996; Singh et al., 2013). Recent work has demonstrated behavioural phenotypes associated with lithium administration in mice lacking IMPase (Impa1) and the extracellular transporter of myo-inositol (Smit1) mice (Agam et al., 2009). A recently described bioavailable IMPase inhibitor, named Ebselen, is also able to rescue pharmacologically-induced mania-like behaviour in mice (Singh et al., 2013). Interestingly, other mood stabilisers, valproic acid and carbamazepine, also demonstrate a common mechanistic action via inositol depletion (Williams et al., 2002).

Both GSK3β and IMPase present compelling evidence for their role in the therapeutic effects of lithium. The mechanisms through which lithium acts on the circadian clock, specifically in vivo effects on tau, are yet to be fully elucidated. Lithium’s pronounced effects on circadian period can also be re-capitulated in vitro on both clock gene and electrical firing rhythms (Abe et al., 2000; Li et al., 2012; McCarthy et al., 2013). In these studies lithium increases the amplitude of molecular rhythms via interactions with Per2 although this action has yet to be demonstrated in vivo. As such the period-lengthening effects of lithium appear the most pervasive feature of lithium upon circadian rhythms. It is known that this action occurs independently of GSK3β, whose inhibition results in shortening of period (Li et al., 2012). The period-lengthening effects of lithium in the circadian system thus remain unclear, and given the literature supporting IMPase and InsP cycling in lithium’s therapeutic effects present themselves as candidate mechanisms.
Figure 1: Simplified phosphatidylinositol (PtdIns)/inositol phosphate (InsP) pathway and the effect of Li⁺: The PtdIns cycle is based on the phosphorylation and addition of diglycerol groups to a basic myo-inositol backbone, represented as a blue hexagon. PtdIns2 (PIP2) (A) is a membrane precursor of the PI3K and InsP cycle. Activation of Gq/11 signalling results in the cleaving of PIP2 from the plasma membrane. This process results in the production of diglycerol (DAG) (activating PKC) and IP3 (B). IP3 stimulates IP3 receptors (IP3R) of the endoplasmic reticulum (ER) releasing Ca²⁺ and activating downstream Ca²⁺-signalling pathways (C). To maintain this signalling pathway IP3 needs to be re-cycled to free myo-inositol (D). This process utilises multiple de-phosphorylation steps with the final step dependent on the action of inositol monophosphatase (IMPase) (5). Li⁺ acts directly to inhibit IMPase and reduces intracellular pools of myo-inositol. It is hypothesized that the therapeutic actions of Li⁺ in BPD may be via down-regulating over-active PtdIns signalling through depletion of myo-inositol. (For review: Fisher et al., 2002; Teo et al., 2009; York et al., 2001).
The InsP/PtdIns cycle has not received major attention within the SCN, although Na⁺/myo-inositol transporter (SMIT) and inositol trisphosphate (IP3) receptors key transport and signalling effectors of this pathway are expressed within the rodent SCN, the latter potentiating phase shifting effects of optic nerve stimulation (Hamada et al., 1999a, 1999b; Inoue et al., 1996). Extracellular myo-inositol is also capable of rescuing lithium-induced suppression of firing rates in SCN slices (Mason and Biello, 1992). These data therefore support a physiological role of the InsP/PtdIns cycle in SCN function and within short-term responses to lithium.

In this study we attempted to dissect the effects on the inositol cycle and IMPase inhibition on the long-term molecular time-keeping within the SCN, primarily the effects on clock period. SCN explants from bioluminescent clock-gene reporter PER2::LUC mice were subjected to long-term bioluminescence recording in the presence of lithium and agents that target IMPase and the inositol cycle; myo-inositol and the specific inhibitors of IMPase L690,330 and L690,488 (Atack, 1997; Atack et al., 1993, 1994; Chen et al., 1998; Sarkar et al., 2005). Treatment with lithium consistently lengthened the period of intact SCN tissue, although the period-lengthening effects of lithium were not attenuated through co-culture with an extracellular source of myo-inositol. Pharmacological targeting of IMPase with the either specific inhibitor L690,330 or L690,488 also failed to lengthen period in the SCN yet at high concentrations dampens clock gene oscillations. This reveals a potential role for inositol cycling in basic SCN pacemaking although the period-lengthening mechanism of lithium remains elusive and opens up difficult questions into how this effect is exerted.
**Methods**

*Animal housing and maintenance:* Male and female PER2::LUC animals were group-housed prior to experimentation in a 12:12 LD cycle at constant temperature ~18°C and humidity ~40% with food and water *ad libitum*. PER2::LUC animals express a fusion protein of the core clock gene PERIOD2 with the enzyme luciferase attached to the 3’ end of the protein product of translation (Yoo et al., 2004). In the presence of beetle luciferin, magnesium and ATP luciferase catalyses the conversion of luciferin, emitting photons of light and allowing the real-time monitoring of protein expression.

*SCN explant preparation:* Male and female animals (2-6 months) were deeply anaesthetised with isofluorane and culled by cervical dislocation. Brains were rapidly removed and care taken to cut the optic nerves with minimum mechanical force before whole-brains were plunged into ice-cold Hank’s balanced salt solution (HBSS; NaHCO\(_3\) supplemented, Sigma, UK) supplemented with 10mgml\(^{-1}\) penicillin-streptomycin (Gibco Invitrogen, Paisley, UK) and 0.01M HEPES (Sigma, UK). Intact brains were blocked at approximately the anterior extremity of the cerebellum and through the basal forebrain using a fresh razor blade. Blocked tissue was attached to a vibratome stage by the caudal face using cyanoacrylate glue and placed in ice-cold HBSS. 250µm hypothalamic slices were prepared using a manual vibratome (Campden Instruments HA752, Loughborough, UK). Slices were maintained in ice-cold HBSS and SCN explants were prepared under a dissection microscope with surgical scalpel blades. Slices were dissected approximately half way between the dorsal tip of the third ventricle and SCN, and at the distal ends of the lateral anterior hypothalamic nuclei with the optic chiasm intact.

*Tissue culture:* Freshly prepared SCN explants were immediately transferred into a sterile culture hood and placed in 35mm culture dishes (Corning, Tewksbury, USA) containing 0.4µm PTFE cell-culture inserts (Millipore, Watford, UK). Each dish contained 1ml luciferin-supplemented sterile neuronal culture media; Dulbecco’s Modified Eagle Medium (DMEM; D-2909 Sigma, Gillingham, UK) 3.5g/L D-glucose (Sigma) 0.035% NaHCO\(_3\) (Sigma) 0.1M HEPES (Sigma) B27 serum-free media (Gibco Invitrogen, UK) 0.1mM beetle luciferin potassium salt (Promega, Southampton, UK). 35mm dishes were pre-lined with autoclaved high vacuum grease (Dow Corning Ltd, Coventry, UK) and...
immediately sealed with a UV-treated borosilicate glass 0.13mm coverslip (VWR, Lutterworth, UK).

Sealed culture dishes were transferred to light-tight incubators maintained at 37°C and 5% CO₂. Bioluminescence was recorded by photon-multiplier tube assemblies (H8259/R7518P, Hamamatsu, Welwyn Garden City, UK) encased within steel chambers. Total photon counts were recorded in 5-minute intervals on PMTMonTL software (Hamamatsu, UK) and stored on a personal computer.

**Drug treatment:** SCN explants were subjected to pharmacological manipulation from the 1st day in culture or after 4 days in culture. Pharmacological agents were made up with the initial culture media in the stated concentration when explants were treated immediately after dissection.

Cultures treated after 96 hours were removed from the incubator and transferred into a sterile culture hood. Fresh 35mm culture dishes were pre-prepared containing fresh culture media with either vehicle or drug. PTFE inserts containing the SCN were carefully removed and placed in the new culture dish and immediately sealed with a new UV-treated coverslip. This protocol resulted in tissues being outside of incubator cabinets for a maximum duration of 15 minutes.

**Pharmacology:** LiCl (Sigma) was dissolved in RNAse-free sterile H₂O (Sigma) and applied to culture media in 2mM and 4mM concentrations, approximating therapeutic concentrations of Li⁺ that are reported to modulate central and peripheral circadian rhythms (Abe et al., 2000; Li et al., 2012; Mason and Biello, 1992). Vehicle controls were treated with the same volume of sterile H₂O or relevant vehicle unless stated.

Myo-inositol (Sigma) was dissolved in RNAse-free sterile H₂O and placed within media at 10mM doses - significantly above estimated concentrations of extracellular myo-inositol in CSF and at which can alter lithium-induced firing rate suppression in SCN tissue (Fisher et al., 2002; Mason and Biello, 1992).

L690, 300 (Tocris, UK) was dissolved in sterile H₂O and dissolved in concentrations of 100µM. L690,488 (Tocris, UK) was dissolved in DMSO and explants cultured in concentrations of 1, 10 and 100µM.
Data analysis: Raw bioluminescence data was exported as a time-series into Excel 2010 (Microsoft) and data was de-trended using subtraction of a 24-hour running average. De-trended data was subjected to smoothing by a 3-hour running average for phase calculation. For analysis, de-trended and smoothed data was exported to GraphPad Prism6 software (GraphPad, USA). Period calculations were made over 72-hour epochs prior to and post treatment. All data 12-hours prior and post treatment was also exclude from tau calculations to avoid artefacts of the treatment process. Period was calculated by manual and automatic methods. Manual calculations were performed by taking the average value of the time passed between y intercepts greater than 18 and less than 30 hours apart. Automatic calculations were performed by fitting de-trended and data with the following equation; a sine function with an added damping constant to account for the rate of damping of the oscillation over time ex vivo:

\[ Y = \text{Amplitude} \times e^{-KX} \times \sin \left(2\pi X / \text{Wavelength}\right) + \text{PhaseShift} \]

Period is reported as the mean of fitted and manually calculated data in all cases. Amplitude changes are reported as the raw value of the deviation of the first peak in culture from 0. Damping rate is reported as the relative change of the 4th peak in culture to the 1st peak.
Results

*Lithium lengthens period in SCN tissue explants:* In order to validate the period-lengthening effects of lithium at doses close to therapeutic relevance, SCN explants were treated with 2mM and 4mM LiCl. Two protocols were used to establish the effects of LiCl, with SCN explants cultured from the start of the experiment with LiCl in culture media, or tissues were treated with LiCl or vehicle after 96 hours in culture (Fig 2). This was performed to identify differences in lithium’s response at high and low amplitude epochs of PER2::LUC oscillations. As expected, lithium consistently lengthens period of SCN explants under both protocols. When SCN explants were cultured with 2mM LiCl immediately after dissection, tau was lengthened by ~0.58h (Fig 2A-C; ± 0.05; p<0.0001).

SCN explants treated with 2mM or 4mM LiCl after 96 hours in culture exhibited molecular oscillations 0.80h and 1.24h (± 0.19; ± 0.29; p<0.05) longer than vehicle treated controls (Fig 2E-G).

Under both treatment conditions lithium-treated slices demonstrated a trend towards increasing the amplitude of oscillations relative to vehicle controls. Amplitude change did not prove to be statistically significant in cultures treated from day 0 (Fig 2D). Increases in amplitude following lithium treatment after 96h in culture were evident in both 2mM (0.33 ± 0.13 fold change) and 4mM doses (0.58 ± 0.12 fold change; p<0.05) relative to changes in vehicle amplitude yet only 4mM treatment groups reached significance threshold (Fig 2H). This result suggests that the effect of lithium in boosting amplitude is more efficacious in cultures treated after 96h and may therefore require lower amplitude initial oscillations.

*Extracellular myo-inositol fails to attenuate the period-lengthening properties of lithium:* In an attempt to offset lithium’s actions in the SCN, lithium treated explants were co-cultured in the presence of high concentrations of myo-inositol. As lithium inhibition of IMPase results in inositol depletion, this experiment was performed to attenuate the effects of LiCl through provision of extracellular sources of myo-inositol, typically depleted by lithium. After 96h in culture without any pharmacological manipulation, SCN explants were placed in fresh media containing 2mM or 4mM LiCl in the presence or absence of 10mM myo-inositol. In the presence of LiCl alone, 2mM treated slices displayed a long
Figure 2: Lithium lengthens period of intact SCN tissue \textit{in vitro} and selectively increase amplitude: A: Protocol used in tissue treatment in figures B-D. Following dissection SCN explants were cultured immediately with 2mM LiCl and left undisturbed for a minimum of 7 days. B: Example trace of raw bioluminescence values of vehicle (grey) and 2mM LiCl treated slices (green). In this example control tissues has a period and amplitude of 24.4h and 5727 A.U., LiCl treated tissue displays a tau and amplitude of 25.3h and 7375 A.U. C: Period of SCN explants cultured with H$_2$O vehicle (n=7) or 2mM LiCl (n=7). LiCl lengthens period of SCN molecular oscillations (H$_2$O; 24.57 ±0.10h; 2mM LiCl; 25.15 ±0.05h; p=0.0001). D: Amplitude of PER2::LUC oscillations were not altered by culture with 2mM lithium from day 0 (H$_2$O; 6835 ± 1706 A.U.; 2mM LiCl; 8906 ± 1086 A.U.; p=0.33). E: Protocol used in tissue treatment in F-H. SCN explants were cultured for 96h without the presence of LiCl then transferred to a culture dish containing fresh media with H$_2$O, 2mM LiCl or 4mM LiCl and left undisturbed for a minimum of 6 days. F: Example H$_2$O (grey) and 2mM LiCl (green) treated raw bioluminescence traces. Grey trace (H$_2$O) shows a period of 24.38h prior and 24.35h post media change. Green trace (2mM LiCl) displays a period of 24.38h before and 24.65h period after LiCl treatment. Blue arrow indicates time of treatment. G: PER2::LUC period following H$_2$O (n=7), 2mM (n=16) or 4mM (n=3) LiCl treatment. LiCl dose-dependently lengthens tau (H$_2$O; 24.22 ± 0.19; LiCl 2mM; 25.02 ± 0.19; LiCl 4mM; 25.46 ± 0.29; ANOVA p=0.02; Tukey post-hoc; *p<0.05). H: Relative change in amplitude following treatment. LiCl treated tissue show a dose-dependent increase in amplitude. Only 4mM LiCl treated tissues reached a significance threshold (H$_2$O; 0.47 ± 0.07 fold change; LiCl 2mM; 0.80 ± 0.13 fold change; LiCl 4mM 1.05 ± 0.12; Kruskall-Wallis p=0.036; Dunn’s post-hoc; *p<0.05).
circadian period of ~25h (Fig 3A,C; 25.09h ± 0.28). In the presence of 10mM myo-inositol the period of PER2::LUC oscillations was subtly shorter, although this difference did not approach significance (-0.10h; p=0.79). In the presence of 4mM LiCl SCN explants exhibited lengthened tau relative to 2mM treated tissues (Fig 3B,D; 25.46 ± 0.28h) yet in the presence of myo-inositol, the period of oscillations increased, although not approaching significance (25.91 ± 0.60). This data reveals that if the period-lengthening properties of lithium manifest through inositol depletion, extracellular provisions are not sufficient to counteract this effect within the SCN.

Reports on myo-inositol uptake suggest that a delay can arise in the transport or physiological effects of myo-inositol (Belmaker et al., 1996; Lubrich and van Calker, 1999; Patishi et al., 1996). To eliminate any potential effects that may be induced by a latency of myo-inositol to transport across the plasma membrane, circadian period of PER2::LUC oscillations was compared across time. Following treatment with 2mM LiCl, tau showed no change between 0-72h vs 72-144h (Fig 3F). 2mM groups with or without myo-inositol exhibited slight increases in period over time (both 0.37h) demonstrating that increasing time in the presence of myo-inositol does not prevent LiCl-dependent tau alterations in SCN tissue.

In 4mM LiCl treated explants in the absence of myo-inositol, little change was observed in tau between 0-72h vs. 72-144h (Fig 3G; 25.41 ± 0.24 vs 25.54 ±0.19h). 4mM LiCl explants in the presence of 10mM myo-inositol however exhibited a reduction in period over 72h-144h in culture (25.77 ± 0.59 vs 24.99 ± 0.29h; p=0.50). This trend did not approach statistical significance although due to a low sample number of 4mM treated explants, this arm of the experiment lacks sufficient power and may necessitate further investigation. Given the observations with 2mM treatments and the variance in the 4mM data, it appears that increasing time in culture with myo-inositol does not appear sufficient to consistently prevent tau alterations.

Changes in amplitude of PER2::LUC oscillations were not rescued by the presence myo-inositol in 2mM cultures, matching observations made in terms of tau. A small decrease in amplitude in 2mM cultures was apparent after media change (0.88 ± 0.17 fold change) yet the presence of myo-inositol resulted in small increase in amplitude of oscillations.
Figure 3: Period-lengthening effects of lithium are not attenuated by presence of extracellular myo-inositol; A: Example raw bioluminescence trace of SCN explant treated with 2mM LiCl with (blue, n=10) and without (green, n=11) the presence of 10mM myo-inositol. Blue trace (+myo-inositol) displays a pre and post treatment tau of 24.17h and 24.85 respectively. Green trace (-myo-inositol) shows a pre and post-treatment tau of 23.88h and 25.05h. B: Example raw bioluminescence trace of SCN explants treated with 4mM LiCl with (blue, n=3) or without (green, n=3) 10mM myo-inositol. Blue trace (+myo-inositol) demonstrates a pre and post treatment tau of 24.38h and 25.75h. Green trace (-myo-inositol) displays a pre and post-treatment tau of 24.43h and 25.05. C: Post-treatment period in slices treated with 2mM LiCl ± 10mM myo-inositol. Presence of myo-inositol fails to prevent period-lengthening effects of 2mM LiCl (LiCl 2mM (post); 25.09 ± 0.28h; LiCl 2mM + myo (post); 24.99 ± 0.18h; p=0.79) D: Period change of 4mM LiCl treated tissues ± myo-inositol. Presence of myo-inositol did not affect period (LiCl 4mM; 25.46 ± 0.28h; LiCl 4mM + myo; 25.91 ± 0.60; p=0.53). E: Fold change in tau after drug application across all treatment groups (LiCl 2mM; 1.03 ± 0.02 fold change; LiCl 2mM + myo; 1.02 ± 0.01; p=0.45; LiCl 4mM; 1.04 ± 0.01 fold change; LiCl 4mM + myo; 1.05 ± 0.02; p=0.53). F-G: Circadian period of PER2::LUC oscillations in 0-72h vs 72-144h in culture following LiCl ± myo treatment. F: Length of time in culture with myo-inositol does not rescue period-lengthening effect of 2mM LiCl (LiCl 2mM day 0-3; 25.09 ± 0.31h; LiCl 2mM day 3-6; 25.43 ± 0.29h; LiCl 2mM + myo day 3-6; 25.99 ± 0.19h; LiCl 2mM + myo day 3-6; 25.39 ± 0.21; p=0.54). G: Tau is reduced but does not approach statistical significance during 72-144h in culture with 4mM LiCl with myo-inositol (LiCl 4mM day 0-3; 25.41 ± 0.24h; LiCl 4mM day 3-6; 25.54 ± 0.19; LiCl 4mM + myo day 3-6; 25.77 ± 0.59; 24.97 ± 0.29; p=0.85). H: Amplitude change of SCN explants treated with LiCl with or without myo-inositol. The presence of myo-inositol had opposing effects in 2mM and 4mM treated explants without approaching statistical significance (LiCl 2mM; 0.88 ± 0.17 fold change; LiCl 2mM + myo; 1.13 ± 0.21; p=0.35; LiCl 4mM; 1.05 ± 0.12; LiCl 4mM + myo; 0.80 ± 0.08; p=0.15). Presence of myo-inositol does not alter amplitude effects of LiCl.
without approaching significance (1.13 ± 0.21; p=0.35). 4mM doses of LiCl appeared to show opposite trends to 2mM concentrations, with a small increase in amplitude (1.05 ± 0.12 fold change) in the presence of 4mM, yet a decrease in amplitude with myo-inositol also present in culture media (0.80 ± 0.08; p=0.15). The opposing effects seen with 2mM and 4mM LiCl concentrations of LiCl make the interpretation of the effect of extra-cellular myo-inositol. Whether there is a concentration-dependent interaction of LiCl and myo-inositol remains unclear with the power of the experiment. A lack of a clear statistical difference with either 2mM or 4mM concentrations suggests that the presence of myo-inositol and inositol depletion by LiCl does not play a major role in the amplitude enhancing effects of lithium.

Targeted inhibition of IMPase does not alter circadian clock period: To dissect the role of IMPase directly in the effects of lithium on the circadian clock, specific inhibitors of IMPase were cultured with PER2::LUC explants. There are two commercially available inhibitors of IMPase, L690,330 and an esterified pro-drug L690,488, the latter of which has increased cell permeability than the highly polar L690,330 (Fig 4B, Fig 5B (Atack, 1997; Atack et al., 1993, 1994). Both of these compounds show a powerful and specific inhibition of IMPase (L690,330; $K_i = 1\mu$M vs. Li$^+$ $K_i = 1$mM; Atack et al., 1993, 1994) function that results in decrease of myo-inositol, IP3 and accumulation of inositol-1-phosphate (IP1; Atack, 1997; Sarkar et al., 2005; Singh et al., 2013).

Initially SCN explants were cultured in the presence of L690,488 for a minimum of 7 days (Fig 4A). The period of PER2::LUC oscillations in the presence were not altered in the presence of either 10 or 100µM concentrations of the drug relative to DMSO controls (Fig 4C). Under an identical treatment protocol 2mM LiCl treated slices display period lengthening of 0.60 hours whereas 10µM L690,488 treated tissues demonstrated a period increase of only 2 minutes, and 100µM explants showed a slight decrease in tau of 4 minutes (p=0.89). To determine if effects of L690,488 on clock function arose with any latency PER2::LUC period was compared over the first 0-72h vs. 0-144h in culture. Both DMSO and 10µM L690,488 cultures showed a slight increase in period of 13 and 24 minutes respectively although again this trend showed no significant treatment group differences (p=0.27). These data reveal that targeted inhibition of IMPase through L690,488 does not affect the period of clock gene oscillations in the SCN.
Figure 4: L690,488 inhibition of IMPase does not affect SCN period, but dampens rhythms at high concentrations: A: L690,488 was added to culture immediately after tissue dissection. B: Molecular structure of L690,488. C: Example bioluminescence trace of DMSO (grey, n=16) and 10µM (dark blue, n=11) and 100µM (light blue, n=7) L690,488 treated tissues. D: Period of SCN explants in the presence or absence of L690,488. Neither 10 nor 100µM doses affected PER2::LUC tau (DMSO; 24.45 ± 0.07h; 10µM; 24.49 ± 0.16h; 100µM; 24.39 ± 0.09h; p=0.84). E: Period of explants cultured in DMSO or 10µM L690,488 across 0-72h vs. 72-144h in culture. Both DMSO and L690,488 treated cultures exhibit a small increase in period over 72-144h without no statistical differences between cohorts (DMSO day 3-6; 24.68 ± 0.16h; L690,488 day 3-6; 24.89 ± 0.09; p=0.27). F: Damping rate of PER2::LUC oscillations. Rhythms are not affected by 10µM L690,488. 100µM application results in rapid damping and cessation of oscillations (DMSO; 0.44 ± 0.02 fold change; 10µM 0.44 ± 0.03 fold change; 100µM; 0.25 ± 0.06 fold change; p=0.0009; Tukey post-hoc; **p<0.01). G: First peak amplitude of DMSO and L690,488 treated cultures. First peak amplitude shows a small but not significant increase in 10µM treated cultures (DMSO; 7273 ± 1789 A.U.; 10µM; 10057 ± 2089 A.U.; p=0.33).
At 100µM concentrations of L690,488, PER2::LUC oscillations exhibited varying rates of damping and ceased altogether after a maximum of 5 oscillations (Fig 4C). Some explants would cease oscillating after as few as two oscillations. When quantified as fold change in amplitude, 10µM doses exhibited no change in damping relative to DMSO controls, yet 100µM doses demonstrated increased rates of damping. The mechanism behind these changes to SCN damping rates at high concentrations of L690,488 are yet to be investigated. This does reveal however that in the absence of inositol phosphates recycling by IMPase, typical pacemaking function of the SCN is severely compromised.

Although L690,488 exhibits greater cellular permeability than L690,330, the latter compound is still able to permeate cells and can increase neuronal inositol monophosphates in vivo (Atack et al., 1993) To confirm findings with L690,488, SCN explants were cultured in the presence of high concentrations (100µM) of L690,330. A small increase in period was observed with L690,330, although this difference did not approach significance (Fig 5 A-D: H2O; 24.53 ± 0.13h vs. L690,330; 24.76 ±0.11h; p=0.19). In both H2O and L690,330 treated explants, tau exhibited a small decrease between 0-72h and 72-144h of approximately similar magnitude (Fig 5E). Separate SCN tissues were treated after 96 hours in culture with L690,330 and again this did not affect the period of oscillations (Fig 5I-J). These data reveal suggests that the presence of L690,330 does not affect the period of SCN tissues.

First peak amplitude was not affected by the presence of L690,330 when cultured were treated from day 1 or after 96 hours in culture (Fig 5F,K). The presence of L690,330 however did result in an increase in the rate of damping (Fig 5G; H2O; 0.46 ± 0.03 fold change; L690,330; 0.37 ± 0.02 fold change; p=0.03. Similar increases in damping of PER2::LUC rhythms were also observed with high concentrations (100µM) of L690,488 although the effect was visibly less pronounced with L690,330. This result suggests at a potentially common effect of IMPase inhibition to dampen amplitude and disrupt SCN function independent of effects on period.
Figure 5: L690,330 does not affect tau or amplitude in SCN explants but increases damping rate of PER2::LUC oscillations: A: Protocol used for C-G, explants were treated with 100µM L690,330 immediately at the start of incubation period. B: Molecular structure of L690,330. C: Raw bioluminescence traces of H2O (grey, n=7) and L690,330 (blue, n=9) treated cultures. D: Period of PER2::LUC oscillations. L690,330 treated tissue demonstrate a slight increase in tau that is not statistically significant (H2O; 24.53 ± 0.13h; L690,330; 24.76 ± 0.11h; p=0.19). E: Period of oscillations between 0-72h and 72-144h in culture. Both H2O and L690,330 cultures exhibited a small period decrease (H2O day 3-6; 24.18 ± 0.17h; L690,330 day 3-6; 24.43 ± 0.22h; p=0.42). F: First-peak amplitude of SCN explants shows no difference between treatment groups (H2O; 7008 ± 1570 A.U.; L690,330; 6471 ± 987 A.U.; p=0.77). G: Damping rate of PER2::LUC oscillations. L690,330 treated explants show a faster damping rate than H2O control (H2O; 0.46 ± 0.03 fold change; L690,330; 0.37 ± 0.02; p=0.03). H: SCN explants were also treated with 100µM L690,330 after 96h culture. I: Example vehicle (grey, n=6) and L690,330 (blue, n=9) treated raw bioluminescence trace. Blue arrow highlights media change. J: No differences in period were observed between treatment groups (H2O; 24.27 ± 0.20h; L690,330; 24.30 ± 0.49; p=0.96). K: Amplitude change following L690,330 treatment. H2O and L690,330 demonstrated similar amplitude after media change (H2O; 0.46 ± 0.11 fold change; L690,330; 0.45 ±0.07 fold change; p=0.90). L690,330 does not appear to affect SCN PER2::LUC period yet increases damping rate.
Discussion

Lithium effects on SCN period are independent of IMPase inhibition and InsP turnover: Lithium is a mood-stabiliser widely prescribed in the treatment and prophylaxis of BPD. Across a broad range of species, including humans, lithium lengthens the period of behavioural rhythms (Klemfuss, 1992; Li et al., 2012; Welsh and Moore-Ede, 1990). Lithium exerts these effects directly upon the molecular clock of the circadian system, extending tau and increasing amplitude of clock gene rhythms the latter of which is in-part regulated through inhibition of GSK3β (Li et al., 2012). As GSK3β inhibition shortens circadian period in vitro, this study focused on the role of InsP cycle and IMPase inhibition on the period-lengthening effects of lithium on the circadian clock. Through pharmacological inhibition of IMPase and manipulation of extracellular myo-inositol, we demonstrate that lithium’s actions on the period or amplitude of SCN clock gene oscillations are not manifested through down-regulation of IMPase or the InsP cycle.

Lithium’s actions on IMPase result in depletion of myo-inositol, IP3 and the accumulation of inositol-1-phosphate (IP1; Lubrich et al., 1997; Allison and Stewart, 1971; Allison et al., 1976; Sarkar et al., 2005). Inositol depletion affects InsP and PtdIns signalosomes and it is through changes to these pathways that lithium’s effects are believed to manifest (Berridge et al., 1989; Harwood, 2005; Teo et al., 2009). As lithium directly targets IMPase, this activity was mimicked through the use of specific inhibitors L690,330 and the cell permeable form L690,488 (Atack et al., 1993, 1994; Chen et al., 1998; Sarkar et al., 2005). Neither drug exerted significant effects on PER2::LUC period or amplitude. At these concentrations both drugs potently inhibit IMPase and result in accumulation of the target of IMPase; IP1 and decreases in IP3 as observed with lithium treatment (Allison et al., 1976; Atack, 1997). One consideration to make would be a potentially bad source or preparation of drugs but given observations made on damping rates of SCN tissue, both inhibitors had a clear biological effect on explants.

Aside from the recycling of intracellular myo-inositol from IP1 via IMPase, cells can acquire myo-inositol from extracellular fluid via SMITs or H+/myo-inositol transporters, although the role of the latter in plasma membrane transport is as yet undefined (Agam et al., 2009; Berry et al., 2003; Di Daniel et al., 2009; Fisher et al., 2002; Fu et al., 2012).
Lithium can also directly affect the activity and expression of SMITs in astrocyte-like cells over time in ex vivo culture (Lubrich and van Calker, 1999). The SCN are enriched with SMITs, and application of myo-inositol can rescue lithium-induced suppression of electrical activity in SCN neurons (Inoue et al., 1996; Mason and Biello, 1992). This reveals that the SCN possess the capacity to rapidly sequester myo-inositol from extracellular sources. As co-culture with high concentrations of myo-inositol failed to attenuate lithium’s effects on SCN pacemaking, despite a capacity to rapidly acquire this substrate, this demonstrates that inositol depletion does not underpin this activity.

Intracellular myo-inositol is highly concentrated in neurons of the CNS with estimations between 2-15mM depending on the specific type or region of neuron (Godfrey et al., 1982; Novak et al., 1999; Patishi et al., 1996; Yildiz et al., 2010). Whole-brain concentrations in mice are approximately 4.8mM and 4.4mM within hypothalamus of the rat (Belmaker et al., 1998; Thurston et al., 1989). Extracellular concentrations of myo-inositol in the CSF are believed to be approximately 50-100-fold lower than are typically reported within neurons and provision of extracellular sources is a common method to supersede lithium’s effects on inositol transport (Fisher et al., 2002; Harwood, 2005; Shetty et al., 1995; Williams et al., 2002). In addition 10mM myo-inositol is sufficient to rescue lithium-induced changes to electrical activity within SCN neurons (Mason and Biello, 1992). 10mM myo-inositol utilised in this study therefore provides a sufficient extracellular source to counteract cellular inositol depletion.

Mammalian cells are only able to acquire myo-inositol from extracellular sources through the activity of SMITs of the plasma membrane – and potentially HMITs - IMPase hydrolysis of inositol monophosphate or finally via direct synthesis from glucose (Fisher et al., 2002; Maeda and Eisenberg, 1980). The enzyme necessary for latter pathway (myo-inositol-1-phosphate synthase) is however, restricted to the cerebral vasculature and is not active within neural tissue (Novak et al., 1999; Wong et al., 1987). This rules out novel synthesis as a pathway to compensate for pharmacological depletion of myo-inositol within the SCN. The experiments performed in this study therefore account for all known pathways mediating novel inositol acquisition within the SCN. The persistence of lithium’s effects in myo-inositol and L690,488/L690,330 treated tissue demonstrates that the InsP
cycle is not the target through which lithium targets the molecular or central circadian pacemaker.

**Amplitude effects of lithium depend on oscillator strength:** In this study the amplitude enhancing effects of LiCl were evident only in SCN tissues cultured for 96h prior to treatment. When applied to cultures immediately after dissection, when at highest amplitude, this effect was less apparent. This suggests that the ability of lithium to enhance rhythm amplitude is dependent on output of the central oscillator. Larger effects of lithium on amplitude on dampened clock gene oscillations has been described in fibroblasts from human BPD patients previously (McCarty et al., 2013). This is the first demonstration of this property in SCN tissue. Low amplitude and unstable behavioural rhythms in BPD patients whereas sleep-wake disruption is also very common (Hudson et al., 1993; Jones et al., 2005; Rock et al., 2014). This represents a potentially interesting consideration in the therapeutic benefits of lithium in BPD, by which lithium may have impose greater benefits via the circadian system in BPD patients with disrupted circadian pacemaking.

Despite amplitude enhancing effects visible with lithium at higher concentrations, it was not possible to establish any role for IMPase inhibition or the InSP cycle within this phenomenon. No changes to clock gene amplitude were evident after L690,488/L690,330 application or following by myo-inositol co-culture. L690,488 cultures did exhibit a non-significant tendency to increase PER2 amplitude but this was not tested on dampened tissues (>96h). Although our data suggest that IMPase inhibition and inositol depletion do not play a role within lithium’s mechanism of action, potential effects on amplitude cannot be dismissed.

**IMPase inhibition impacts upon tissue damping:** Both L690,488 and L690,330, demonstrated clear effect on the damping rate of clock gene rhythms of the SCN. At 100µM doses the magnitude of changes in oscillator amplitude in culture were significantly reduced and was particularly marked with L690,488. This can be interpreted as IMPase inhibition enhancing the rate of tissue level de-synchronisation, or instead this may represent a lethal effect on cellular and tissue function (Aton et al., 2005; Guilding et al., 2013; Maywood et al., 2006). Given the gradual rate of damping, it seems plausible
that alterations to InsP signalling via IMPase inhibition may alter intracellular signalling pathways critical to the function of the SCN network.

Disruption to InsP or PtdIns signalling can affect various aspects of neuronal function including intracellular signal transmission, modulation of ion channels and neurotransmitter release (Gamper and Shapiro, 2007; Irvine and Schell, 2001). The major downstream targets of InsP signalling are IP$_3$ receptor-dependent ER-Ca$^{2+}$ release and protein kinase C (PKC) activation by cleaved diglycerol (DAG; Berridge, 2009; Pasquali et al., 2010). Within the SCN PKC interacts with core elements of the circadian clocks, mediating phase shifts, whereas IP3 activation also modulates phase shifts and optic-nerve stimulation-induced electrical activity (Bonsall and Lall, 2013; Hamada et al., 1999b; Robles et al., 2010). Calcium oscillations derived from intracellular stores are also critical to neuronal and network rhythms within the SCN and thapsigargin, an inhibitor of the ER-Ca$^{2+}$ pump (SERCA), can reduce the magnitude and adjust the phase of PER2::LUC oscillations (Enoki et al., 2012; Ikeda et al., 2003; Noguchi et al., 2012).

A recent study using virally transfected DREADDs within SCN tissue revealed a critical effect of Gq-signalling pathways upon the spatiotemporal organisation and pacemaking of the SCN network (Brancaccio et al., 2013). Induction of Gq-signalling pathways activates phospholipase C (PLC), the initial step in the cleavage of phosphatidylinositol-2-phosphate (PIP2) into IP3 and DAG (Mizuno and Itoh, 2009). Blockade of inositol turnover through L690,488 and L690,330 inhibition of IMPase may disrupt the available pool of myo-inositol that constitutes the substrate of this critical pathway. This may explain changes to the damping rates and therefore robustness of SCN tissues in the presence of IMPase inhibitors. Additional inositol phosphates and phosphoinositide signalling molecules may also be affected by IMPase inhibition, although the specific nature are difficult to speculate upon the diverse cellular pathways affected and overall number of signalling molecules (Irvine and Schell, 2001).

**A behavioural effect of lithium independent of GSK3β or InsP pathways:** The effect of lithium on free-running period is impressively broad-ranging across species; yet nearly all reported behavioural correlates of lithium appear attributable to inhibition of GSK3β or InsP dependent pathways. Having demonstrated that IMPase is and inositol depletion...
does not alter circadian pacemaking, this presents an interesting problem. The profound cellular and behavioural effects of lithium on the circadian system must arise through a separate biochemical mechanism. This opens up further questions into how distinct pathways outside of GSK3β and IMPase signalosomes may underlie lithium’s activity upon physiology and behaviour across the CNS.

Although inhibition of IMPase or GSK3β alone fails to lengthen clock period, a pleiotropic effect of simultaneous blockade may induce different effects on circadian pacemaking. Both InsP and GSK3β signalling activate a broad range of downstream targets and share common and distinct downstream targets and physiological effects (Pasquali et al., 2010). Physiological effects such as tau lengthening may therefore be absent when IMPase or GSK3β are targeted alone. No study has yet addressed possible pleiotropic effects of dual inhibition on the circadian system. This represents a final experiment to rule out lithium’s effects on tau via these signalling pathways.

Although most of lithium’s therapeutic actions have been ascribed to these two major targets, there remain potential candidates for lithium’s actions within the SCN. These targets however do not play as clear a role within CNS function. Although the major action of lithium ion GSK3β is believed to function through direct inhibition (Klein and Melton, 1996; O’Brien and Klein, 2009), upstream interaction with a β-arrestin-2-AKT signalling complex also mimics some of the behavioural actions of lithium via upregulation of AKT, that results in further inactivation of GSK3β (Beaulieu et al., 2004b, 2008). Lithium’s wider effects upon AKT signalling may therefore be more relevant to molecular and network period lengthening. Given that circadian transcription factors (PER2) regulate AKT, this represents a relevant avenue of investigation (Yang et al., 2010). Intriguingly, heightened AKT signalling within central circadian neurons of Drosophila also results in the lengthening of behavioural rhythms (Zheng and Sehgal, 2010).

In addition to β-arrestin-2-AKT complexes, therapeutically relevant targets of lithium include IMPase-like phosphomonoestersases and other metabolic enzymes. These targets are involved in glycogen metabolism; fructose-1,6-bisphosphate (FBPase), phosphoglucomutase (PGM); sulfotransferase activity; bisphosphate nucleotidase (BPNase); and other inositol phosphatases; inositol polyphosphate-1-phosphotase
(IPPase; Gould et al., 2004b; Quiroz et al., 2004). As IPPase is involved in recycling of inositol from inositol-phosphates, then inhibiting IMPase would be expected to manifest similarly to IPPase making it an unlikely target given the results of this study (Fisher et al., 2002; Inhorn and Majerus, 1988).

Other targets have generally been overlooked in understanding the molecular actions of lithium given the prominent role GSK3β and IMPase within CNS function and have instead been associated with the side-effects of lithium toxicity (Spiegelberg et al., 1999). Both FBPase and PGM are expressed within the CNS (Löffler et al., 2001), and lithium can alter glycogen levels in the brain, although this has only been shown to directly affect PGM within astrocytes (Souza et al., 2010), the major site of glycogen synthesis within the CNS. Speculatively, alterations to glycogen processing may affect SCN function through changes to cellular redox states. The molecular clock of the circadian system is coupled with cellular metabolism and redox states through NAD-dependent histone deacetylase activity (Nakahata et al., 2009; Ramsey et al., 2009). The inter-dependent nature of this relationship exists at the level of the SCN and can impact upon electrical and age-dependent output of the SCN (Chang and Guarente, 2013; Wang et al., 2012). A systematic approach challenging the currently described therapeutic targets of lithium in the circadian system represents the only solution to this question with AKT signalling representing the most promising avenue.

Implications for the therapeutic actions of lithium and the pursuit of lithium alternatives

Our findings add to the complexity that surrounds the hunt to establish the seemingly multi-faceted and diverse action of lithium within the CNS. These data are relevant towards an understanding of lithium’s actions across the brain and body. It would overstate the significance of this study to suggest that any major re-consideration of lithium’s therapeutic mechanism in BPD is required, given the data in support of IMPase and GSK3β. Yet there remains a lack of agreement between these schools of thinking, on the relative contribution of GSK3β and IMPase in the beneficial effects of lithium (Agam et al., 2009; O’Brien and Klein, 2009; Pasquali et al., 2010). Much emphasis is often placed upon these two signalling pathways when the physiological effects and benefits of lithium are clearly not limited to individual cellular targets.
The results of this study also have potential implications in the search for pharmacological alternatives to lithium. There is growing evidence of a genetic association that defines the effectiveness of lithium in the treatment of BPD (Grof, 2010; Rybakowski, 2013; Schulze et al., 2010). Recent genetic studies have implicated specific clock genes within the sensitivity and effectiveness of lithium in BPD prophylaxis (Campos-de-Sousa et al., 2010; McCarthy et al., 2013; Rybakowski et al., 2014). These findings therefore implicate the circadian system within the therapeutic actions of lithium. In addition, as the association between circadian rhythms and BPD appears of ever-increasing relevance across clinical and laboratory-based studies (Chung et al., 2014; McCarthy and Welsh, 2012; Wulff et al., 2010), impacts upon on the circadian clock must therefore be considered in the development of novel pharmacological interventions for BPD. Our data suggests that novel pharmaceuticals that target IMPase and InsP cycling may fail to encompass beneficial actions on the circadian system. Whether effects on circadian period and biological timekeeping in general are relevant to the activity of lithium remains to be seen. Until the mechanisms that underlie these properties are fully described the answer to this question will remain elusive.
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General Discussion
General Discussion

Research Context

The work undertaken over the past three years for this thesis has been focused upon improving understanding of the neurobiological association of circadian rhythms and mood disorders. The relationship between circadian disruption and neuropsychiatric disorders is well established in the literature, with much attention now directed towards elucidating the rhythmic mechanisms within the CNS that directly influence behaviour (Foster et al., 2013; Gonzalez, 2014; Landgraf et al., 2014; Schnell et al., 2014). Although this work is relevant across the wider spectrum of neuropsychiatric disorders, the goal of these investigations has been aimed specifically towards bipolar disorder (BPD). BPD is a condition that affects between 35 and 105 million people worldwide (0.5-1.5%), with that number approximately doubling when sub-clinical manifestations are considered (Merikangas, 2007; Wittchen et al., 2011). These disorders represent a huge economic and sociological burden in addition to the emotional and human impacts on the immediate friends and family of sufferers. In a broad context, more than a third of all people living within the EU are afflicted with some form of clinically diagnosed brain disorder (Wittchen et al., 2011). The economic burden of these various conditions is staggering, estimated at €798 billion annually within the EU and with €43 billion of these costs arising from affective disorders (Gustavsson et al., 2011).

A lack of both understanding and effective management tools are attached to affective disorders. The drive to improve on this knowledge basis is complicated by a polygenic and multi-factorial aetiology alongside the inherent difficulty in establishing the role of environmental influences on disease development (Craddock and Sklar, 2013; Phillips and Kupfer, 2013). Long-term, a clear goal shared across clinical and laboratory research is to improve our basic understanding of these conditions in order to provide reliable therapeutic and sociological solutions for patients. For this to happen requires a leap forward in our understanding of the fundamental roots of these conditions. As has been documented up to this point, changes to sleep and circadian rhythms appear an important pathophysiological symptom shared across affective disorders (Foster et al.,
2013; Gonzalez, 2014; Harvey, 2008; Schnell et al., 2014). Providing a clearer picture of the role played by sleep and circadian systems upon mood behaviours and the pathology of affective disorders represents an important step in this process.

**Major findings and their context within field of research**

Using the *Myk/+* mouse model that mimics behavioural and mechanistic phenotypes of mania-like behaviour, an in-depth circadian, metabolic and light-dependent behavioural profile has been generated. A multi-factorial characterisation of the role of central circadian pacemaking that may underlie these phenotypes has also been successfully acquired to include molecular, synaptic and electrical output of the *Myk/+* SCN. Finally the biochemical interactions of the mood stabiliser lithium with molecular pacemaking of the SCN were investigated with regards to the known cellular target IMPase and the inositol phosphate cycle.

**Myk/+ behavioural and neurophysiological characterisation**

Although reports of a circadian phenotype were made during the initial characterisation of *Myk/+* animals as a model of mania, this study has built upon on these findings that reveal a range of novel behaviours that are informative in the context of current understanding of circadian-mood pathology (Kirshenbaum et al., 2011). Some of these behaviours were particularly distinct including long alpha durations and the ability to exhibit changes in tau. The lability and continuum of phenotypes expressed by these animals also represents an unusual malleability of the circadian system. In relation to other clock gene models of affective behaviours, deficits in the amplitude of free-running rhythms appear particularly relevant given the seeming consistency with which this phenotype arises.

In addition to deficits in the regulation of behavioural rhythms, *Myk/+* animals also exhibited a number of changes to light-dependent behaviours. These phenotypes included altered phase re-setting, which represented a further phenotype shared across clock models. *Myk/+* animals also exhibited increased light-phase activity and light-time ingestion, seemingly dependent on deficits in the suppression of locomotor activity by
light. *In vivo* experiments further revealed changes in the metabolic profile of *Myk/+* animals; increased basal metabolic rate and drinking, with a concomitant reductions in overall feeding activity. Again these metabolic phenotypes appear commonplace across various other clock gene models that have been discussed.

Although yet to be verified, a noteworthy observation made during wheel-running experiments revealed a potentially important insight into *Myk/+* behaviour. Over time in individually-housed cages, some animals exhibited profound reductions in locomotor activity that ended in almost complete cessation of activity. This suggested *Myk/+* animals may represent a model that can separately express both depression and mania-like mood states and therefore a powerful tool to study the basis of these behaviours.

**Altered central pacemaking in *Myk/+* animals**

To understand the role of the central circadian system that contributed to these behaviours, multi-level characterisation of the *Myk/+* central circadian system was attempted through distinct *in vitro* and *ex vivo* approaches. Whole-cell electrophysiology, Ca$^{2+}$ imaging and PER2::LUC bioluminescence recordings allowed molecular, electrical and synaptic dissection of the *Myk/+* SCN. The *Myk/+* circadian pacemaker was defined by a dampened electrical output that appears as a result of altered intercellular synchrony and network architecture. Interestingly these deficits arose seemingly independent of overt changes to the intracellular TTFL, the basic rhythm-generating machinery of the circadian system. These experiments therefore revealed that an altered SCN output appears as a major factor in altered circadian and light-responsive *Myk/+* behaviour.

**Mechanisms of lithium’s actions in the SCN**

The final chapter of this work approached the question of circadian rhythms and BPD from a different perspective. Using PER2::LUC bioluminescence SCN culture an attempt was made to identify the biochemical target of lithium within the circadian system that results in tau lengthening *in vivo* across species. The behaviourally relevant targets inhibited by lithium at therapeutic concentrations are GSK3β and IMPase (Hallcher and Sherman, 1980; Klein et al. 1996; Pasquali et al, 2010). The period lengthening effects of
lithium however appear not to act through changes to IMPase and the inositol phosphate cycle, although pharmacological targeting can reduce amplitude of SCN molecular output. The biochemical effects of lithium on molecular and behavioural rhythms appear more complex than simply acting through inhibition of these molecular targets and serves to highlight the diverse physiological pathways through which lithium may function in the CNS and a potential role for inositol phosphate cycling in SCN output.

**Myk/+ phenotype - relevance to clock models of affective behaviours**

Having established a circadian behavioural and neurophysiological profile of the *Myk/+* genotype it is important to consider these results in relation to other clock gene models exhibiting altered mood states. By drawing comparison between these studies, common traits and mechanisms across different strains can be identified. Creating a profile of typical circadian and behavioural deficits across different models will aid the identification of the major circuits and mood states that are associated with circadian disruption.

Interestingly a broad array of phenotypic similarities are apparent between *Myk/+* animals and the *ClockΔ19* model of mania. Both strains exhibit a similar repertoire of mania-like behaviours including hyperactivity, reduced sleep, heightened reward preference, reduced helplessness and lithium responses (Kirshenbaum et al., 2011; Roybal et al., 2007). From a circadian perspective these animals also share lengthened tau, reduced free-running amplitude and altered phase re-setting to light, although with distinct PRC shapes (Vitaterna et al., 2006). *ClockΔ19* similarity to *Myk/+* continue at the neurophysiological level with deficits reported within the SCN; dampened molecular output *in situ* and absent electrical rhythms in isolated neurons (Herzog et al., 1998; Vitaterna et al., 2006). When organotypic SCN explants are prepared from *ClockΔ19* animals however, network factors are sufficient to maintain electrical rhythms *in vitro* (Nakamura et al., 2002). Although both animals possess a central SCN deficit, the contrasting electrical and molecular properties may be relevant in how changes to circadian pacemaking are imparted across the CNS.
Overt behavioural similarities retain a particular relevance to Myk/+ animals as the VTA is established as a critical mediator of mania-like behaviour in ClockΔ19 animals, and recently in another clock model Rev-erba−/− (Chung et al., 2014; Coque et al., 2011; Dzirasa et al., 2011; Mukherjee et al., 2010). Characterising VTA function in Myk/+ animals would establish if similar deficits arise within dopaminergic systems, which appears promising given the range of shared phenotypes, and help towards identifying common roots of behavioural changes in ClockΔ19, Myk/+ and other pre-clinical circadian models.

In addition to ClockΔ19, the behavioural and physiological characteristics of Myk/+ animals exhibit a number of phenotypic similarities with a range of other mouse models. Individual strains generally exhibit distinct behavioural characteristics, yet certain common phenotypes appear to arise. Myk/+ animals exhibit a clear hyperactive phenotype across the diurnal cycle in an open-field that also manifests in extended alpha during circadian experiments. Hyperlocomotor behaviour is reported in other clock gene lesioned animals (ClockΔ19, CK1ε−/−, Bmal1−/−; Bryant et al., 2012; Kondratova et al., 2010; Roybal et al., 2007) with reduced anxiety phenotypes also common with Myk/+ (ClockΔ19, Afh, Rev-erba−/−; Chung et al., 2014; Keers et al., 2012; Roybal et al., 2007). The phenotype shared most frequently between models however is a reduction in helplessness behaviour defined by forced swim and tail suspension tests (ClockΔ19, Bmal1−/−, Per2Brdm−/−, Afh, Rev-erba; Chung et al., 2014; Hampp et al., 2008; Keers et al., 2012; Leliavski et al., 2014; Roybal et al., 2007). These behavioural profiles suggest that altered circadian regulation is most widely associated with mania-like phenotypes and more specifically psychomotor and anxiety deficits. The circuits underlying these behaviours therefore represent a priority to be targeted in establishing the how circadian rhythms may influence on these phenotypes. It would be of great interest to identify if the VTA is a common site of pathological and rhythmic disruption underlying these shared phenotypes given the role of dopamine in locomotor and reward behaviours (Di Chiara et al., 1995; Cousins et al., 2009).

One issue that arises across different studies of clock gene mutants however is a lack of continuity in the range of behavioural tests that are applied during characterisation of affective states. This emphasises a need to apply systematic behavioural profiling and
common criteria when assessing novel and existing models. Applying standardised experimental paradigms and promoting the publication of negative results alongside full circadian endophenotype descriptions will help to clarify common behaviours that may be regulated by circadian systems across pre-clinical models of affective behaviours.

**Circadian deficits in affective models; central vs local clock control**

The specific circadian phenotype also remains a critical factor to connect specific affective behaviours with clock phenotypes. The change in free-running amplitude and rhythm instability in *Myk/+* animals appear with great frequency in other clock models including *ClockΔ19, Bmal1*+/−, *Per2*Brdm−/− and reduced amplitude drinking rhythms in *Afh* (Bunger et al., 2000; Guilding et al., 2013; Roybal et al., 2007; Zheng et al., 1999). The consistency of low amplitude behavioural rhythms suggests that poor central regulation of pacemaking is an important factor mediating behaviour in these models. Despite a common circadian behavioural deficit, shared mood phenotypes are not apparent. These data would suggest that a reduced control of circadian behaviour can influence diverse affective behaviours rather than specific mood centres, although a wider array of behavioural paradigms across these different examples would be required to validate this supposition.

The physiological basis of central circadian pacemaking across models is also beneficial to build an understanding of the precise role and impact of SCN output on mood. *Myk/+* animals exhibit a reduced electrical output from the SCN although with an overtly intact molecular pacemaker. Many other clock gene models possess molecular lesions within the TTFL that directly impact upon single cell and network molecular oscillations (Feillet et al., 2006; Guilding et al., 2013; Herzog et al., 1998; Ko et al., 2010; Liu et al., 2007; Vitaterna et al., 2006). Across this literature however, many examples have yet to establish multi-level characterisation of central pacemaking to include electrical and synaptic output. It is particularly critical to determine whether loss of basic molecular pacemaking or altered central output defines an animal’s circadian phenotype. Such differences in characteristics may impose divergent circadian regulation in mood centres of the CNS and underpin contrasting affective phenotypes between models.
This leads us into a re-occurring question in the study of clocks and mood, regarding the role of central timing, the SCN, against the role of changes in local rhythmicity in regions accessory to the SCN (Landgraf et al., 2014; McCarthy and Welsh, 2011; Menet and Rosbash, 2011). Many of the clock models discussed possess molecular lesions within the TTFL that affect cellular timekeeping, which would be expected to manifest in changes in basic timekeeping across all rhythmic brain regions (Herzog et al., 2000; Ko et al., 2010). Due to the overtly intact Myk/+ molecular oscillator, physiological regulation of circadian rhythms downstream may be through a different mechanism, with the dampened output signal of the SCN imparting greater influence on affective circuitry.

The relevance of the role of output signal from the SCN is perhaps demonstrated from a recent study on circadian control of memory performance. This work revealed that the efferent output signal from the SCN determines memory performance, a property that is rescued in arrhythmic animals when the anatomical connections and therefore output signal of the SCN are ablated (Fernandez et al., 2014). The specific output of the SCN is therefore critical to transmit functional information to downstream central targets. It is a well-defined property of the SCN that network properties can compensate for genetic deficits and underlying changes to local and SCN-derived output cannot be predicted based on genetic lesions (Liu et al., 2007). Clarifying the output of the central pacemaker as performed in Myk/+ animals is important to understand how changes to SCN output determine specific mood physiology and affective behaviours.

Given various associations of clock genes within bipolar populations and the appearance of sleep-wake disruption prior to mood episodes questions also persist in relation to the role of circadian rhythms in the aetiology of mood disorders (Brietzke et al., 2012; Etain et al., 2011; Landgraf et al., 2014; Zeschel et al., 2013). Do changes to clocks contribute to pathology or are changes to sleep and circadian rhythms a consequence of wider dysregulation within the CNS? The Myk/+ SCN phenotype reveals insights into this question also. As the long-term pacemaking of the SCN appears stable and robust in ex vivo PER2::LUC culture, deficits observed in behavioural and electrical output may be imposed upstream of the SCN. Although yet to be confirmed empirically, these findings suggest that in certain circumstances the SCN may be influenced by altered signalling
from the wider CNS. BPD and neuropsychiatric conditions are widely characterised by changes to specific neurotransmitter signalling pathways (Cousins et al., 2009; Mahmood and Silverstone, 2001; Wulff et al., 2010). If the SCN can be influenced in this manner, understanding which circuits and neurochemicals can impart this influence and how to manage impacts on rhythmic physiology may prove important. The Myk/+ therefore provides a novel insight into how changes to circadian pacemaking may arise in the CNS. Determining how output from the SCN translates to other regions of the CNS and how the central pacemaker may be influenced from upstream signals represent an interesting progression from this work. Whether targeting the SCN and the influence of aberrant rhythms on Myk/+ mood phenotypes present a viable route of therapeutic intervention would be revealing.

**Altered phase alignment and light responses in Myk/+ mood**

In addition to free-running deficits Myk/+ animals also demonstrated altered phase shifting to light, a phenotype shared with ClockΔ19, Afh, Rev-erbα/- and PerzBrdm/- (Albrecht et al., 2001; Guilding et al., 2013; Preitner et al., 2002; Vitaterna et al., 2006). Although the specific re-setting phenotypes between models are distinct, the role of light in the behavioural repertoire of these animals has not been widely considered. In addition, Myk/+ animals exhibit accelerated re-entrainment to large shifts of the LD cycle that simulate jet lag. Light and the correct alignment of sleep and circadian rhythms with light-dark cycles appear as a central mediator of mood with growing frequency in the literature (Foster et al., 2013; LeGates et al., 2014). The consistency of these findings should be considered in the context of their potential impacts on the affective behaviours demonstrated in these animals.

Light and correct phase alignment with LD cycles is a necessary consideration within the spectrum of affective disorders, although how light exposure differentially impacts upon circadian and non-circadian pathways isn’t fully apparent. Altered light has pronounced effects of affective behaviours in rodents and in humans SAD is believed attributable to seasonal changes in day-length (Ashkenazy et al., 2009; Dulcis et al., 2013; LeGates et al., 2012; Magnusson and Boivin, 2003). Patients suffering from affective disorders
demonstrate altered phasing of both sleep-wake rhythms and physiological measures of the circadian system such as melatonin, cortisol and body temperature (Emens et al., 2009; Mansour et al., 2005; Souqtre et al., 1989; Wong et al., 2000). This suggests that in these patients the circadian system may be continually mis-aligned with environmental conditions. Long-term phase mis-alignment induced through shift work can manifest in negative mood states as well as wider impacts on health and physiology including increased prevalence of obesity (Foster et al., 2013; Katz et al., 2001; Knutsson, 2003; Levandovski et al., 2011; Roenneberg et al., 2012 Schnell et al., 2014). Rapid changes in light-dark cycles through jet lag can also affect mood and widely manifests in depressed mood states (Srinivasan et al., 2009). Sleep deprivation and bright light exposure that arise concomitantly under such conditions can also induce episodes of mania and alter behaviour in non-affected humans, including increased risk taking and alterations to mood (Alcehson et al., 2007; Selvi et al., 2007).

The Myk/+ model therefore possesses a range of phenotypes including altered phase re-setting and re-entrainment to changes of the light cycle, that are directly relevant to environmental influences that affect mood in humans and more specifically in sufferers of affective disorders. As altered phase re-setting is expressed by various clock gene models described above, this may be an important factor in the presentation of different mood states. Identifying how phase alignment and changes to light-dark cycles impacts on daily mood presentation represent an interesting direction that may be shared across circadian models of affective behaviours. This may also represent a common phenotype through which Myk/+ and other circadian-compromised animal models mimic areas of human affective disorders. As an additional consideration from these findings, most behavioural experiments are generally performed in animals maintained under LD rather than constant conditions. The role of altered phase alignment may be an important consideration when attempting to characterise mood phenotypes in animal models given potential circadian variation in mood states and chronic phase mis-alignment (Wirz-Jutice et al., 2008).

Altered light-dependent behaviours were also observed in non-circadian phenotypes in Myk/+ animals. Increased light-time locomotor and ingestion activity were apparent and appear due to deficits in negative masking by light. This non-circadian element of light
may also be relevant to the presentation of mood behaviours (LeGates et al., 2014). Negative masking is dependent on both visual and non-visual phototransduction from the retina (Mrosovsky and Hattar, 2003; Mrosovsky and Thompson, 2008; Panda et al., 2002a, 2003). The Myk/+ mutation resides within the NKA α3 subunit and these isoforms are expressed along iPRGCs, critical in the transmission of photic information to the SCN and the wider CNS (Dobretsov and Stimers, 2005; Lucas et al., 2014; Schmidt et al., 2011; Specht and Sweadner, 1984). Changes in function of iPRGCs may alter transmission of photic information and potentially influencing mood. Outside of the SCN iPRGCs innervate regions of the CNS that are strongly associated with mood such as the lateral habenula (LHb) and the amygdala (Hattar et al., 2006; Sakhi et al., 2014; Schmidt et al., 2011). Both of these regions play important roles in emotional and aversive behaviour and memory with multiple interactions across key regions of the brain’s reward pathways including the ventral tegmental area (VTA) and nucleus accumbens (Lecca et al., 2014; Li et al., 2013; Roozendaal et al., 2009; Russo and Nestler, 2013; Stamatakis and Stuber, 2012; Stamatakis et al., 2013). Given the observed changes in locomotor suppression by light there appears a deficit in basic transduction or translation of photic information in Myk/+ animals. If these changes do arise via altered signal propagation from iPRGCs then this represents a further area of consideration in Myk/+ animals and an important pathways in other models of affective disorders.

Open questions and future directions

The acquisition of novel data rarely fails to open up further questions than those originally posed and following in this tradition, many issues persist and important questions remain unanswered across all three chapters. The most pressing issue in relation to chapters 1 and 2 is addressing the underlying cause of the changes to central circadian dysfunction in Myk/+ animals. Despite demonstrating clear abnormalities in SCN output the mechanism of these changes has yet to be determined.
Molecular rhythms vs network rhythms

Due to a lack of overt changes to molecular rhythms *ex vivo*, dampened electrical output within the SCN was postulated to potentially arise from altered afferent signalling. Yet why changes to both electrical and synaptic properties were apparent in acute preparations, while molecular rhythms exhibited little change *ex vivo* requires explanation. The reason may a technical one, arising from differences in methods of slice preparation and maintenance. In acute preparations used in electrophysiological studies, changes in electrical and network properties of the SCN are maintained in slices and can reflect genetic or environmentally imposed changes to electrical and synaptic output, such as neuropeptide deficiency or photoperiod encoding (Brown and Piggins, 2009; Farajnia et al., 2014; Mrugala et al., 2000; Ohta et al., 2005; VanderLeest et al., 2007). In a thorough characterisation of preparation artefacts, VanderLeest and colleagues demonstrated that this form of slice preparation has no overt effects on SCN phase (VanderLeest et al., 2009). Protocols applied during preparation of SCN explants for long-term bioluminescence recording however can induce changes in the phasing of peaks in *Per1::LUC* and more importantly can induce rhythms from arrhythmic animals (Yoshikawa et al., 2005). This example demonstrates that preparation for long-term culture can potentially mask the initial state of SCN oscillations that may arise *in vitro*. As no major differences in the initial amplitude of *PER2::LUC* rhythms from *MykP2* animals were apparent, preparation methods may play a role in masking the initial condition of the SCN following dissection.

What should be stressed however is if methodological issues may contribute to a lack of amplitude differences in the *MykP2* SCN, this does not affect conclusions made in relation to tau and damping rates of SCN explants. These parameters are not direct measures of the initial state of the SCN following dissection, but rather reflect the pacemaking properties of the isolated tissue. The *MykP2* SCN therefore still maintains rhythmic properties comparable to *+/+* animals in these phenotypes. To improve on these findings and establish whether changes to molecular rhythms may arise in *MykP2* animals then *in situ* quantification of clock genes may be required. Also fine details of *Per1::LUC* and *PER2::LUC* spatiotemporal architecture can be defined *ex vivo* by using high resolution...
single-cell PER2::LUC imaging protocols (Evans et al., 2011; Inagaki et al., 2007). Applying this technique would improve resolution of the initial state of the MykP2 SCN following preparation and validate findings of the molecular architecture in these animals.

**Afferent inputs on to the SCN – A role for dopamine**

If external perturbations determine altered electrical and synaptic activity of Myk/+ animals then it is important to suggest potential mechanisms for these actions. Due to specific observations of Myk/+ behaviour it seems that dopaminergic centres represent promising mediators of altered mood behaviour that may provide aberrant feedback that affects pacemaking. Myk/+ animals exhibit a hyperactive phenotype and heightened d-amphetamine response indicative of altered DA systems (Cousins et al., 2009; Kirshenbaum et al., 2011). Changes in the negative masking behaviours of Myk/+ animals also suggest involvement as dopamine pathways; negative masking effects can be attenuated through methamphetamine or loss of D2 receptors (Doi et al., 2006; Vivanco et al., 2013). NKA α3 subunits co-localise with tyrosine hydroxylase neurons of the VTA highlighting that the Myk/+ mutation may directly influence neuronal activity within mesolimbic dopaminergic circuitry (Bøttger et al., 2011). Given these associations, the VTA represents an important region to pursue towards establishing the mechanisms of altered behaviour and potential arousal-based feedback on to the SCN.

To establish the influence of upstream signals upon the SCN empirically, certain options are available. *In vivo* recordings from the Myk/+ SCN would provide a powerful insight of electrical activity in freely moving animals. This method can allow direct observations of the impact of locomotor activity on electrical output and waveform of the SCN and may aid in confirming or rejecting this hypothesis, although this does represent a technically demanding procedure (Houben et al., 2014; van Oosterhout et al., 2012; Yamazaki et al., 1998). Scheduled exercise is a technique that restricts the access to running wheels to distinct epochs across the day. This paradigm can enhance pacemaking and entrainment in behaviourally compromised animals and may provide a less invasive tool to establish how locomotion and arousal circuits influence Myk/+ circadian behaviour (Hughes and Piggins, 2012). The potential influence on circadian timekeeping can be investigated...
through manipulation of DA centres by antipsychotics, such as haloperidol, on wheel-running activity and subsequent SCN output.

**Intrinsic factors of the Myk/+ mutation within the SCN**

Although heightened afferent signalling within the SCN appears as a potential factor defining the Myk/+ SCN phenotype, a potential intrinsic deficit underlying dampened output cannot be dismissed. Inherent deficits within the SCN should be considered as whole-cell data provided evidence of certain changes to single SCN neurons; a general increase in InRes across the Myk/+ SCN. This trend was mainly apparent in spontaneously active SCN neurons (IF and RF cells). This suggests that InRes may be physiologically relevant in neurons firing action potentials. Due to a low Na⁺ affinity and shallow voltage dependence, the NKA α3 subunit is believed to act as a reserve pump to extrude high Na⁺ loads during action potential firing (Azarias et al., 2013; Dobretsov and Stimers, 2005; Munzer et al., 1994; Zahler et al., 1997). If the NKA α3 is expressed within SCN cells then the Myk/+ mutation may theoretically result in the delayed clearance of intracellular Na⁺ and extracellular K⁺. This property of α3 function has been demonstrated in hippocampal neurons in vitro (Azarias et al., 2013). Increased latency in K⁺ clearance may alter the driving force of K⁺ ions across the plasma membrane and therefore an increase in InRes. Generally NKA α3 function is attributed to high frequency firing while the Myk/+ SCN achieving maximum firing rate of ~8Hz (Dobretsov and Stimers, 2005). This would seemingly appear low to account for effects on Na⁺ loading but without further study of the role of NKA α isoforms on SCN membrane properties, the relative contribution to electrical activity cannot be assumed.

A further consideration may be a role of the NKA as a signal receptor within the SCN. The NKA is inhibited by cardiotonic steroids such as ouabain and the proteoglycan agrin, with the former activating intracellular signalling cascades via extracellular binding to NKA pumps (Silva and Soares-da-Silva, 2012; Tidow et al., 2011). Signalling pathways activated by ouabain are diverse and relevant to SCN function including ERK, AKT and PKC, with α3 subunits possessing particularly strong associations with intracellular Ca²⁺ signalling via IP3 and Na⁺/Ca²⁺ exchangers (Kim et al., 2008; Miyakawa-Naito et al., 2003; Tian and Xie, 2008). Reduction in the activity of agrin and changes in ouabain binding can both affect
Myk/+ mood state and therefore play a known physiological role in Myk/+ animals (Kirshenbaum et al., 2011, 2012). Phosphorylated ERK and AKT are both upregulated in Myk/+ hippocampal neurons and ERK pathways are critical effectors integrating light information within the SCN (Coogan and Piggins, 2004). NKA pumps activated by ouabain are capable of inducing cellular Ca\(^{2+}\) oscillations in cells representing a further potential interaction with circadian clockwork, given the critical role of Ca\(^{2+}\) in both membrane excitability and molecular pacemaking in the SCN (Brancaccio et al., 2013; Colwell, 2011; Ikeda et al., 2003; Khodus et al., 2011; Miyakawa-Naito et al., 2003; Silva and Soares-da-Silva, 2012; Tian and Xie, 2008).

To improve understanding of intrinsic deficits within the Myk/+ and a possible defining role in network output, work can be directed towards elucidating how pump activity is altered in the Myk/+ SCN. Electrophysiological isolation of the NKA current in Myk/+ neurons may provide insights towards the mutation’s effects on the membrane, although this is complicated by the difficulty of isolating NKA α3 currents from other isoforms (Wang and Huang, 2004). Imaging of intracellular Na\(^+\) levels during action potential firing could identify changes in Na\(^+\) load in Myk/+ neurons, although this would require higher temporal resolution than utilised in Ca\(^{2+}\) imaging methods in this study. To identify potential changes in NKA signalling pathways, in situ quantification of p-ERK and p-AKT pathways represent a logical starting point given previous findings in Myk/+ hippocampal neurons. Interestingly application of concentrations of ouabain that target α2 and α3 NKA subunits rapidly reduce amplitude of WT PER2::LUC SCN rhythms ex vivo revealing that ouabain and NKA inhibition do impact upon SCN molecular pacemaking (unpublished observations).

**Myk/+ phenotype within current theoretical models of circadian pacemaking**

Moving on from hypotheses of the roots of Myk/+ SCN dysfunction, an important consideration to make is how these data fit into current theories of network models of the SCN. In the field of circadian biology theoretical models are widely developed to explain empirical observations of both cellular and network pacemaking (Roenneberg et al., 2008). Traditionally the cells and network of the SCN are modelled as limit-cycle
oscillators, these forms of oscillations that are more sensitive to phase shifting and perturbation at lower amplitudes (Abraham et al., 2010; Forger and Peskin, 2003). Recently however this idea has been challenged to suggest that at the network level the SCN actually behaves inversely to these expectations, with higher amplitude oscillations facilitating larger amplitude phase shifts (Gu et al., 2014; Ramkisoensing et al., 2014). This model has been largely developed from in vivo experimental paradigms using long and short photoperiods (Meijer et al., 2007). In this model large phase advances in behaviour under long photoperiods are correlated with restricted alpha bouts, low amplitude electrical rhythms and an increase excitatory GABAergic tone during the day in vitro (Farajnia et al., 2014; Gu et al., 2014; VanderLeest et al., 2007). This model also demonstrates that despite large amplitude behavioural shifts, the electrical activity of the SCN is less adept at re-setting its phase, and heightened behavioural shifts arise from the presence of a running wheel (Ramkisoensing et al., 2014).

Observations of low amplitude electrical rhythms and heightened phase advances to light suggest that the Myk/+ behavioural and physiological phenotype contradict the theoretical underpinnings of this SCN model. Increased excitatory GABA tone is also observed in Myk/+ yet at a different phase of the circadian cycle and changes to PRC shape not just amplitude are also apparent. In light of other Myk/+ behavioural phenotypes however, Myk/+ traits agree with the principles of this model. Specific differences may be attributable to increased locomotor activity and long alpha durations in Myk/+ animals. Behavioural phase shifts in response to light in this described network model are heavily influenced by access to a running-wheel and locomotor feedback (Ramkisoensing et al., 2014). The increased wheel-running activity of Myk/+ animals across the circadian cycle would therefore be expected to impact on the magnitude of behavioural shifts, explaining heightened early night re-setting despite low amplitude electrical rhythms. Alpha duration may also explain changes to PRC shape, with similar changes observed in rats with extended alpha durations, further emphasising the role of alpha and locomotor feedback in defining phase shifting and network properties in rodents (Honma et al., 1984). It would be of interest to define whether behavioural shifts are matched by reduced shifts of the SCN as described in this model, but this would necessitate in vivo electrical recordings.
Locomotor activity generates inhibitory feedback upon the SCN across the entire circadian cycle via NPY, GABA and serotonin. The neurophysiological substrate of increased alpha durations on the properties of the SCN may therefore be explained by aberrantly timed feedback, resulting in the distinct phenotype of the Myk/+ model (Besing et al., 2012; Morin and Allen, 2006; van Oosterhout et al., 2012; Schaap and Meijer, 2001; Yamazaki et al., 1998). Future studies that are directed towards modelling the SCN network may therefore benefit from taking some of these findings into consideration, specifically in relation to how arousal and locomotor activity may impact upon SCN network features and phase shifting responses.

**Intracellular targets of lithium**

In relation to chapter 3 and the role of lithium on circadian rhythms, the outstanding questions are more apparent given the nature of the results acquired. This study was initiated to identify the intracellular targets of lithium that results in period lengthening in cells, tissues and behaviour (Klemfuss, 1992; Li et al., 2012; Welsh and Moore-Ede, 1990). As a mood stabiliser with unique characteristics it is not clear whether this property may be relevant within the therapeutic actions of lithium (Campos-de-Sousa et al., 2010; Can et al., 2014; Cipriani et al., 2013; Rybakowski et al., 2014; Tondo et al., 1997). Given the conclusions drawn in chapter 3 from pharmacological manipulations of PER2::LUC explants ex vivo, the molecular target of lithium appears separate from the hypothesised IMPase and inositol phosphate cycling.

The obvious question therefore remains to identify the pathways through which lithium exerts influence on cellular and behavioural timekeeping. Although this chapter produced a negative result, these findings still hold relevance given the widely ascribed role of IMPase and GSK3β in lithium’s impacts on behaviour (Agam et al., 2009; Harwood, 2005; O’Brien and Klein, 2009; Pasquali et al., 2010; Rowe et al., 2007). Our data confirms that lithium’s actions on the SCN manifest through alternative intracellular pathways. At times in the literature perhaps places too much emphasis upon these specific pathways as the basis of lithium’s effects. As our study suggests, perhaps lithium’s effects are more diverse at therapeutic concentrations given its broad range of possible cellular targets and may go
some way to explaining the lack of concordance between IMPase and GSK3β schools of thought.

Continuing work on lithium’s interactions does hold clinical relevance given that it is currently unclear whether any therapeutic benefits of lithium may act through the circadian system (Campos-de-Sousa et al., 2010; McCarthy et al., 2013; Rybakowski et al., 2014). Lithium possesses distinct properties among currently available pharmaceuticals prescribed in the management of bipolar disorder and effects on the circadian system are equally distinct among available drugs. Attempts to understand how lithium influences the circadian system is therefore of continued interest to both neuropsychiatric and circadian fields.

**Further considerations: metabolism, clocks and lithium**

Metabolic assessment of Myk/+ animals revealed profound alterations to BMR and ingestion behaviours alongside reduced body weight. It was noted that in addition to Myk/+ animals, many clock gene models of altered affective behaviour also exhibit altered metabolic phenotypes including ClockΔ19, Rev-erbα−/−, Bmal1−/−, Afh and altered food entrainable behaviours in Per2Brdm animals (Duez and Staels, 2009; Feillet et al., 2006; Guilding et al., 2013; Kondratov et al., 2006; Marcheva et al., 2010; Turek et al., 2005). As metabolic abnormalities appear with such consistency, these phenotypes present themselves as interesting phenomena that has yet to be given much credence in the literature. As described previously, BPD is a heritable condition which appears polygenic in nature, determined by the contribution of a large number of genes that represent risk factors in the development of episodes (Craddock and Sklar, 2013). Mood disorders exhibit clear links to circadian rhythm disruption, alongside an epidemiological association with metabolic syndrome (Fagiolini et al., 2005; Taylor and MacQueen, 2006). Inter-dependence of circadian and metabolic physiology at both cellular and systemic level are also apparent and together may represent a tripartite genetic relationship with altered mood behaviours (Asher and Schibler, 2011; Nakahata et al., 2009; Ramsey et al., 2009; Wang et al., 2012).
Clock genes exhibit polymorphisms that appear as risk factors in the development of BPD while others demonstrate associations with the prophylactic and circadian effects of lithium, highlighting a potential genetic basis of these links (Etain et al., 2011; McCarthy et al., 2013; Nievergelt et al., 2016; Rybakowski et al., 2014; Shi et al., 2008). Studies on the genetic association of metabolic genes in BPD are currently lacking although mitochondrial dysfunction is associated to BPD (Iwamoto et al., 2005; Kato and Kato, 2000). As BPD is a heterogeneous disorder within which patients differentially respond to certain treatments, perhaps circadian and metabolic-associated genes represent a distinct group of risk-factors that may characterise certain manifestations or therapeutic responses of BPD.

The use of lithium in treating BPD is widespread although only select patients respond positively to lithium (Grof, 2010). Lithium-response clusters within families and patients that respond exhibit an increased chance of a BPD relative that also demonstrate lithium responsiveness (Alda et al., 2005; Grof et al., 1994, 2002). Lithium responders are widely considered therefore as a genetically distinct sub-population of BPD patients (Alda et al., 2005; Grof, 2010; Schulze et al., 2010). As circadian rhythms and metabolism possess such strong physiological associations and appear concomitantly in clock models of altered mood, clock and metabolic disruption may represent a similar genetic sub-population. In light of the link between circadian systems and lithium, these genetic factors may even form a part of lithium-responsive sub-groups. Using both clock and metabolic abnormalities as biomarkers could aid in defining sub-groups of patients that respond better to specific clinical interventions such as chronotherapy or lithium. It should be stressed that this suggestion is speculative without clear genetic associations in human patients, yet given observations in Myk/+ and other pre-clinical models make these observations in pre-clinical models of noteworthy consideration.

**Myk/+ as a switching model of bipolar disorder**

A final point that presented itself from this work is the potential of the Myk/+ model to exhibit both mood aspects of BPD. Wheel-running experiments highlighted that individual animals displayed large reductions in wheel-running activity over time that may represent a behavioural switch to depression-like phenotypes. To validate these observations
behavioural experiments used to define mania-like phenotypes in Myk/+ animals’ should be performed on animals exhibiting this hypolocomotor behaviour. Defining the specific conditions that induce this type of behaviour may be revealing on the environmental influences that are prominent in changes to mood behaviour. If the Myk/+ model can be validated as a representation of both mania and depression-like episodes, it would represent a powerful tool to study the neurobiology that defines the change between distinct behaviours in BPD. If animals can be readily-induced into different mood states, then investigations into neuronal activity and gene expression patterns in major mood centres of the CNS would be possible.

**Future directions of the field**

Recent years has seen much research attention being applied to the role of sleep and circadian regulation in affective disorders. The identification of animal models with altered circadian timing that exhibit altered mood behaviours has allowed the first steps towards understanding the neurobiological associations of circadian rhythm disruption to be made (Chung et al., 2014; Coque et al., 2011; Roybal et al., 2007). Many of the questions posed here in relation to this study on Myk/+ animals also represent the broader issues within this field. From this perspective I think that the most pressing issues can be broken down into two major questions that have yet to be fully answered:

1. Do changes to circadian regulation rhythms directly contribute to the aetiology and pathophysiology of mood disorders?
2. Does targeting the circadian system represent a viable solution in the management of neuropsychiatric disorders?

Although reports of circadian disruption are widely reported in clinical behavioural studies and within the neurobiology of laboratory models, unequivocal evidence that circadian mechanisms underpin changes in mood is lacking. Recent papers have begun to demonstrate this principal within the VTA although further validation and improved techniques are required to display that circadian mechanisms drive altered affective behaviour (Chung et al., 2014; Coque et al., 2011; Mukherjee et al., 2010). Generally studies are limited in their scope as they often rely upon complete loss of specific genetic
elements that, even if a key circadian clock component, does not confer specificity to clock function due to the broad array of interactions made by clock components outside the TTFL (Miller et al., 2007; Panda et al., 2002b).

Application of new techniques within existing and novel animal models represents a critical tool in providing many of the answers to these problems. Given recent advances in the specificity of neurobiological tools available such as optogenetics, viral targeting and transgenic lines allowing targeted, conditional manipulation of cellular elements, there are now powerful new methods that can be applied to hone in on underlying circadian mechanisms. To benefit this search, continuing work towards identifying functional changes throughout the CNS will help to define a map of the circuitry of circadian and mood interactions.

By targeting the rhythmic regulation of physiology across candidate regions using spatially specific tools, the role of rhythmicity across the CNS in mood behaviours can be elucidated. If effects can be translated from specific structures through to the level of behaviour then circadian system may present themselves as viable targets for intervention in affective disorders. Given the speed with which the beneficial effects of chronotherapeutics can manifest upon mood this pursuit retains some level of promise. Given the current availability of novel pharmaceuticals that target the circadian clockwork, these tools may prove beneficial to alleviate neuropsychiatric conditions (Meng et al., 2008; Solt et al., 2012). Animal models provide a powerful first step to determine if such manipulations may provide any such benefits.

**Conclusions**

Work on the Myk/+ mouse has provided a multi-level and detailed characterisation of both behavioural and physiological profiles in this model of the manic phase of BPD. These profiles have revealed novel insights into how circadian pacemaking may be altered in models of affective disorders, namely by specific changes to network-dependent central timing and interactions with light. This work can be built upon in future by determining how these deficits influence central pathways outside the SCN that underlie altered mood states in Myk/+ animals. In addition to the characterisation of the circadian
system in the Myk/+ model, the role of lithium on circadian rhythms was revealed to act through distinct pathways outside the widely-recognised cellular targets IMPase and GSK3β. These data reinforce the complex nature of lithium’s cellular actions and how these may manifest across the brain. Taken together, this work adds to the continuing effort to understand how circadian rhythm disruption arises within the behaviour and therapeutic interventions across affective conditions such as bipolar disorder. Elucidating the role of the biological timing in neuropsychiatric disease represents an area of great importance given the profound associations that appear in clinical and pre-clinical literature. Hopefully the insights from this investigation will prove useful towards our understanding into the behaviour, physiology and pharmacological interactions of the circadian systems in mood disorders.
References


